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PPQB**

MAURO MACEDO DE OLIVEIRA

**DERIVADOS DA DIBENZALACETONA COMO POTENCIAIS
AGENTES CITOTÓXICOS, ANSIOLÍTICOS E REVERSORES DA
RESISTÊNCIA BACTERIANA: ENSAIOS *IN SILICO*, BIOLÓGICOS E
ESPECTROSCÓPICOS.**

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Tese apresentada ao Programa de Pós-Graduação em Química Biológica, da Universidade Regional do Cariri, como requisito parcial para obtenção do Título de Doutor em Química Biológica.

Orientador: Prof. Dr. Carlos Emídio Sampaio Nogueira

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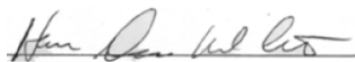
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Dedico:

Primeiramente a Deus, por todas as graças que me tem dado e pela força que me dá nos momentos difíceis.

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“O sucesso é ir de fracasso em fracasso sem perder o entusiasmo.”.

Autor desconhecido

RESUMO

Várias doenças têm causado preocupação por todo o mundo, em especial o câncer, infecções por organismos resistentes a múltiplas drogas e desordens relacionadas à ansiedade. Por isso a busca por novos compostos bioativos tem motivado os cientistas. Nesse contexto destaca-se a classe de compostos denominada de chalconas e seus derivados, dentre os quais os análogos da dibenzalacetona. Compostos orgânicos formados por duas regiões reativas α e α' que promovem uma grande deslocalização dos elétrons π , chamam atenção por suas atividades biológicas. Neste trabalho nós sintetizamos três derivados da dibenzalacetona, (1*E*,4*E*)-1,5-di(tiofen-2-il)penta-1,4-dien-3-ona, (1*E*,4*E*)-1,5-bis(4-etoxifenil)penta-1,4-dien-3-ona e (1*E*,4*E*)-1,5-bis(4-clorofenil)penta-1,4-dien-3-ona, denominados por DB tiofeno, DB etoxi e DB cloro, respectivamente. Aqui é reportado a caracterização espectroscópica das três moléculas bem como suas atribuições vibracionais em que se utilizou a Teoria do Funcional de Densidade (DFT) com o funcional de correlação de troca B3LYP. Em testes preliminares, o DB tiofeno e o DB cloro apontaram para uma provável atividade citotóxica contra células tumorais de cólon HCT-116, a primeira ainda indicou atividade moderada contra a linhagem de câncer de próstata PC3 e a segunda, baixa atividade contra essa mesma linhagem. Ensaio de modulação da resistência aos antibióticos em cepas multirresistentes de *S. aureus* (1199B e K2068) revelaram que os três compostos foram capazes de inibir o efluxo do substrato EtBr pela bomba de efluxo NorA (redução da CIM em 29,3%, 37,0% e 37,0%, respectivamente), enquanto apenas o DB tiofeno e o DB cloro inibiram o efluxo de EtBr mediado pela MepA (redução da CIM em 43,9% e 37,0%), demonstrando que são potenciais inibidores dessas bombas de efluxo. Ainda, o DB tiofeno exibiu uma notável capacidade de inibir o efluxo de norfloxacino mediado pela NorA em quase 30,0%, e de ciprofloxacino mediado pela MepA pela metade, colocando-se como um provável candidato a adjuvante para esses antibióticos. Estudos de docagem molecular indicaram que a ação inibidora das bombas de efluxo pelos derivados da dibenzalacetona ocorre provavelmente por competição ao mesmo sítio de ligação dos antibióticos. DB tiofeno e DB cloro também provocaram deficiência motora em *zebrafish* (*Danio rerio*), o que sugere que esses compostos promoveram um efeito sedativo. Por outro lado, a não alteração da atividade locomotora pelo DB etoxi pode indicar potencial ação antinociceptiva. Foi demonstrado que esse efeito sedativo se deu através da atividade ansiolítica semelhante aos benzodiazepínicos com mecanismo de ação GABAérgica. Ademais, testes *in silico* de semelhança com drogas sugeriram bom potencial dos referidos compostos para uso como medicação oral.

Palavras-Chave: Dibenzalacetona, Inibidores de bomba de efluxo, Ansiolítico, Citotoxicidade, Espectroscopia vibracional, Docagem.

ABSTRACT

Several illnesses have caused concern around the world, in particular cancer, infections by multidrug-resistant strains and anxiety disorders. That is why the search for new bioactive compounds has motivated scientists. In this context, the class of compounds denominated as chalcones and their derivatives stands out, among which are the dibenzalacetone derivatives. Organic compounds formed by two α and α' reactive regions that provide a large relocation of π -electrons, call attention for their biological activities. In this work, we synthesized three dibenzalacetone derivatives, (1*E*,4*E*)-1,5-di(thiophen-2-yl)penta-1,4-dien-3-one, (1*E*,4*E*)-1,5-bis(4-ethoxyphenyl)penta-1,4-dien-3-one and (1*E*,4*E*)-1,5-bis(4-chlorophenyl)penta-1,4-dien-3-one, named by DB thiophene, DB ethoxy and DB chlorine, respectively. Here the spectroscopic characterization of the three molecules is reported, as well as their vibrational attributions using the Density Functional Theory (DFT) with the B3LYP exchange correlation functional. In preliminary assays, DB thiophene and DB chlorine displayed cytotoxic activity against colon tumor cells HCT-116, the first one still indicated moderate activity against the PC3 prostate cancer lineage and the second one, low activity against this same lineage. Antibiotic resistance modulation assays using multiresistant strains of *S. aureus* (1199B and K2068) revealed that the three compounds were able to inhibit the EtBr efflux by NorA efflux pump (MIC reduction by 29.3%, 37.0% and 37.0%, respectively), while only DB thiophene and DB chlorine inhibited MepA-mediated EtBr efflux (MIC reduction by 43.9% and 37.0%), demonstrating that they are potential inhibitors of these efflux pumps. In addition, DB thiophene exhibited a remarkable ability to inhibit NorA-mediated norfloxacin efflux (MIC reduction by 30.0%) and MepA-mediated ciprofloxacin (MIC reduction by half), posing itself as a likely adjuvant candidate for these antibiotics. Molecular docking studies indicated that the inhibitory action of efflux pumps by dibenzalacetone derivatives probably occurs by competition at the same binding site as the substrates. DB thiophene and DB chlorine also caused motor impairment in *zebrafish* (*Danio rerio*), which suggests that these compounds provide a sedative effect. On the other hand, the non-alteration of locomotor activity by DB ethoxy may indicate potential antinociceptive action. This sedative effect was shown to occur through benzodiazepine-like anxiolytic activity with a GABAergic mechanism of action. Furthermore, *in silico* drug-likeness test suggested good potential of said compounds as oral medication.

Keywords: Dibenzalacetone, Efflux pump inhibitors, Anxiolytic, Cytotoxicity, Vibrational spectroscopy, Docking.

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LISTA DE ABREVIATURAS E SIGLAS

- RMN – Ressonância magnética nuclear
- EM – Espectroscopia de massa
- IV – Infravermelho
- DFT – Teoria do Funcional da Densidade
- CIM – Concentração inibitória mínima
- CLSI – Instituto de padrões clínicos e laboratoriais
- MTT – Brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio
- DNA – Ácido desoxirribonucleico
- ATP – Trifosfato de adenosina
- AGS – Adenocarcinoma gástrico
- IC₅₀ – Concentração inibitória de 50%
- FASII – Via biossintética de ácidos graxos II
- FtsZ – Proteína bacteriana envolvida na divisão celular
- PTP – Proteínas tirosina fosfatases
- PTK – Proteínas tirosina quinases
- MSF – Superfamília dos facilitadores majoritários
- SMR – Pequena família de resistência a múltiplas drogas
- MATE – Família de extrusão de multidrogas e compostos tóxicos
- RND – Família resistência à nodulação celular
- ABC – Superfamília de cassetes de ligação de ATP
- NorA – Proteína de efluxo de múltiplas drogas contida em linhagens de *S. aureus*
- MepA – Proteína de efluxo de múltiplas drogas contida em linhagens de *S. aureus*
- EtBr – Brometo de etídio
- norA* – Gene que codifica a proteína NorA
- mepA* – Gene que codifica a proteína MepA
- Tet(A) – Proteína de efluxo de tetraciclina contida em linhagens de *E. coli*
- Bmr - Proteína de efluxo de múltiplas drogas contida em linhagens de *B. subtilis*
- NOR – Norfloxacino
- CIP – Ciprofloxacino
- CPZ – Clorpromazina
- CCCP - Carbonil cianeto m-clorofenilhidrazona
- EPI – Inibidor de bomba de efluxo

GABA_A – Receptor do ácido γ -aminobutírico A

nAChRs – Receptores de acetilcolina nicotínica

5HT₃ – Receptores de serotonina

GlyR – Receptores de glicina

BDzs – Banzodiazepínicos

FT – Transformada de Fourier

AG – Algoritmo genético

AGL – Algoritmo genético lamarckiano

SVM – Máquina de vetor de suporte

RF – Floresta aleatória

ANN – Rede neural artificial

DL – Aprendizagem profunda

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INTRODUÇÃO GERAL

A preocupação mundial com diversas desordens médicas, tais como câncer (BRAY et al., 2018), infecções resistentes a medicamentos (TACCONELLI; PEZZANI, 2019) e distúrbios relacionados ao sistema nervoso central tal como a ansiedade (LIU et al., 2015), tem motivado cientistas do mundo todo na identificação de novos compostos químicos bioativos. Por outro lado, o processo para desenvolvimento completo de uma droga pode levar muitos anos, é complexo, bastante custoso e geralmente está sujeito a uma enorme pressão por parte das indústrias farmacêuticas bem como dos médicos e pacientes para agilizar esse processo. Por sorte a era pós-genômica propiciou um aumento gigantesco de dados biológicos que, associados ao desenvolvimento da biologia e da química computacional, foram cruciais para uma revolução sem precedentes na química medicinal. Métodos empíricos foram substituídos por abordagens muito mais sofisticadas e multifacetadas, que melhoraram bastante a eficiência na descoberta de medicamentos, resultando em uma aceleração significativa do processo (WU et al., 2019).

As chalconas compreendem uma classe de compostos muito importante na busca por moléculas bioativas pois possuem uma estrutura química privilegiada, de fácil preparo e com um amplo espectro de atividades biológicas. Isto se deve provavelmente pela sua pequena estrutura com uma α,β -insaturação, com muitos hidrogênios substituíveis e por possuírem características de aceptadores de *Michael*¹, que as tornam tolerantes à diferentes moléculas biológicas permitindo que elas se liguem de forma rápida (RANI et al., 2019; ZHUANG et al., 2017). O amplo espectro das bioatividades das chalconas e seus análogos inclui, por exemplo, atividade anti-inflamatória (CHENG et al., 2008; LI et al., 2017), atividade antimicrobiana (INAMULLAH et al., 2017; UŠJAK et al., 2019), atividade ansiolítica (SALEHI et al., 2021), atividade antioxidante (AOKI et al., 2008; BELSARE et al., 2010), atividade anticâncer e citotóxica (MIRZAEI; KEIGHOBADI; EMAMI, 2017; MODZELEWSKA et al., 2006), atividade antifúngica (ELSOHLY et al., 2001; MOHAMMED; JAMALIS, 2017), atividade antimalárica (DOMÍNGUEZ et al., 2005; YADAV et al., 2012), atividade antiprotozoária (MELLO et al., 2018), atividade antituberculose (UMAA et al., 2013), atividade anticonvulsivante (SIDDIQUI et al., 2010; VERMA; SRIVASTAVA; PANDEY, 2018), atividade antidiabética (EMAYAVARAMBAN et al., 2013), atividade anti-hipertensiva (AVILA-VILLARREAL et al., 2013) etc. Apesar de suas importantes propriedades

¹ Os compostos aceptadores de *Michael* compreendem os eletrófilos e geralmente são biologicamente ativos. A estrutura da chalcona contém um grupo funcional cetona α,β insaturado percebido como um potencial aceptador de Michael.

biológicas, as chalconas também demonstram utilidade em materiais com propriedades óticas não lineares e eletroativos fluorescentes tais como corantes fluorescentes e diodos emissores de luz (NOHUT MAŞLAKCI et al., 2018).

Quando se realiza uma reação de condensação com dois equivalentes de aldeídos em relação a uma cetona que possui duas regiões reativas α e α' , obtém-se derivados que na literatura recebem diferentes denominações, por exemplo, derivados da dibenzalacetona (ARBUZOV et al., 1969; KIRAN et al., 2006; RAJASHEKAR et al., 2012; SANTOS et al., 2020), bis-chalconas (BALE et al., 2018; REDDY et al., 2014; SHETTIGAR et al., 2006), curcuminóides (LIANG et al., 2009) ou simplesmente derivados de chalconas (SALEH, 2017). Essas moléculas apresentam uma porção dienona com uma extensa deslocalização dos elétrons π , o que facilita ainda mais a polarizabilidade desses compostos em relação às chalconas com apenas uma porção enona. Assim como as chalconas simples (com uma porção enona), esses derivados também apresentam características multifuncionais com diversas aplicações médicas e tecnológicas (BALE et al., 2018; NOHUT MAŞLAKCI et al., 2018).

Apesar de extensos estudos do perfil biológico das chalconas ainda existe um grande esforço na obtenção de derivados que possam potencializar a ação desses compostos. Chalconas com anéis heterocíclicos, por exemplo, foram identificadas como potenciais agentes contra diversos tipos de câncer (MARTINEZ-CIFUENTES et al., 2015; SHARMA; KUMAR; KUMAR, 2013), assim como as bis-chalconas simétricas se mostraram eficientes em impedir o efluxo de drogas pela proteína transportadora ABCG2 que é um dos maiores obstáculos para o tratamento de câncer com quimioterapia (WINTER et al., 2014).

O aumento da resistência aos antimicrobianos constitui um problema de saúde global que emergiu desde o início da era dos antibióticos. Um patógeno pode ser naturalmente resistente ou ele pode adquirir essa resistência a uma droga que anteriormente era sensível, um processo que facilmente ocorre nos hospitais e que os tornam terreno fértil para floras cada vez mais resistentes (MAYERS et al., 2017). Os mecanismos de efluxos de múltiplas drogas tais como NorA e MepA, já identificadas em cepas de *S. aureus* (COSTA S. S. et al., 2013), se mostram como um obstáculo à saúde pública na medida em que cada vez mais alguns organismos se tornam resistentes aos tratamentos convencionais e promovem infecções bacterianas preocupantes (REZENDE-JÚNIOR et al., 2020). Embora várias classes de compostos, incluindo as chalconas, já tenham sido identificadas com sendo adjuvantes na ação inibidora de bombas de efluxo, a química das chalconas ainda parece ser uma das áreas mais

importantes para gerar novas oportunidades na concepção e descoberta de novos compostos bioativos (RANI et al., 2019).

Outra preocupação que emerge atualmente, refere-se aos transtornos de ansiedade que afetam grupos de diversas idades ao redor do mundo. Os benzodiazepínicos (BZDs) compreendem a classe de medicamentos mais comum no tratamento dos transtornos de ansiedade, porém causam efeitos adversos tais como sedação, relaxamento muscular, amnésia e potencial de dependência. Assim, os cientistas buscam há anos por agentes efetivos no combate a ansiedade que não causem efeitos colaterais (LIU et al., 2015). Já se sabe, por exemplo, que algumas chalconas tais como a isoliquiritigenina e a buteína, possuem ação ansiolítica muito similar ao Diazepam em modelos animais com alta afinidade no sítio dos BZDs em receptores GABA_A (JAMAL; ANSARI; RIZVI, 2008; SALEHI et al., 2021). Portanto, as chalconas e seus derivados compõem uma classe de substâncias bastante promissoras no combate aos distúrbios do sistema nervoso central, em especial, aqueles relacionados à ansiedade (MATHEW et al., 2019).

- OBJETIVOS E QUESTIONAMENTOS

Tendo em vista a vasta literatura científica contemplando as potencialidades das chalconas na química medicinal, percebe-se que mesmo com o aumento recente na síntese de vários derivados, ainda é unânime entre os especialistas que é possível o desenvolvimento de estruturas moleculares análogas às chalconas ainda mais eficazes, e com o adicional de serem de baixo custo, então, é possível atuar no desenvolvimento de derivados da dibenzalacetona sintéticos com esse propósito. Um questionamento que emerge logo na sequência é: Quais atividades biológicas seriam relevantes no contexto da identificação de chalconas bioativas? Essa resposta veio em função da vontade de contribuir na descoberta de algum composto para o enfrentamento de alguns tipos de câncer, que é uma doença grave e que acomete milhares de pessoas todos os anos, do aproveitamento da vasta expertise do departamento no estudo de infecções com bactérias resistentes à múltiplas drogas, bem como, eventualmente, da disponibilidade para realização de outras atividades biológicas de interesse. Portanto, foi realizado o estudo da atividade antimicrobiana dos derivados da dibenzalacetona sintéticos e sua ação moduladora com antibióticos convencionais em cepas multirresistentes pelo mecanismo de bomba de efluxo. Em seguida, conduziu-se a investigação tanto da atividade citotóxica frente às células tumorais de cólon, próstata e glioblastoma, quanto em relação à atividade locomotora e ansiolítica em modelos de *zebrafish* (*Danio rerio*).

Em paralelo às investigações das atividades biológicas, desvendou-se toda a estrutura dos compostos obtidos, bem como fez-se uma completa varredura nas suas características espectroscópicas vibracionais. Essa investigação é importante na elucidação dos modos vibracionais das moléculas contribuindo para a literatura científica especializada e no entendimento de algumas de suas características. Para isso recorreu-se às técnicas de modelagem molecular necessárias na obtenção da geometria otimizada para posterior cálculo dos modos normais de vibração. Já para provar a pureza dos compostos sintetizados recorreu-se a cromatografia gasosa com espectroscopia de massa. Contudo, ainda era necessário fazer a convergência entre suas características estruturais com eventuais atividades biológicas, de forma a tentar propor algum mecanismo de ação. Para isso utilizou-se técnicas de quimioinformática, que é uma área que combina campos científicos da química, ciências da computação e ciências da informação para transformar dados químicos e biológicos em conhecimento que apoia a tomada de decisão.

Portanto este trabalho teve como objetivos sintetizar três derivados da dibenzalacetona e investigar suas atividades: Citotóxica frente às cepas HCT-116 (carcinoma do cólon), SNB (glioblastoma) e PC3 (próstata); antimicrobiana e moduladora da ação de antibióticos convencionais em cepas multirresistentes de *S. aureus*; locomotora e ansiolítica em modelos de *zebrafish* (*Danio rerio*). Também se propôs a apresentar o *design* e a elucidação estrutural dessas moléculas bem como uma completa caracterização espectroscópica vibracional com modelos *in silico* feitos a partir de estudos de mecânica molecular e química quântica, realizando uma comparação com as medidas experimentais de espectroscopia de infravermelho e Raman. Além disso, realizar simulações de docagem molecular dos compostos em modelos biológicos representando as proteínas transportadoras das bombas de efluxo NorA e MepA, e o receptor GABA_A, para um melhor entendimento dos mecanismos presentes nessas interações.

Os compostos objetos desse estudo são: (1*E*,4*E*)-1,5-di(tiofen-2-il)penta-1,4-dien-3-ona, (1*E*,4*E*)-1,5-bis(4-etoxifenil)penta-1,4-dien-3-ona e (1*E*,4*E*)-1,5-bis(4-clorofenil)penta-1,4-dien-3-ona, que foram chamados de DB tiofeno, DB etoxi e DB cloro, respectivamente.

- ESTRATÉGIAS DE PESQUISA

Este trabalho é de natureza aplicada, com uma abordagem quantitativa e é classificado quanto aos seus objetivos como uma pesquisa explicativa. Esse tipo de pesquisa ocupa-se de encontrar as explicações para a ocorrência de determinados fatos ou fenômenos. As ciências da natureza utilizam com muita frequência esse tipo de pesquisa que envolve o pesquisador num

nível elevado de responsabilidade para com os resultados obtidos (SANTOS, 2007). Quanto aos procedimentos utilizados nesse trabalho destacam-se: a pesquisa bibliográfica – que é o domínio do estado da arte sobre determinado assunto, e a pesquisa experimental – que se caracteriza pela manipulação direta das variáveis que influenciam o objeto de estudo. A manipulação dessas variáveis permite relacionar as causas e os efeitos de determinado fenômeno (CERVO; BERVIAN; SILVA, 2007).

Existem diversas estratégias, catalisadores e condições para a síntese de chalconas (ZHUANG et al., 2017), uma das mais utilizadas é a reação de condensação via catálise ácida ou básica. Devido à baixa complexidade e custo, os derivados da dibenzalacetona desse estudo foram sintetizados pela condensação de Claisen–Schmidt (CLAISEN; CLAPARÈDE, 1881; SCHMIDT, 1881) com catalisador básica, utilizando dois equivalentes do aldeído apropriado em relação a propan-2-ona de forma que houvesse uma dupla condensação e a formação de uma porção dienona. A estratégia empregada na confirmação da estrutura química dos compostos obtidos se deu através da espectroscopia de ressonância magnética nuclear (^1H e ^{13}C RMN) bem como da espectroscopia de massa (EM). Ambas são técnicas consagradas da análise instrumental e bastante utilizadas na caracterização de compostos orgânicos.

O estudo da espectroscopia vibracional ocorre numa faixa bem larga do espectro das radiações eletromagnéticas, nessa faixa se dão as transições de níveis de energia vibracional e rotacional que fornecem várias informações estruturais das moléculas tais como ângulos e comprimentos de ligação. Para o estudo com esse tipo de transição energética, foram empregadas nesse trabalho, as técnicas de espectroscopia de Infravermelho (IV) e espectroscopia Raman, ambas com transformada de Fourier. Essas duas técnicas estão intimamente relacionadas pois se originam no mesmo nível de transição vibracional das moléculas, porém devido à forma como ocorrem as excitações energéticas em cada uma, elas devem ser utilizadas de forma complementar. Em outras palavras, uma ligação apolar pode ser Raman ativa, mas IV inativa. No entanto, uma ligação polar pode ser IV ativa e Raman inativa ou ambas ativas (YADAV, 2013).

Investigações estruturais mais aprofundadas requerem, além de dados experimentais de IV e Raman, cálculos teóricos de modelagem molecular e química quântica *in silico*. Para realização dos cálculos teóricos, recorreu-se à Teoria do Funcional da Densidade (*Density Functional Theory* – DFT) por apresentar ganhos na velocidade computacional e no espaço de memória em relação aos métodos tradicionais *ab initio* e aos métodos semi-empíricos (HINCHLIFFE, 2008). Nos cálculos de DFT empregou-se o funcional híbrido de correlação

Lee-Yang-Parr B3LYP (BECKE, 1993; LEE; YANG; PARR, 1988) combinado com o conjunto base 6-31G* (JOHNSON III; RUSSELL, 2020). Essa escolha deu-se porque o campo de força do B3LYP produz espectros com excelente concordância com os dados experimentais (STEPHENS et al., 1994).

Para determinação da atividade antimicrobiana e moduladora da ação de antibióticos empregou-se o método da microdiluição seriada em caldo de crescimento. Esse método foi escolhido por ser bastante utilizado para a avaliação quantitativa *in vitro* da atividade antimicrobiana pela mensuração da concentração inibitória mínima – CIM (BALOUIRI; SADIKI; IBNSOUDA, 2016). Seguiu-se os padrões aprovados pelo Instituto de Padrões Clínicos e Laboratoriais (*Clinical and Laboratory Standards Institute – CLSI*) (WIKLER, 2006) que garantem que o ensaio seja feito de forma padronizada afim de avaliar a relevância clínica dos resultados. Na avaliação da citotoxicidade dos compostos foi empregado o ensaio colorimétrico rápido com Brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT) para crescimento e sobrevivência celular (MOSMANN, 1983). Nos ensaios de atividade locomotora foi utilizado o teste de campo aberto e para atividade ansiolítica o teste claro/escuro, ambas em modelo de *zebrafish* (*Danio rerio*) (BENNEH et al., 2017; MAGALHÃES et al., 2017).

Para o estudo de docagem molecular foi necessário inicialmente construir modelos de homologia das proteínas transmembranares NorA e MepA pois suas estruturas cristalizadas ainda não estavam disponíveis nos bancos de dados. No caso da estrutura proteica do receptor GABA_A, uma vez que este já existia na literatura na forma cristalizada, foi preciso apenas prepará-la para a docagem. Empregou-se tanto *AutoDock4* (MORRIS et al., 2010) quanto o *AutoDock Vina* (TROTT; OLSON, 2010) nos cálculos de docagem molecular. Para a avaliação do potencial dos compostos em se tornar um medicamento, conduziu-se uma análise *in silico* das propriedades físico-químicas, testes de farmacocinética, semelhança com drogas e compatibilidade com química medicinal no *SwissADME* (DAINA; MICHIELIN; ZOETE, 2017), uma plataforma que fornece modelos de previsão on-line de forma livre.

- ESTRUTURA DA TESE

A presente tese foi dividida em cinco capítulos, no primeiro consta a fundamentação teórica dos conceitos importantes para o trabalho, nele buscou-se criar condições para que o leitor possa situar-se quanto ao estado da arte relativo ao tema. Inicia-se com a caracterização da classe de compostos denominada de chalconas e suas formas de obtenção, em seguida é

apresentada uma classe análoga das chalconas denominada de derivados da dibenzalacetona ressaltando suas principais atividades biológicas encontradas da literatura. Procurou-se enfatizar as atividades citotóxica/anticâncer, antibacteriana/moduladora da ação dos antibióticos e ansiolítica pois eram essas que integravam os objetivos da tese. Nesse capítulo também constam conceitos de espectroscopia vibracional (Infravermelho e Raman), espectroscopia de massa, de ressonância magnética nuclear, cálculos computacionais relativos à Teoria do Funcional da Densidade e de docagem molecular. O segundo capítulo é composto pelo artigo já publicado na revista *Journal of Molecular Structure* número 1215 no ano de 2020, intitulado “*Spectroscopic characterization and efflux pump modulation of a thiophene curcumin derivative*”. Nele relata-se a obtenção do DB tiofeno, sua completa caracterização espectroscópica, avaliação do seu desempenho como inibidor das bombas de efluxo NorA e MepA, e estudos de docagem molecular do composto em um modelo de homologia da proteína transportadora da bomba de efluxo NorA. No terceiro capítulo acrescentou-se um artigo também já publicado no ano de 2021 na revista *Journal of Molecular Structure* número 1231, cujo título é “*Full Spectroscopic Characterization and Cytotoxicity Activity of Synthetic Dibenzalacetone Derivatives*”. Nessa publicação constam as sínteses dos compostos DB etoxi e DB cloro, toda caracterização espectroscópica, a avaliação das suas atividades citotóxicas bem como um estudo *in silico* de semelhança com drogas para denotar seu potencial de se tornarem fármacos. O quarto capítulo é composto por um terceiro artigo cujo título é “*Cytotoxicity Activity, Efflux Pump Inhibition, Anxiolytic-Like Effect and Molecular Docking Studies of Synthetic Bis-chalcones*”. Nesse artigo os três derivados da dibenzalacetona (DB tiofeno, DB etoxi e DB cloro) tiveram suas atividades citotóxica, ansiolítica e moduladora de bomba de efluxo (NorA e MepA) compiladas e avaliadas, tal como seus mecanismos de interação, tanto em modelos de homologia das proteínas NorA e MepA quanto na proteína do receptor GABA_A, examinados por estudos de docagem molecular. Por fim, no capítulo cinco, são expostas as considerações finais evidenciando os principais achados e conclusões dessa tese. Outrossim, são sugeridos nessa parte alguns caminhos para investigações futuras bem como outros trabalhos que podem ser motivados por essa tese.

CAPÍTULO 1

1. FUNDAMENTAÇÃO TEÓRICA

1.1. CHALCONAS

As chalconas constituem um grupo de compostos com esqueleto estrutural comum formado por 1,3-difenil-2-propen-1-ona, uma fenilestiril cetona que existe como isômeros *cis* e *trans*, sendo o isômero *trans* termodinamicamente mais estável (Figura 1). A palavra “chalcona” deriva do Grego “*chalcos*” que significa bronze, por causa da cor da maioria das chalconas naturais que são amarelas, laranjas ou marrons. Seu formato geral contém dois anéis aromáticos que estão ligados por meio de uma carbonila e outros dois átomos de carbono α , β -insaturados, formando uma porção enona. O anel aromático ligado diretamente à carbonila é chamado de A e o outro é chamado de B (GOMES et al., 2017; RANI et al., 2019; SINGH et al., 2020; ZHUANG et al., 2017).

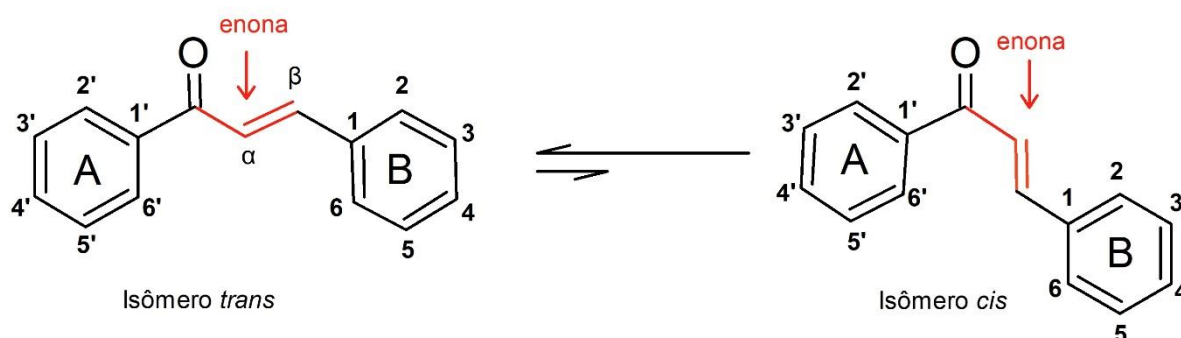


Figura 1. Esqueleto estrutural comum das chalconas *cis* e *trans* com numeração dos carbonos.

A síntese de chalconas em laboratório remonta ao século XIX e o isolamento da primeira chalcona natural só ocorrera após o ano de 1910. As chalconas naturais são encontradas numa ampla variedade de árvores e plantas, principalmente como pigmentos das pétalas, mas também nas cascas, folhas, frutos e raízes (NI; MENG; SIKORSKI, 2004). Normalmente as chalconas naturais são sólidos cristalinos e possuem substituintes nos anéis tais como hidroxilas, metilas e prenilas, mas também existem na forma de dihidrochalconas, dímeros (bichalconas) e glicosídeos (RAMMOHAN et al., 2020).

1.1.1. Síntese química das chalconas

As chalconas são normalmente preparadas através de reações de condensação catalisada por ácidos ou bases. Apesar da simplicidade na obtenção da estrutura básica, diversas técnicas e procedimentos vem sendo desenvolvidos ao longo dos anos. Além disso, sua estrutura privilegiada permite uma variedade de substituições o que possibilita a produção de diferentes compostos. Aqui serão retratadas apenas as principais estratégias na síntese do esqueleto básico.

1.1.1.1. Condensação de Claisen–Schmidt

Este tipo de reação recebe o nome dos dois cientistas que primeiro demonstraram a condensação da acetona com o benzaldeído (Figura 2), e da acetona com o aldeído furfural, na presença de um catalizador (CLAISEN; CLAPARÈDE, 1881; SCHMIDT, 1881). Esse catalizador tanto pode ser uma base forte, quando a chalcona é gerada via desidratação aldólica envolvendo um enolato sobre uma carbonila, quanto um ácido forte, quando a chalcona é gerada via mecanismo enol. O rendimento desse tipo de reação depende dos reagentes e do catalizador, podendo variar de 10% até quase 100%. As bases normalmente utilizadas são terc-butóxido de potássio, hidróxido de potássio e hidróxido de sódio dissolvidos em metanol ou etanol em temperatura ambiente. Em algumas situações pode ser necessário o aumento da temperatura para melhorar o rendimento da reação (ZHUANG et al., 2017).

A forma mais comum de realizar essa reação é em fase líquida utilizando solventes polares, porém, a condensação em fase sólida também pode ser realizada em alguns casos. Nessa situação a cetona liga-se à uma resina, e então é tratada com o benzaldeído. Finalmente as chalconas são liberadas da resina por tratamento com ácido trifluoroacético (RAMMOHAN et al., 2020). Existem formas alternativas de obtenção de chalconas por condensação de Claisen–Schmidt. Algumas em condições de ausência de solvente tais como o método da moagem (RATEB; ZOHD, 2009), método da irradiação por micro-ondas (KAKATI; SARMA, 2011) e o método da irradiação com ultrassom (CHTOUROU et al., 2010). Essas técnicas possuem uma abordagem sustentável por não utilizarem solvente e por apresentarem bons rendimentos a temperatura ambiente.

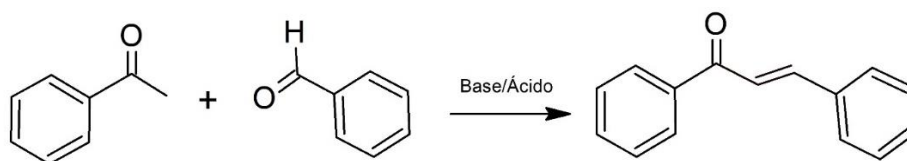


Figura 2. Condensação de Claisen-Schmidt de chalconas.

1.1.1.2. Reações de acoplamento cruzado

Muitas vezes a condensação de Claisen–Schmidt resulta numa mistura complexa de subprodutos que tornam custoso a purificação das chalconas. Nesses casos existem alternativas já conhecidas para a síntese das chalconas como as reações de acoplamento cruzado.

1.1.1.2.1. Acoplamento de Suzuki

Essa reação foi descrita pela primeira vez em 1979 por Akira Suzuki. Trata de um tipo de acoplamento cruzado utilizando catalizadores de paládio que produz a formação de ligações carbono-carbono bastante poderosas. Eddarir e colaboradores foram os primeiros a utilizar esse tipo de reação para obtenção de chalconas. Nesse trabalho são relatadas duas rotas para obtenção das chalconas (Figura 3), uma envolvendo o acoplamento entre ácidos cinâmicos ativados e ácidos fenilborônicos, e outra compreendendo o acoplamento entre ácidos benzóicos ativados e ácidos fenilvinilborônicos (EDDARIR et al., 2003).

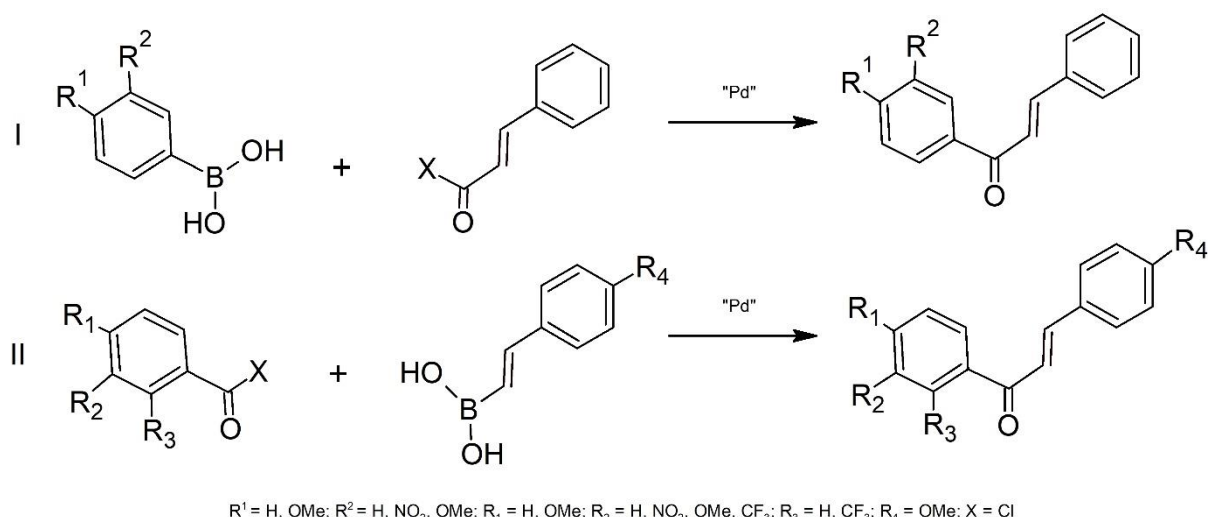


Figura 3. Esquemas para obtenção de chalconas através do acoplamento de Suzuki sob diferentes condições e diversos catalizadores de paládio relatadas por Eddarir et al. (2003).

1.1.1.2.2. Acoplamento de Heck

O acoplamento de Heck consiste na reação entre halogenetos de arila, benzila e estirila com compostos olefínicos na presença de uma amina impedida² e uma certa quantidade de catalizador de paládio para formar derivados vinílicos nos quais o grupo aril, benzil ou estiril substitui um hidrogênio vinílico da olefina original. A temperatura de reação gira em torno de 100 °C e os rendimentos são geralmente bons (HECK; NOLLEY, 1972). Estruturalmente as chalconas são estilbenos³, e podem também ser obtidas de forma eficiente pela reação clássica de Heck. Como mostrado na Figura 4, um ácido arilborônico ou um iodeto de arila pode reagir com uma cetona insaturada na presença de uma base e um catalisador de paládio obtendo o esqueleto básico das chalconas (RAMMOHAN et al., 2020).

²Aminas terciárias que possuem o par de elétrons não compartilhado do átomo de nitrogênio impedido estericamente.

³Hidrocarboneto consistindo de uma ligação dupla trans eteno substituída com um grupo fenila em ambos os átomos de carbono da ligação dupla.

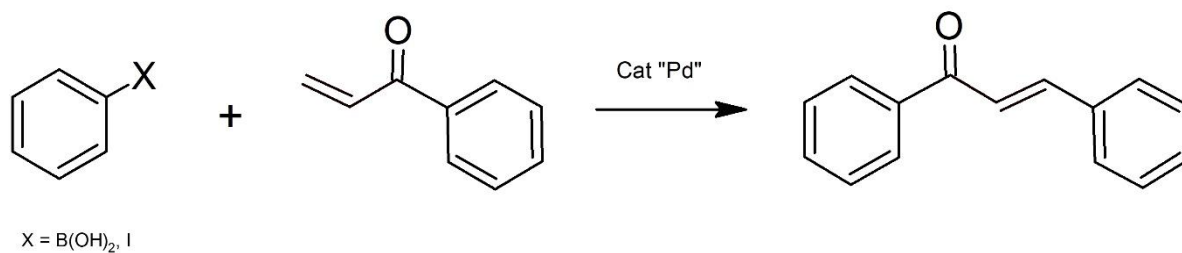


Figura 4. Síntese de chalconas através do acoplamento de Heck.

1.1.1.2.3. Reação de Wittig

Ramirez e Dershowitz obtiveram pela primeira vez a benzalacetofenona (1,3-difenil-2-propen-1-ona) a partir de trifenilbenzoilmetileno fosforano e benzaldeído (Figura 5) dissolvidos em tetrahydrofurano ao longo de 30 h sob refluxo, como também ao longo de 3 dias em benzeno sob refluxo, obtendo rendimentos moderados em ambas. Essas reações foram interpretadas como sendo análogas à olefinação de Wittig (RAMIREZ; DERSHOWITZ, 1957). Mais tarde a cinética da reação bem como seu rendimento foram bastante melhorados utilizando irradiação de micro-ondas (XU; CHEN; HUANG, 1995).

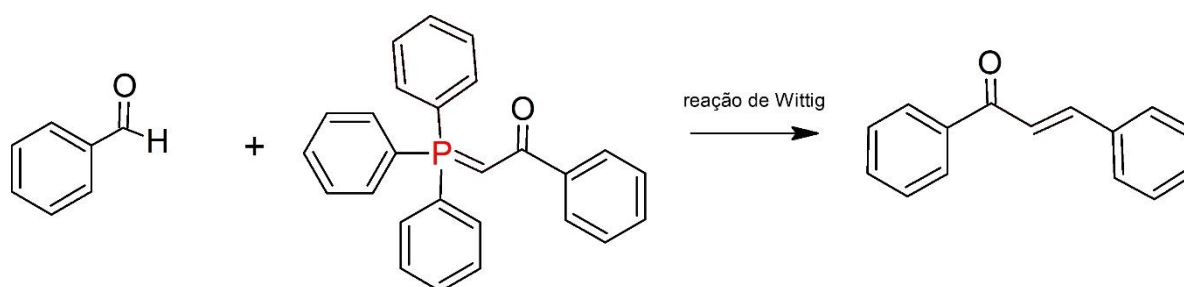


Figura 5. Reação de Wittig para síntese de chalcona.

1.1.1.2.4. Olefinação de Julia-Kocienski

Apesar de haver certa imprevisibilidade no resultado estereoquímico das transformações, a reação de Julia-Kocienski tornou-se indispensável como alternativa para olefinação de compostos orgânicos (AÍSSA, 2009). A reação original publicada por Marc Julia em 1973 é baseada num processo de eliminação redutiva em duas etapas de β -aciloxi-aryl sulfonas (JULIA; PARIS, 1973). Já a reação de Julia modificada, conhecida como Julia-Kocienski, envolve o acoplamento direto de heteroarilsulfonas com compostos de carbonila em etapa única (BAUDIN et al., 1991). Então esse tipo de reação se tornou bastante versátil e foi sendo utilizada para a síntese de diversos compostos biologicamente ativos, até que Kumar e

colaboradores foram os pioneiros na produção de diversas chalconas e flavonoides através da olefinação de Julia-Kocienski com excelentes rendimentos. Eles desenvolveram um novo reagente (2- (Benzo[d]tiazol-2-il-sulfonil)-1-feniletanonas) capaz de reagir com aldeídos na presença de uma base para formar chalconas (Figura 6) (KUMAR et al., 2010).

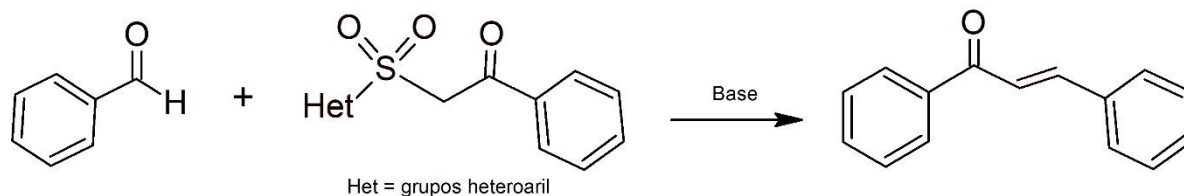


Figura 6. Olefinação Julia-Kocienski para síntese de chalconas.

1.1.1.3. Outras estratégias para síntese de chalconas

Existem outras estratégias para a síntese de chalconas. Dentre elas destacam-se: a acilação de Friedel-Crafts, onde ocorre a acilação de um éter aromático com cloreto de cinamoil na presença de um ácido de Lewis forte (*e.g.* cloreto de alumínio); e o rearranjo do fenilcinamato de Photo-Fries, que é a fotossíntese de chalconas onde acontece o rearranjo de um fenilcinamato para uma hidroxi-aril cetona na presença de ácidos de Lewis (Figura 7) (ZHUANG et al., 2017).

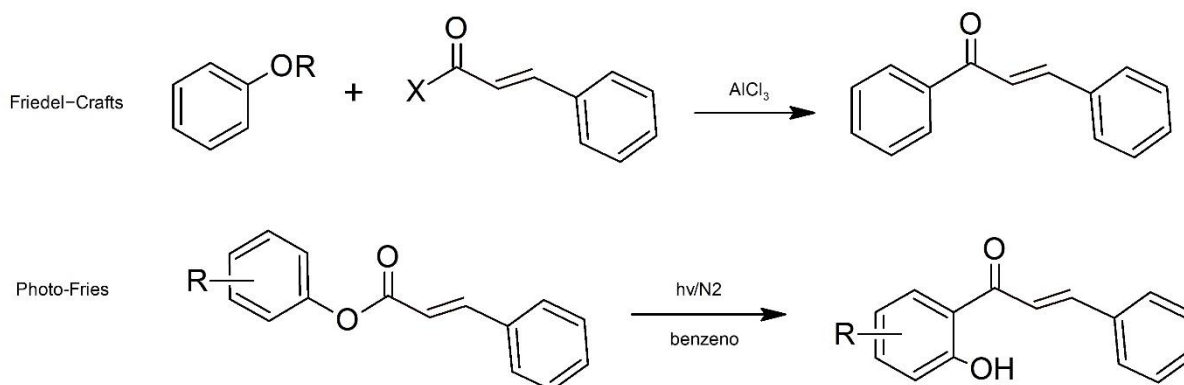


Figura 7. Síntese de chalconas pelas reações Friedel-Crafts e Photo-Fries.

1.2. DERIVADOS DA DIBENZALACETONA

Os estudos com chalconas evoluíram e as modificações estruturais foram acontecendo com o objetivo de potencializar eventuais propriedades. Dentre as principais modificações observadas destaca-se o aumento do sistema conjugado de duplas ligações das chalconas resultando em derivados que, como já relatado na introdução deste trabalho, podem ser

denominados como derivados da dibenzalacetona, bis-chalconas ou simplesmente derivados das chalconas. Outra modificação que merece ser ressaltada é a adição de anéis heterocíclicos na estrutura. Esses compostos podem ter uma estrutura análoga à curcumina, portanto, não raro, encontra-se também a denominação de curcuminóide em alguns trabalhos.

As chalconas e seus derivados apresentam uma extensa deslocalização de elétrons devido a presença de grupos doadores de elétrons fortes (D) e aceitadores de elétrons fortes (A) ligados por pontes de elétrons π . As chalconas simples, com apenas uma porção enona, geralmente apresentam configuração assimétrica do tipo D- π -A, já os derivados da dibenzalacetona, devido sua porção dienona, possuem uma deslocalização eletrônica ainda maior com configuração típica D- π -A- π -D. Além da presença de grupos A e D, a planaridade desse tipo de molécula também ajuda na deslocalização eletrônica favorecendo ainda mais na polarizabilidade e influenciando fortemente algumas propriedades desses compostos (REDDY et al., 2014).

Os derivados da dibenzalacetona são cromóforos que podem ser obtidas por uma dupla reação de condensação contendo uma cetona com duas regiões reativas α e α' com dois equivalentes de um aldeído (NOHUT MAŞLAKCI et al., 2018).

A Figura 8 mostra o mecanismo de reação de dupla condensação de Claisen–Schmidt com catalisador básico via desidratação aldólica, em que a acetona (propanona) reage com dois equivalentes de um aldeído aromático para produzir os derivados da dibenzalacetona.

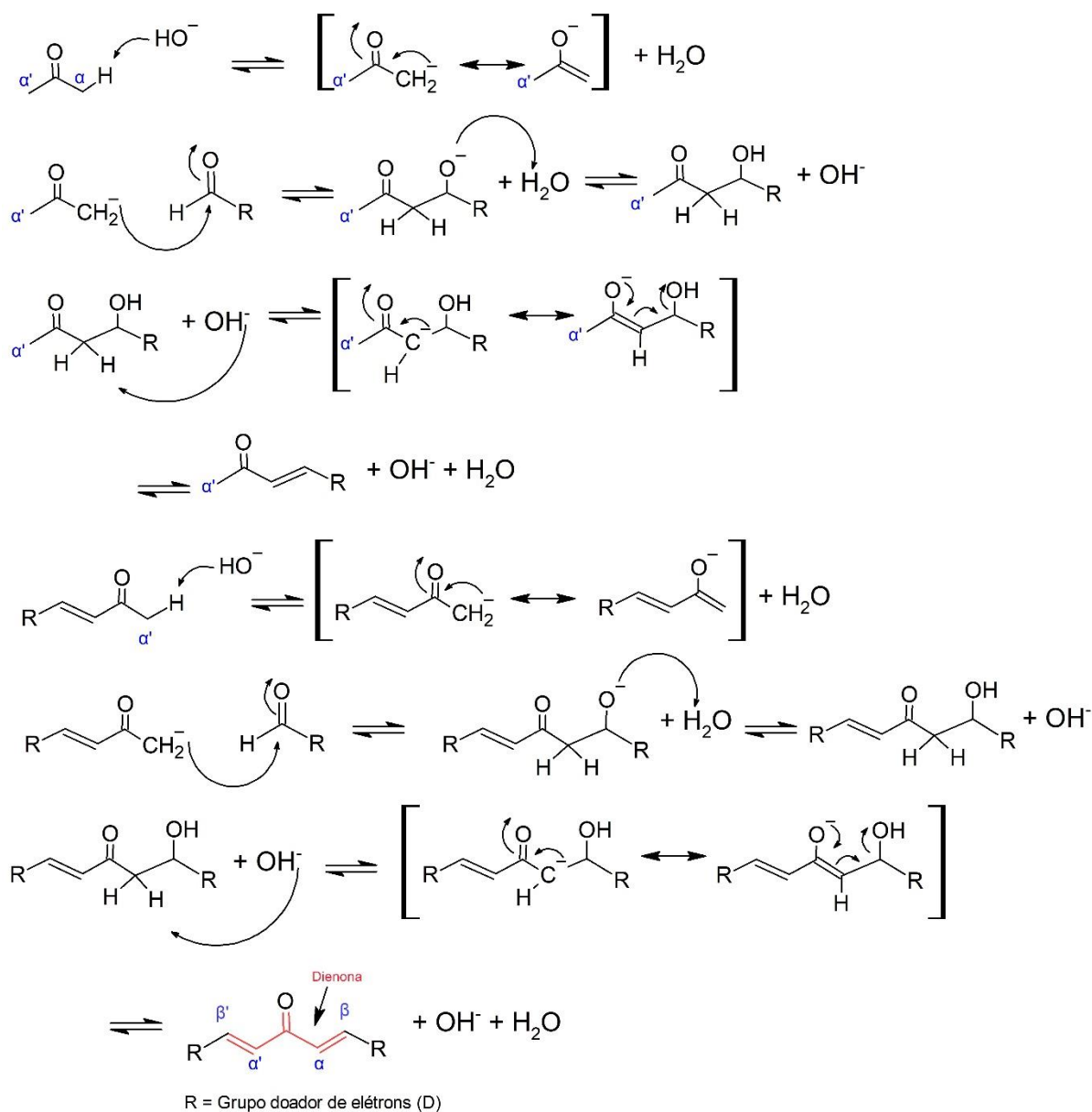


Figura 8. Mecanismo de reação para síntese dos derivados da dibenzalacetona utilizando catalisador básico. (D) normalmente é um anel aromático substituído ou não, ou um anel heterocíclico.

1.3. ALGUMAS PROPRIEDADES DAS CHALCONAS E DERIVADOS

Em geral as chalconas são mais estáveis quimicamente que os flavonóides e isoflavonóides. São solúveis em álcoois, soluções aquosas ácidas e básicas bem como solventes orgânicos tais como acetona, clorofórmio e diclorometano. Em soluções alcalinas elas apresentam uma coloração vermelha ou laranja. Chalconas são convertidas em flavonóis pela sua oxidação utilizando peróxido de hidrogênio em solução metanólica de hidróxido de sódio. As flavononas podem ser facilmente obtidas através da ciclização de chalconas tratadas com

ácido bromídrico em ácido acético glacial. Nesta isomerização pode ocorrer desmetilação parcial e desbenzilação. As chalconas quando aquecidas com traços de iodo em dimetilsulfóxido produzem as flavonas correspondentes (RAMMOHAN et al., 2020).

Devido ao seu sistema de duplas ligações conjugadas, e dependendo dos substituintes nos anéis benzeno, as chalconas podem apresentar propriedades fluorescentes. Compostos com propriedades fluorescentes despertam bastante interesse em função das diversas aplicações que eles podem ter (*e.g.* sondas químicas, materiais eletrocromicos, corantes fluorescentes, aditivos para células solares, marcadores no diagnóstico e na ação de fármacos). Os principais parâmetros envolvidos no estudo da fluorescência e que estão relacionados com a densidade eletrônica ao redor da molécula são: Os comprimentos de onda de absorção ($Abs \lambda_{nm}$) e emissão ($Emi \lambda_{nm}$), o coeficiente de extinção (ϵ) e o rendimento quântico (ϕ) (RAMMOHAN et al., 2020; ZHUANG et al., 2017).

1.4. ATIVIDADES BIOLÓGICAS DAS CHALCONAS E DERIVADOS

Como já mencionado, as chalconas e seus derivados são uma classe de compostos que possuem amplo espectro de atividades biológicas com reconhecido potencial farmacológico. A Figura 9 mostra algumas das atividades biológicas das chalconas e seus derivados. Os prováveis fatores que tornam esses compostos tão versáteis biologicamente já foram mencionados na parte introdutória desse trabalho. A seguir discorreremos sobre três das principais atividades biológicas das chalconas e derivados: Atividade anticâncer, atividade antibacteriana e atividade ansiolítica.

1.4.1. Atividade anticâncer

Câncer é uma doença com elevado risco de morte caracterizada pela multiplicação não controlada de células. Atualmente o câncer é considerado um dos principais problemas de saúde pública no mundo, aparecendo como a quarta principal causa de morte antes dos 70 anos de idade (BRAY et al., 2018). No Brasil, estima-se que a incidência de câncer no triênio 2020 – 2022 deva chegar a 625 mil novos casos por ano. O câncer de pele não melanoma será o mais incidente, seguido de mama, próstata, cólon, reto, pulmão e estômago (MINISTÉRIO DA SAÚDE - INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2019). Embora os tratamentos químicos para diversos tipos de câncer tenham evoluído muito, suas limitações e efeitos colaterais ainda motivam muitas pesquisas na busca de novos agentes anticâncer que sejam mais seguros e sobretudo mais seletivos (SINGH; ANAND; KUMAR, 2014).

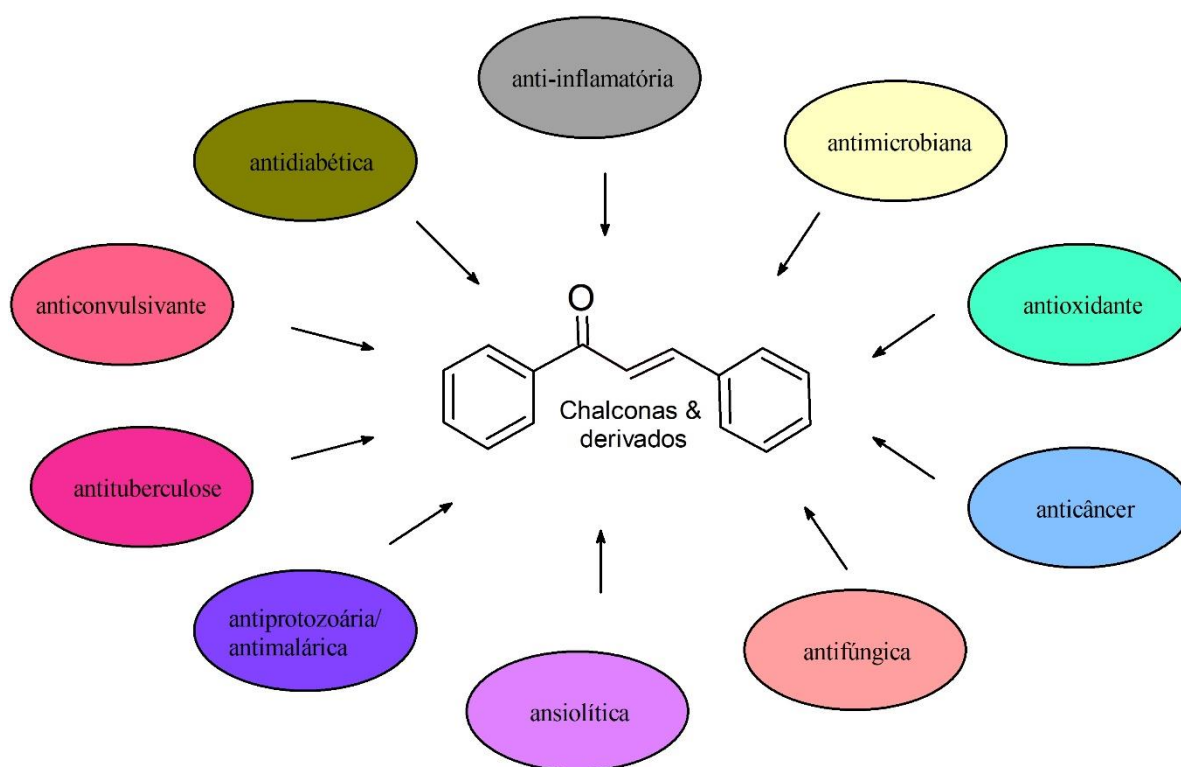


Figura 9. Principais atividades biológicas identificadas em chalconas e seus derivados.

Muitas chalconas, tanto de origem natural quanto sintética, bem como seus derivados, são frequentemente identificados com efeito anticâncer em diversas linhagens de células. A manipulação dos seus anéis aromáticos, por exemplo, a substituição destes por anéis heterocíclicos ou por derivados do ferroceno, bem como a conjugação com outras estruturas farmacologicamente ativas têm sido reportadas como estratégias que aumentam as propriedades anticâncer das chalconas (RANI et al., 2019).

Os primeiros relatos de atividade anticâncer das chalconas apareceram na década de 70, onde uma série de chalconas cloro/nitro substituídas apresentaram atividade citotóxica em várias células tumorais (DORE; VIEL, 1974). Contudo essa classe de compostos só ganhou notoriedade cerca de 20 anos depois, e desde então, só cresce o interesse científico nas chalconas como inibidoras do crescimento de células tumorais (DUCKI, 2007).

Diversas chalconas possuem efeito citotóxico contra diferentes linhagens de células tumorais, porém a forma como as células tumorais são combatidas é bastante variado. As chalconas podem atuar impedindo a proliferação celular inibindo a catepsina K, uma enzima da classe das proteases de cisteína lisossomal que se expressa significativamente no câncer de mama humano e quando suprimida ocasiona a redução da propagação do tumor; inibindo a tubulina, proteína globular responsável pela mitose, motilidade, arquitetura do citoesqueleto,

transporte intracelular e secreção; inibindo a fosfatase CDC25B que desempenha papel importante na progressão do ciclo celular; inibindo a expressão da topoisomerase que ocasiona a parada do ciclo celular; inibindo glicoproteínas autócrinas e parácrinas (Wnt) que são importante no desenvolvimento do tecido mamário e na carcinogênese; e inibindo o receptor de tirosina quinases ErbB que desempenham um papel importante na fisiologia normal das células como também no câncer. Além disso as chalconas podem atuar como inibidores do meio hormonal, inibindo as 5 α -redutases, a translocação do receptor andrógeno e a síntese dos hormônios sexuais, todos esses relacionados ao câncer de próstata; como inibidores da desacetilação proteica e inibidores da degradação da proteína p53, também chamada de supressora tumoral p53, que é uma proteína que regula o ciclo celular e atua como supressor de tumor, prevenindo o câncer. A p53 tem um papel importante na atividade anticâncer e medeia o reparo do DNA, apoptose, inibição da angiogênese, estabilidade genômica e ativação gênica. A proteína p53 é degradado por vários compostos, tais como o MDM2 e o Sirtuin-1. Por fim, também existem chalconas que atuam na inibição da via de sinalização JAK/STAT bem como na inibição da angiogênese, que é o processo de vascularização dos tumores (MAHAPATRA; BHARTI; ASATI, 2015).

Quando não atuam diretamente na redução do crescimento das células malignas, as chalconas também podem agir como moduladoras do efeito de outras drogas. As células humanas naturalmente possuem mecanismos de exportação para uma grande variedade de substratos, incluindo produtos metabólicos, drogas, toxinas, lipídios endógenos, peptídeos, nucleotídeos, esteróis etc. Sendo assim, as células tumorais podem adquirir resistência aos quimioterápicos através do efluxo dessas drogas diminuindo assim sua eficácia sobre o tumor. Para que ocorra esse processo são necessárias proteínas transmembranares que expulsam os fármacos através da membrana celular. Dentre os principais tipos de mecanismos de efluxo encontra-se a família transportadores de cassetes de ligação de ATP (ABC). Nessa família são conhecidas até agora três tipos de proteínas de efluxo que parecem ser responsáveis pela resistência a múltiplas drogas em células humanas: a glicoproteína-p (ABCB1, MDR1), as proteínas de resistência do câncer de mama (BCRP, ABCG2, MXR, ABCP) e a proteína 1 associada à resistência a múltiplas drogas (MRP1, ABCC1). Nesse caso, as chalconas não atuam diretamente na diminuição do câncer, elas atuam de forma a impedir que as células malignas sejam resistentes à quimioterapia. Já foram identificadas diversas chalconas eficazes na inibição da ABCG2 humana, na BCRP e nas glicoproteínas-p ABCB1 e MDR1 (MAHAPATRA; BHARTI; ASATI, 2015).

Recentemente, uma série de chalconas foi sintetizada e testada *in vitro* contra uma linhagem de câncer de fígado (HepG2), todas demonstraram significativa atividade citotóxica, no entanto aquelas com anéis substituídos por cloro ou hidroxila na posição *para* detiveram um maior potencial (PATIL; KHAN; ZANGADE, 2020). Já as bis-chalconas 2,6-di(4-fluorobenzilideno)ciclohexanona e (2E,6E)-2,6-bis(4-trifluorometilfenilmetilideno)ciclohexanona obtiveram resultados satisfatórios contra as linhagens PC3 (câncer de próstata), HeLa (câncer cervical), MCF-7 (câncer de mama) e 3T3 (fibroblasto normal), entretanto nenhuma delas obteve IC₅₀ menor que as drogas padrão doxorrubicina e cicloheximida (LOTFY et al., 2017). Em outro trabalho, cinco compostos também derivadas da benzilidina ciclohexanona, com estrutura análoga à cúrcuma, inibiram o crescimento de células cancerosas de adenocarcinoma gástrico (AGS) e carcinoma de células escamosas do esôfago (KYSE30) através da indução de apoptose e parada do ciclo celular na fase G1. Todos os compostos obtiveram resultados melhores que a cúrcuma sugerindo que os curcuminóides menos polares possuem uma maior citotoxicidade *in vitro* (ALIBEIKI et al., 2017).

Bis-chalconas contendo uma porção terpenóide também se mostraram ativas na antiproliferação das linhagens SF-295 (sistema nervoso central), HCT-116 (câncer de cólon) e OVCAR-8 (câncer de mama). Esse estudo revelou que os melhores resultados foram obtidos contra a células HCT-116 e que a substituição de um grupo nitro em qualquer posição do anel benzeno foi decisivo no aumento da atividade citotóxica, enquanto que o tipo de ciclohexeno, encontrado na porção terpeno, não afeta significativamente essa atividade (LIMA et al., 2019). Outro composto desse tipo, com um cloro na posição *para* do anel aromático, foi capaz de inibir o crescimento do sarcoma S-180 pela ação antiangiogênica e anti-inflamatória (LEMES et al., 2018). Derivados de chalconas também já foram identificados como sendo inibidores específicos das proteínas transportadoras ABCG2 e ABCB1 superexpressadas em algumas linhagens de câncer resistentes a múltiplas drogas (CAI et al., 2020).

1.4.2. Atividade antibacteriana

Embora o advento dos antibióticos tenha proporcionado uma revolução no século XX, quase todos os grupos importantes de antibióticos vêm perdendo eficácia por causa do aumento da resistência microbiana. O uso desenfreado, o mau uso e o abuso dessas drogas vêm acelerando a evolução desses organismos, resultando numa maior ocorrência de complicações em infecções que outrora seriam facilmente tratadas. Muitos organismos responsáveis por infecções comuns (*e.g.* infecções do trato urinário, pneumonia e infecções da corrente

sanguínea) estão cada vez mais resistindo às terapias com antibióticos convencionais. Daí essa situação apresenta-se com um gravíssimo problema de saúde pública em todo o mundo. Desde então, o desenvolvimento de novos antibióticos tem se configurado como uma das formas de enfrentar tal situação. Porém, ele também tem sido acompanhado pelo aumento constante de cepas bacterianas resistentes a antibióticos e pela diversidade de mecanismos usados pelas bactérias para superar o efeito letal desses compostos (BALOUIRI; SADIKI; IBNSOUDA, 2016; COSTA S. S. et al., 2013; ZHANG et al., 2018).

Nesse contexto as chalconas e seus derivados têm atraído muito interesse como agentes antibacterianos, que se reflete na vasta literatura científica produzida nas últimas décadas, porém os mecanismos de ação ainda não estão completamente compreendidos. Os principais mecanismos de ação das chalconas como antibacterianos já reportados na literatura são pela inibição da via biossintética de ácidos graxos tipo II (FASII), pela interferência na replicação do DNA bacteriano, pela inibição da proteína FtsZ, pela inibição dos fatores de virulência ou pela inibição das proteínas tirosina fosfatases. Além disto, as chalconas podem potencializar a ação de antibióticos pela inibição de mecanismos de resistência, tais como as bombas de efluxo (DAN; DAI, 2020).

1.4.2.1. Inibição da via biossintética de ácidos graxos tipo II (FASII)

Os ácidos graxos são estruturas simples que desempenham um papel fundamental nas células. Eles são o principal constituinte da membrana celular bem como intermediário no metabolismo bacteriano. O sistema FASII existente nas bactérias usa uma série de proteínas monofuncionais que são codificadas por genes discretos para catalisar cada etapa da via biossintética. Esse sistema é capaz de produzir diversos produtos para o metabolismo celular tais como ácidos graxos insaturados, ácidos graxos de cadeia *iso* e *anteiso* ramificada e ácidos graxos hidroxilados. Ácidos micólicos, produzidos pela FASII, são longos ácidos graxos que desempenham uma função essencial na arquitetura e permeabilidade da parede celular de micobactérias. Portanto o sistema FASII tem sido amplamente explorado como alvo para a ação antibacteriana (WANG; MA, 2013).

Em estudo recente, três derivados de chalconas tiveram excelentes resultados na ação contra uma cepa de *M. tuberculosis* (H₃₇Rv) com modo de ligação semelhantes aos inibidores FASII. Nesse mesmo estudo, ficou demonstrado também que esses compostos não foram tóxicos aos megacariócitos humanos nem às células B murinas (RAMESH et al., 2020). Antes disso, em 2012, uma série de chalconas e derivados mostraram inibição da FASII em

micobactérias variando de moderada a boa, tendo como melhor resultado uma inibição do crescimento microbiano de 88% em uma concentração da chalcona de 100 μ M (ANAND et al., 2012).

1.4.2.2. Inibição da replicação do DNA bacteriano

A enzima DNA girase é uma topoisomerase do tipo II presente em células procarióticas que desempenham papel essencial na replicação do DNA. Como a DNA girase bacteriana está ausente em humanos, ela é um alvo atraente para a ação antibacteriana. As fluoroquinolonas são uma classe de antibióticos de muito sucesso com esse tipo de ação (DAN; DAI, 2020).

Em 2014, três chalconas à base de quinolonas se mostraram significativamente ativas ($IC_{50} \leq 0,125$ mg/mL) na ação antibacteriana em cepas de *S. aureus*. Nesse estudo ficou demonstrado que duas dessas três chalconas inibiram fortemente a DNA girase da *S. aureus*, o que sugere ter sido esse o mecanismo de ação desses compostos (ABDULLAH et al., 2014).

1.4.2.3. Inibição da proteína FtsZ

O processo de divisão celular é sem dúvida vital à sobrevivência e crescimento de organismos procarióticos. Em bactérias e *archaea* esse processo geralmente ocorre por meio de um mecanismo denominado "fissão binária", em que um septo é colocado numa posição de divisão, e uma célula separe-se em duas novas células filhas contendo uma cópia dos cromossomos e outros componentes necessários para a viabilidade e funcionalidade celular. Em *E. coli*, esse processo é comandado por pelo menos 12 proteínas que atuam no sítio da divisão. Entre elas destaca-se a proteína filamentosamente sensível a temperatura mutante Z (FtsZ) que exercem uma função central e tem sido a mais estudada entre elas. A FtsZ sofre montagem dinâmica em um anel contrátil (anel Z) na célula intermediária, que marca o local do futuro septo. O anel Z consiste em protofilamentos de subunidades da FtsZ polimerizados cuja formação é o primeiro passo conhecido na divisão do citoplasma. A montagem da proteína FtsZ no anel é necessária para o recrutamento e interação entre as outras proteínas envolvidas na divisão celular. O *ftsZ* é o gene responsável pela transcrição dessa proteína e está presente em quase todas as espécies de bactérias. A proteína FtsZ é homóloga da tubulina eucariótica; a estrutura tridimensional de ambas é notadamente semelhante, apesar do baixo nível de compatibilidade dos aminoácidos. Isto posto, a mesma estratégia utilizada na terapia contra o câncer pela inibição da tubulina, pode ser usada como base no desenvolvimento de inibidores da FtsZ (BOBEREK et al., 2012; TRIPATHY; SAHU, 2019).

O cinamaldeído foi identificado como sendo capaz de ligar-se a proteína FtsZ, perturbando a formação do anel Z e inibindo sua dinâmica de montagem. Isto sugere que o cinamaldeído, uma pequena molécula de origem vegetal, é um composto com potencial para o *design* de drogas que atuem como agente anti-FtsZ (DOMADIA et al., 2007). A partir daí uma série de chalconas derivadas do cinamaldeído foram sintetizadas e exibiram atividade antibacteriana com melhor eficiência contra *S. aureus* ATCC25923, especialmente os compostos 3, 8 e 10 que exerceram atividade superior ou comparável a todos os medicamentos de referência. Esses compostos também mostraram um melhor resultado na atividade inibidora da divisão celular contra *S. aureus* ATCC25923 através do mecanismo anti-FtsZ, confirmados pelos ensaios de dispersão de luz e de GTPase (LI et al., 2015).

1.4.2.4. Inibição do fator de virulência

As bactérias compreendem um grande grupo de espécies capazes de infectar animais e plantas. A grande maioria desses patógenos possui uma capacidade limitada de infectar espécies hospedeiras, e são os chamados fatores de virulência, envolvidos nessa interação, que determinam o grau de patogenicidade dos diferentes agentes infecciosos. Fatores de virulência estão frequentemente envolvidos na interação direta com os tecidos do hospedeiro ou na ocultação da superfície bacteriana frente aos mecanismos de defesa do organismo infectado. Atenuar esses fatores é uma das abordagens utilizadas atualmente no desenvolvimento de novos agentes antimicrobianos (WU; WANG; JENNINGS, 2008).

A pouco tempo, quatro compostos contendo esqueleto típico das chalconas foram sintetizados e modularam significativamente alguns dos mais importantes fatores de virulência quando aplicados em concentrações subinibitórias; como a produção de biofilme e motilidade de *A. baumannii* e *P. aeruginosa*, e a produção de piocianina em *P. aeruginosa* (UŠJAK et al., 2019). Previamente, a chalcona 1,3-difenil-2-propen-1-ona foi capaz de inibir substancialmente a atividade da SrtA (enzima responsável pela ancoragem das proteínas de superfície relacionadas à virulência) com um IC₅₀ de 53,15 µM, e a atividade da alpha hemolisina Hla (citotoxina formadora de poros) com um IC₅₀ de 17,63 µM. Além disso, demonstrou reduzir a proteína A (SpA) em bactérias intactas, a ligação à fibronectina e a formação de biofilme (ZHANG et al., 2017).

1.4.2.5. Inibição das proteínas tirosina fosfatases (PTP)

As proteínas tirosina fosfatases (PTP), junto com as proteínas tirosina quinases (PTK), controlam o nível de fosforilação dos seus substratos alvo. Elas cumprem um papel crucial na

transdução de sinal de muitos eventos celulares, incluindo resposta imune, metabolismo, crescimento e transcrição gênica. Essas proteínas são secretas na célula hospedeira e atenuam as defesas imunológicas (DAN; DAI, 2020). Chalconas contendo grupos 1-naftil ou 2-naftil no lugar do anel B do esqueleto básico das chalconas foram ativas na inibição da PTP A em cepas de *M. tuberculosis* (CHIARADIA et al., 2008). Mais tarde, quatro derivados de chalconas inibiram a proteína YopH, uma PTP importante na patogênese do gênero *Yersinia*. Essas bactérias estão relacionadas principalmente com a ocorrência de síndromes gastrointestinais podendo levar a sepse (MARTINS et al., 2013).

1.4.2.6. Inibição das bombas de efluxo

Bombas de efluxo são proteínas transmembranares capazes de expulsar substratos do interior da célula para o meio externo. Esse processo de eliminação de compostos é importante na resistência bacteriana aos antibióticos, tanto intrínseca quanto adquirida. As bombas de efluxo possuem duas formas distintas pela qual obtêm energia; as bombas de efluxo primárias retiram energia da hidrólise do ATP (trifosfato de adenosina), enquanto as secundárias retiram energia de gradientes químicos formados por íons H^+ ou Na^+ . De acordo com a estrutura e a forma de obtenção de energia, as bombas de efluxo em procariontes Gram-positivas e Gram-negativas são divididas em: Superfamília dos Facilitadores Majoritários - MFS; Pequena Família de Resistência a Múltiplas Drogas – SMR; Família de Extrusão de Multidrogas e Compostos Tóxicos – MATE; Família Resistência à Nodulação Divisão Celular - RND; Superfamília de Cassetes de Ligação de ATP – ABC (SHARMA; GUPTA; PATHANIA, 2019).

S. aureus são cocos Gram-positivos que exibem uma grande variedade de mecanismos de resistência a antibióticos e por isso são responsáveis por muitos casos de infecções fatais. Nessa espécie já foram identificados 14 tipos de bombas de efluxo de múltiplas drogas codificadas tanto em cromossomos quanto nos plasmídeos. Dentre elas, as mais estudadas são as da família MSF tendo a bomba de efluxo NorA como exemplo mais representativo. Cepas de *S. aureus* resistentes a metilina geralmente superexpressam essa bomba de efluxo. Outra bomba de efluxo, a MepA, pertencente à família MATE, também é expressada por cepas mutantes de *S. aureus* (LAMUT et al., 2019).

A inibição das bombas de efluxo pode ser alcançada por várias estratégias; pela redução da expressão do gene da bomba; pela interrupção da montagem da bomba; pela redução da ligação do substrato por meios competitivos ou não competitivos; e pela perturbação da fonte

de energia necessária. Compostos que são capazes de realizar tais inibições são chamadas de inibidores de bomba de efluxo – EPI (SCHINDLER; JACINTO; KAATZ, 2013).

1.4.2.6.1. NorA

NorA é um sistema de efluxo resistente à fluoroquinolonas que é capaz de expulsar uma série de antibióticos e outras substâncias tais como norfloxacino (NOR), ciprofloxacino (CIP), brometo de etídio (EtBr) e compostos quaternários de amônia. O gene cromossomal que codifica a bomba de efluxo NorA é chamado de *norA*, foi identificado pela primeira vez em 1986 em uma cepa resistente a fluoroquinolonas. A proteína da NorA possui 388 aminoácidos compreendidos em 12 segmentos transmembranares e compartilha 44% de semelhança com a bomba de efluxo de múltiplas drogas Bmr do *B. subtilis* e 24% com a bomba de efluxo de tetraciclina Tet(A) da *E. coli*. A NorA usa a força motriz de prótons para energizar o transporte de compostos antimicrobianos através da membrana celular, por meio de um mecanismo de antiporta H^+ /droga (Figura 10). Nele ocorre o transporte do substrato e do co-substrato em direções opostas (COSTA S. S. et al., 2013; ZÁRATE et al., 2019).

Estudos demonstraram que o efluxo de EtBr e outros antibióticos, realizadas pela NorA, é sensível à clorpromazina (CPZ) e ao carbonil cianeto m-clorofenilhidrazona (CCCP). Esses compostos inibem o efluxo da NorA diminuindo a resistência aos antibióticos (KAATZ et al., 2003; MALLÉA et al., 2002; PAGÈS; MASI; BARBE, 2005; SABATINI et al., 2008).

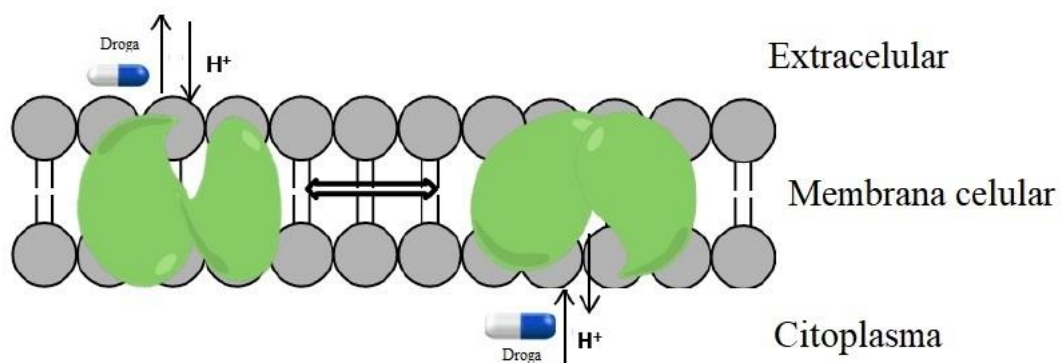


Figura 10. Efluxo de múltiplas drogas por meio do mecanismo de antiporta H^+ /droga encontrado em bombas de efluxo do tipo MFS. Adaptado de Zárate et al. (2019).

Alguns estudos podem ser encontrados na literatura relacionando especificamente a inibição da bomba de efluxo NorA com chalconas e derivados. Em artigo recente, chalconas naturais isoladas das flores de *A. brachypoda*, se mostraram capazes de inibir a bomba de efluxo

NorA em cepas de *S. aureus* quando usadas em combinação com norfloxacino (REZENDE-JÚNIOR et al., 2020). Em outro estudo, curcuminóide sintético (1E,4E)-1,5-Di(tiofen-2-il)penta-1,4-dien-3-ona, contendo dois anéis tiofeno, atuou como um EPI da bomba NorA também em combinação com o norfloxacino (OLIVEIRA et al., 2020). Alguns anos antes, uma série de chalconas foram testadas como inibidoras da NorA, e duas delas, demonstraram potencial de inibição comparável ao do já conhecido EPI, reserpina (HOLLER et al., 2012).

1.4.2.6.2. MepA

A MepA pertence à Família de Extrusão de Multidrogas e Compostos Tóxicos – MATE, e foi identificada pela primeira vez em cepas de *S. aureus* em 2005 por Kaatz e colaboradores. Ela é capaz de extrudar vários biocidas e agentes antimicrobianos clinicamente relevantes. O gene responsável pela codificação dessa proteína é chamado de *mepA* (KAATZ; MCALEESE; SEO, 2005). Os transportadores transmembranares do tipo MATE usam gradientes H^+ e/ou de Na^+ para conduzir o efluxo de compostos poliaromáticos e drogas catiônicas (DU et al., 2018). A proteína da MepA possui 451 aminoácidos em 12 segmentos transmembranares e apresenta identidade de 26% com os transportadores MATE CdeA de *C. difficile* e 21% com NorM de *V. parahaemolyticus* (COSTA S. S. et al., 2013).

A quantidade de estudos de modulação da bomba de efluxo MepA com chalconas não é tão extensa, contudo podemos citar duas publicações onde uma chalcona e uma bis-chalcona contendo anel tiofeno, foram capazes de inibir a MepA de forma mais eficiente que o tradicional EPI, CPZ, em combinação com o antibiótico ciprofloxacino (OLIVEIRA et al., 2020; SILVA et al., 2021).

1.4.3. Atividade ansiolítica

A ansiedade e a depressão são as respostas mais comuns dos organismos humanos ao estresse repetitivo. A primeira está relacionada à avaliação de risco potencial e envolve a incerteza quanto a uma ameaça, é caracterizada por um estado de angústia mais difuso com sintomas de hiperexcitação e preocupações inúteis. O receptor do ácido γ -aminobutírico A ($GABA_A$) está relacionado com a modulação dos níveis de ansiedade, insônia e memória. Os receptores $GABA_A$ são proteínas de membrana heteropentamérica que formam um canal de íons Cl^- que regula a maioria das inibições de neurotransmissões no sistema nervoso central dos mamíferos. O receptor $GABA_A$ faz parte da superfamília de canais de íons controlados por ligante que também incluem os receptores de acetilcolina nicotínica (nAChRs), de serotonina (5HT₃) e de glicina (GlyR). São formados por vários tipos de subunidades (α , β , γ , δ e ϵ)

organizadas pseudo-simetricamente em torno do canal condutor de íons central. Seu ligante endógeno é o ácido γ -aminobutírico (GABA). Déficits na neurotransmissão mediadas pelo GABA estão associadas a ocorrência de distúrbios convulsivos tais como epilepsia, transtornos de ansiedade, esquizofrenia e transtorno obsessivo compulsivo. Estudos farmacológicos sugerem os benzodiazepínicos (BZDs) e outros moduladores positivos do receptor $GABA_A$ reduzem a ansiedade enquanto os antagonistas do receptor $GABA_A$ competitivos e não competitivos e os agonistas inversos dos BZDs são ansiogênicos (BARNARD; DARLISON; SEEBURG, 1987; LIU et al., 2015; MÖHLER, 2012; OLSEN; TOBIN, 1990). Os BZDs se ligam ao receptor $GABA_A$ num sítio diferente do sítio ortostérico e exerce modulação alostérica modificando a capacidade do receptor $GABA_A$ de ativar seu sítio endógeno (MARDER, 2012; OLSEN, 2015).

Já se sabe que alguns flavonóides (MARDER, 2012) e chalconas (SALEHI et al., 2021) podem atuar no receptor $GABA_A$ com efeitos similares aos BZDs. Por exemplo, a chalcona isoliquiritigenina apresentou modulação alostérica positiva nos receptores $GABA_A$ com efeitos hipnóticos (CHO et al., 2011), antidepressivos (WANG et al., 2008) e ansiolíticos (JAMAL; ANSARI; RIZVI, 2008). No estudo de Jamal, Ansari e Rizvi (2008), a buteína também apresentou ação ansiolítica sem efeitos colaterais sedativos. Em outro estudo, Wang et al. (2008) identificaram efeitos antidepressivos na buteína bem como da isoliquiritina. Também existem diversos relatos de efeitos antinociceptivos de vários derivados de chalconas em ratos como modelo animal (CORRÊA et al., 2001; DE CAMPOS-BUZZI et al., 2006; MOHAMAD et al., 2010; VIANA; BANDEIRA; MATOS, 2003). Mais recentemente, estudos de atividade ansiolítica têm sido conduzidos com zebrafish (*Danio rerio*) como modelo animal pois seu genótipo tem 70% de homologia com os seus receptores de neurotransmissores dos mamíferos (CORRÊA et al., 2001). Nesse modelo, as chalconas (2*E*,4*E*)-1-(2-hidroxi-3,4,6-trimetoxifenil)-5-fenilpenta-2,4-dien-1-ona (FERREIRA et al., 2019) e N-{(4'-[(*E*)-3-(4-fluorofenil)-1-(fenil)prop-2-en-1-ona]} (XAVIER et al., 2020) demonstraram forte evidência de suas propriedades ansiolíticas, sendo que a primeira com ação comprovadamente mediada por receptores $GABA_A$.

1.5. ESPECTROSCOPIA MOLECULAR

1.5.1. Espectroscopia vibracional

O espectro vibracional foi estudado pela primeira vez na absorção de bandas na região do infravermelho (IV), que foi a primeira região não-visível das radiações eletromagnéticas a

ser descoberta. O astrônomo William Herschel foi o responsável por tal revelação em 1800. Porém, apenas com o surgimento da teoria quântica foi possível compreender a origem dos espectros moleculares na região do visível, ultravioleta (UV) e infravermelho (IV). Na primeira conferência Solvay em 1911, N. Bjerrum introduziu a ideia de que as moléculas estão associadas a três tipos de energia de diferentes magnitudes - vibracional, rotacional e translacional - o que poderia fornecer recursos separados em espectros. A equação de onda de Schrödinger conseguiu resolver com grande sucesso as características do espectro de IV. Ela mostrou que a região de IV médio ($4000 - 400 \text{ cm}^{-1}$) correspondia às transições compreendidas entre vibracional e rotacional de níveis de energia mais próximos; O espectro de IV próximo ($14000 - 4000 \text{ cm}^{-1}$) originava-se de sobretons ou combinações dos modos vibracionais fundamentais e o espectro IV distante ($400 - 10 \text{ cm}^{-1}$) advinha de vibrações fundamentais de baixa frequência, por exemplo, modos de rede cristalina de sólidos ou rotacionais puros na fase gasosa. Mais tarde, em 1928, também se descobriu o fenômeno de espalhamento inelástico de fótons de luz, chamado de Efeito Raman em homenagem ao físico Chandrasekhara Venkata Raman. Esse método consiste numa segunda via para estudar as faixas de frequência associadas as vibrações e rotações moleculares. Uma das principais aplicações da espectroscopia vibracional está relacionada com a análise de estruturas moleculares, uma vez que bandas de absorção individuais estão associadas com grupos funcionais dentro de moléculas orgânicas (GRIFFITHS; CHALMERS, 2002).

As vibrações moleculares podem variar desde o simples movimento entre dois átomos acoplados em moléculas diatômicas até movimentações mais complexas em grandes moléculas multifuncionais. O número teórico de bandas corresponde ao número de vibrações fundamentais e podem ser calculados a partir dos graus de liberdade da molécula. Cada átomo possui três graus de liberdade correspondentes às três coordenadas cartesianas (x, y e z). O número de graus de liberdade de uma molécula é igual ao número total de graus de liberdade dos átomos individuais, portando uma molécula contendo N átomos possui $3N$ graus de liberdade. No caso de moléculas não-lineares, três dos graus de liberdade descrevem a rotação e três descrevem a translação. Assim tem-se um total de $3N - 6$ graus de liberdade. Já em moléculas lineares, apenas 2 graus de rotação (porque a rotação sobre seu eixo de linearidade não muda as posições dos átomos) e 3 de translação são descritos, totalizando $3N - 5$ graus de liberdade (YADAV, 2013).

Cada modo de vibração envolve deslocamentos praticamente harmônicos dos átomos em relação a sua posição de equilíbrio. Para cada modo, todos os átomos vibram em uma

determinada frequência característica, ν_i . A energia potencial, $V(r)$, de um oscilador harmônico é função da distância entre os átomos, r . Em qualquer modo vibracional simples os átomos vibram em movimento harmônico e os estados de energia vibracional podem ser calculados conforme a equação 1 (GRIFFITHS; CHALMERS, 2002).

$$V_{iv} = h\nu_i \left(n_i + \frac{1}{2} \right) \quad (1)$$

h é a constante de Planck, ν_i é a frequência do modo de vibração natural e n_i é o número quântico no modo vibracional i ($n_i=1,2,3\dots$).

Já a frequência natural de vibração de um oscilador harmônico é determinada pela constante de força elástica K (rigidez da ligação química) e pelas massas (m_1 e m_2) dos átomos unidos. A frequência natural de vibração é dada pela equação 2 que é derivada da lei de Hooke (GRIFFITHS; CHALMERS, 2002).

$$\nu_i = \frac{1}{2\pi} \cdot \sqrt{\frac{K}{\mu}} \quad (2)$$

$$\mu = \frac{m_1 \cdot m_2}{m_1 + m_2} \text{ (massa reduzida)}$$

1.5.1.1. Espectroscopia no infravermelho (IV)

Quando as moléculas absorvem radiação IV são excitadas para um estado energético superior, assim como ocorre em outros processos de absorção em que a energia é quantizada. Absorção da radiação IV corresponde a energia da ordem de 8 a 40 kJ/mol que são capazes de aumentar a amplitude dos movimentos vibracionais das ligações covalentes nas moléculas. Contudo nem todas essas ligações são capazes de absorver radiação IV mesmo em frequências exatamente iguais à do movimento vibracional, apenas as ligações com momento de dipolo que variam no tempo são capazes de absorver radiação no infravermelho. Assim uma ligação simétrica que tenha grupos idênticos ou quase idênticos em cada ponta não absorverá radiação IV. O espectro de IV pode servir como uma espécie de "impressão digital" da molécula, sendo denominada de *fingerprint* a região abaixo de 1500 cm^{-1} , pois cada composto tem um padrão de absorção único nesta região tornando impossível sobreposição de bandas, já acima disto, compostos parecidos podem apresentar espectros semelhantes. As absorções de cada tipo de ligação covalente são encontradas apenas em uma pequena faixa do espectro vibracional, fora dela, provavelmente aquele pico deve ser atribuído à alguma outra ligação (PAVIA et al., 2010).

Na Figura 11 estão resumidas algumas faixas do espectro de IV onde ocorrem estiramentos de ligações químicas típicas.

Frequência (cm ⁻¹)						
4000	2500	2000	1800	1650	1550	650
C - H O - H N - H	C ≡ C C ≡ N X=C=Y (C,O,N,S)	Poucas faixas	C = O	C = N C = C N = O	C - C N = O	C - O C - N C - Cl

Figura 11. Regiões aproximadas em que alguns tipos de ligações químicas absorvem no IV e produzem estiramentos. Adaptado de Pavia et al. (2010).

Os modos vibracionais mais simples que dão origem a absorção no IV são o estiramento e o dobramento. Contudo outros tipos mais complexos são também ativos para radiação IV. No estiramento a distância entre os átomos aumenta e diminui, porém eles continuam no mesmo eixo da ligação. Sua energia é baseada na lei de Hooke e é dada por $E = \frac{1}{2}k_b(r - r_0)^2$, em que k_b representa o parâmetro de rigidez da ligação química e r_0 é a posição de equilíbrio da ligação. O estiramento pode ocorrer de duas maneiras: simétrico – quando o movimento dos átomos em relação ao átomo comum (ou central) está simultaneamente na mesma direção ao longo do eixo da ligação; ou antissimétrico – quando um átomo se aproxima do átomo comum enquanto o outro se afasta dele. Já no dobramento, as posições dos átomos mudam em relação aos seus eixos originais de ligações. As vibrações de dobramento podem ocorrer no mesmo plano ou não, e são classificadas de quatro maneiras: *Scissoring* – o movimento dos átomos ocorre na direção oposta, com mudança no ângulo que eles formam com o átomo central; *Rocking* – os átomos se movimentam na mesma direção em relação aos eixos de ligação; *Wagging* – dois átomos se movem simultaneamente acima e abaixo do plano em relação ao átomo comum; e *Twisting* – um dos átomos sobe e o outro desce em relação ao plano do átomo comum. A energia dos modos de dobramento também é baseada na lei de Hooke como $E = \frac{1}{2}k_\theta(\theta - \theta_0)^2$, sendo k_θ o parâmetro de rigidez angular da ligação e θ_0 o ângulo de equilíbrio. Conforme mencionado anteriormente, existem outros tipos de modos normais que podem ser ativos no IV, um exemplo disso são os modos de torção ou diedral, cuja energia é usada principalmente para corrigir a energia total. A energia do modo de torção deve ser adicionada aos termos de estiramento, dobramento e às interações não-ligadas para fazer com que a energia total esteja de acordo com

os experimentos ou cálculos quânticos rigorosos. Alguns autores tratam a energia dos modos de torção da mesma forma como os modos de dobramento, uma função simples do tipo $E = \frac{1}{2}k_{\chi}(\chi - \chi_0)$, em que k_{χ} é o parâmetro de rigidez do ângulo de torção e χ_0 é o ângulo de torção de equilíbrio. Esses parâmetros são determinados a partir do ajuste da curva. Cada modo de torção possui parâmetros únicos atribuídos para cada quarteto de átomos com base nos seus tipos, por exemplo, C-C-C-C, C-O-C-N e H-C-C-H (HINCHLIFFE, 2008; YADAV, 2013). A Figura 12 mostra exemplos de modos vibracionais de estiramento e dobramento de um grupo CH_2 e de torção de um grupo C-C-C-C.

O equipamento que obtém o espectro de absorção no infravermelho é chamado de “espectrômetro de infravermelho”. Dois tipos de espectrômetros são comumente utilizados nas análises químicas: os instrumentos dispersivos e os por transformada de Fourier (FT). Os do primeiro tipo produzem uma radiação no infravermelho que é dividida em dois feixes idênticos, um dos feixes passa pela mostra e o outro serve como referência. Então os feixes chegam em um monocromador que dispersa cada um deles em um espectro contínuo de frequências de luz infravermelha de forma alternada e os envia a uma grade de difração. Essa rede de difração varia o comprimento de onda da radiação que chega a um detector. Este por sua vez sente a razão entre as intensidades dos feixes de referência e o da amostra de forma que se identifica quais frequências foram absorvidas e quais não foram. Em seguida o sinal é amplificado e registrado, então obtém-se o espectro no domínio da frequência. Já o equipamento com FT produz um padrão chamado “interferograma”, que é complexo, mas nele estão contidas todas as frequências que formam o espectro infravermelho. O interferograma consiste num gráfico de intensidade *versus* tempo, daí aplica-se a operação matemática conhecida como transformada de Fourier para convertê-lo num espectro que seja um gráfico de intensidade *versus* frequência (PAVIA et al., 2010).

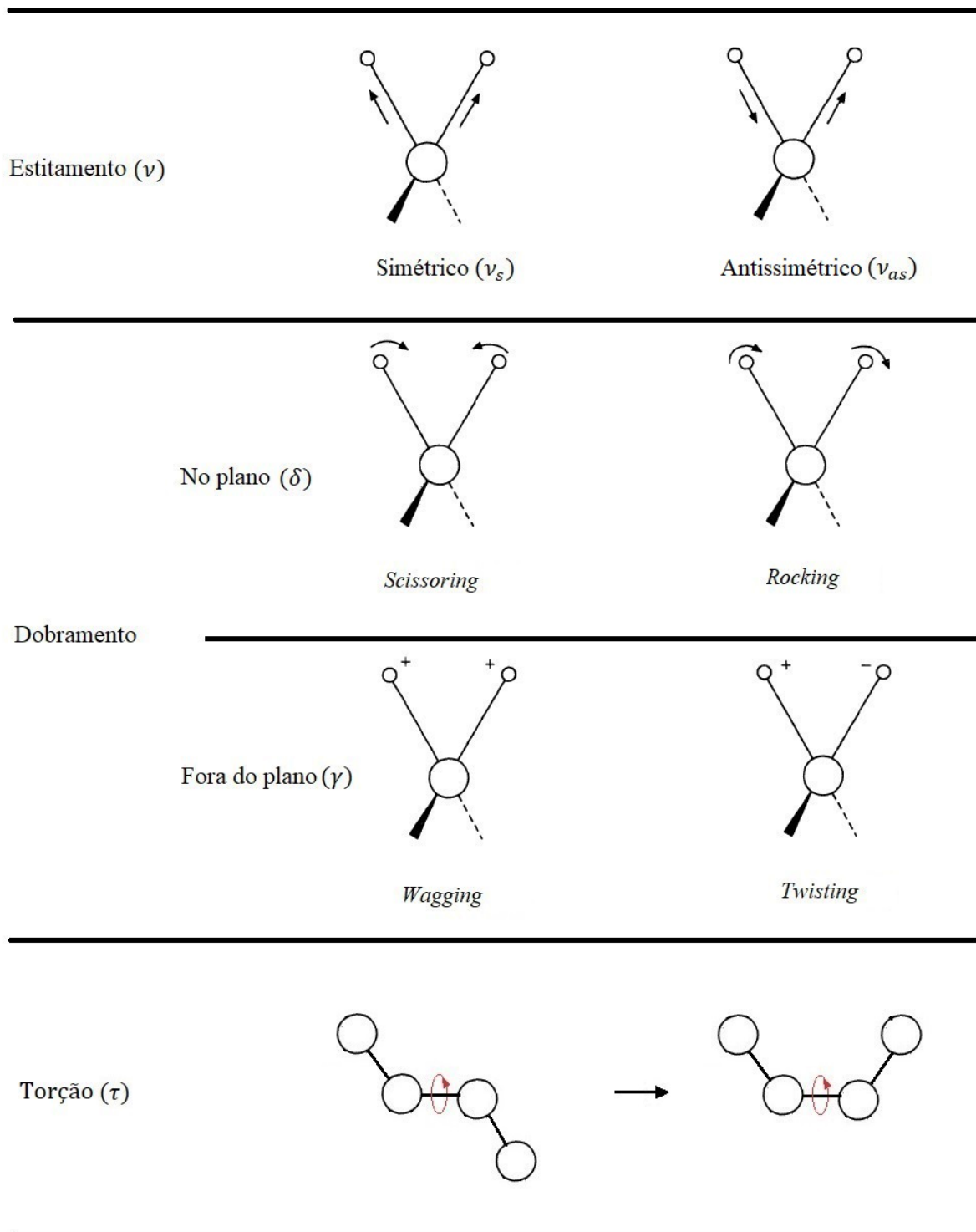


Figura 12. Exemplos de modos vibracionais de estiramento e dobramento de um grupo CH_2 e torção de um grupo C-C-C-C . Os sinais + e - indicam movimentos perpendiculares ao plano do papel. Adaptado de Yadav (2013).

1.5.1.2. Espectroscopia Raman

Quando uma amostra transparente é irradiada por um feixe de luz monocromática com número de onda $\tilde{\nu}_0$, a maioria da radiação passará pela amostra sem nenhuma alteração, enquanto uma pequena porção de luz será espalhada. Uma análise do feixe de luz espalhado mostrará que uma boa parcela da radiação possui o mesmo número de onda da radiação incidente (Espalhamento Rayleigh), porém ele também conterá componentes discretos com número de onda diferentes, ou seja, aparecerão novas linhas no espectro da radiação espalhada em pares posicionados simetricamente distantes das linhas de Rayleigh. Essas linhas terão número de onda, $\tilde{\nu}' = \tilde{\nu}_0 \mp \tilde{\nu}_M$, em que o número de onda $\tilde{\nu}_M$ corresponde às transições de níveis de energia vibracional ou rotacional (raramente eletrônica) de um sistema molecular. O aparecimento dessas linhas no feixe de luz espalhada é chamado de Efeito Raman ou Espalhamento Raman (GRIFFITHS; CHALMERS, 2002). O par de linhas espectrais simetricamente deslocados da linha Rayleigh são chamados de linha *Stokes*, quando possuem frequência menor que a radiação incidente, e linha *anti-Stokes*, quando possui frequência maior. Quando um *laser* interage com os elétrons de uma molécula causando polarização, a energia salta para um estado denominado virtual. No processo de Rayleigh não há nenhuma mudança de energia e, conseqüentemente, a luz retorna ao mesmo estado. Já no espalhamento Raman, ocorre que, ou o estado vibracional fundamental (m) leva à absorção de energia pela molécula e sua promoção a um estado vibracional excitado de alta energia (n), ou devido à energia térmica, algumas moléculas podem estar presentes em um estado excitado como (n) e ao espalhar-se retornam ao estado fundamental (m) (SMITH; DENT, 2005). A Figura 13 resume os processos de espalhamento Rayleigh e Raman.

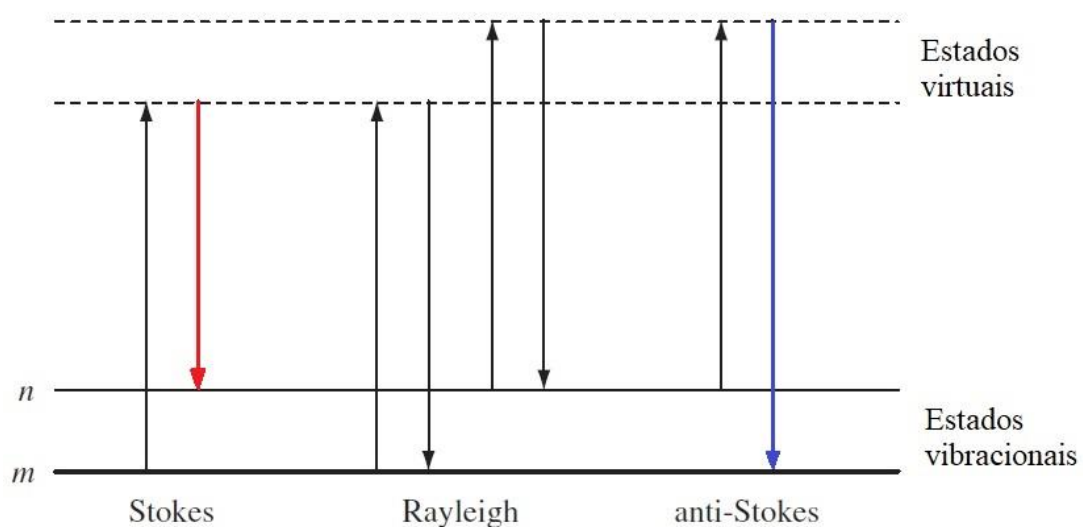


Figura 13. Diagrama dos processos de espalhamento Rayleigh e Raman. Adaptado de Smith e Dent (2005).

Pode-se observar que a espectroscopia de IV e Raman utilizam a radiação de forma diferente. Na espectroscopia de IV a energia direcionada para a amostra varia numa faixa de frequências, e quando uma certa frequência incidente corresponde exatamente ao modo de vibração da molécula, ocorre a absorção promovendo-a a um estado vibracional excitado. Contrariamente, a espectroscopia Raman utiliza uma única frequência de radiação que ao incidir sobre a molécula espalha-se e feixes com energia diferente da radiação incidente são detectados. A distância entre a frequência da radiação incidente e da radiação espalhada ($\Delta\nu$) corresponde à absorção ou emissão no IV. Contudo os espectros de IV e Raman não são idênticos, ao passo que no Raman a radiação interage com a molécula polarizando (distorcendo) as nuvens eletrônicas e produzindo um estado de energia capaz de movimentar os núcleos atômicos (vibração), na radiação IV, a vibração molecular está relacionada com o momento dipolo oscilante das ligações químicas. Por isso pode acontecer de um modo de vibração ser ativo no Raman e não ser ativo no IV e vice-versa. Sendo assim, a espectroscopia Raman não é uma técnica que substitui a espectroscopia de infravermelho ou o contrário, na verdade, cada uma delas possui grande importância no estudo dos modos vibracionais, e por vezes, podem ser técnicas complementares (SMITH; DENT, 2005).

De forma simplificada um modo vibracional será ativo no espectro de infravermelho quando $\left(\frac{\partial\mu_i}{\partial Q_k}\right)_0 \neq 0$, e será ativo no espectro Raman quando $\left(\frac{\partial\alpha_{ij}}{\partial Q_k}\right)_0 \neq 0$, em que μ_i é o momento dipolo, α_{ij} é o tensor de polarizabilidade, e Q_k é a k -ésima coordenada normal associada ao modo de vibração de número de onda $\tilde{\nu}_k$ (GRIFFITHS; CHALMERS, 2002).

A instrumentação para realização da espectroscopia Raman é em princípio simples. Um intenso feixe de radiação monocromática passa através de uma amostra e a luz espalhada em ângulos retos com o feixe incidente é analisada por um espectrofotômetro. Filtros são usados para obter uma radiação monocromática. O espectrógrafo usado na espectroscopia Raman deve ter grande poder de coleta, portanto, prismas especiais de alta resolução e foco curto são utilizados (YADAV, 2013).

1.5.2. Espectroscopia de ressonância magnética nuclear (RMN)

A ressonância magnética nuclear (RMN) é um método espectroscópico muito importante na identificação de compostos químicos. Enquanto a espectroscopia de IV mostra os grupos funcionais presentes num composto, na RMN são os núcleos atômicos que são estudados, sendo os mais comuns, hidrogênio e carbono. A RMN oferece informações sobre o

número de átomos magnéticos distintos do isótopo estudado. Então a junção de técnicas como RMN e IV é normalmente suficiente para a determinação da estrutura de um composto desconhecido (PAVIA et al., 2010).

A RMN resulta da ocorrência de transição de níveis energéticos correspondentes às diferentes orientações permitidas do spin nuclear quando o núcleo se encontra sujeito à ação de um campo magnético. Essas diferenças de energia só são percebidas quando esse campo magnético é muito intenso. A primeira experiência de RMN foi concebida com o objetivo de se medir o spin nuclear, no entanto, o núcleo de um elemento não dava origem a apenas um sinal, mas dava origem a vários sinais numa região vizinha daquele átomo. Por certo, a ressonância de um núcleo é afetada pela estrutura molecular onde aquele átomo está inserido. Um equipamento que realiza a RMN é constituído basicamente de uma fonte de campo magnético numa região onde é colocada a amostra associada a uma fonte de radiofrequência que emite radiação através de um circuito espiral envolvendo a amostra. A forma de apresentar os espectros consiste em identificar a posição do sinal de ressonância pelo seu desvio em relação a posição do sinal de um núcleo selecionado como referência (ROCHA GONSALVES; PINHO E MELO, 2007).

1.5.3. Espectroscopia de massa (EM)

Os pilares para a técnica analítica denominada de espectroscopia de massa (EM) foram os estudos da relação massa/carga de J. J. Thomson e os estudos da deflexão magnética dos raios catódicos de Wien. Contudo esse método apenas se desenvolveu nos últimos 50 anos quando foram construídos equipamentos baratos e confiáveis. A espectroscopia de massa é bastante importante para a biotecnologia no exame da sequência de proteínas, oligonucleotídeos e polissacarídeos. Também pode ser usada no desenvolvimento de fármacos, testes clínicos, inclusive para marcadores de muitos tipos de câncer, e detecção de drogas ilícitas ou anabolizantes. A amostra estudada por EM pode ser gás, líquida ou sólida (PAVIA et al., 2010).

Um aparelho de EM possui cinco compartimentos: A unidade de entrada de amostra – responsável por trazer a amostra do meio exterior para um ambiente com pressão reduzida bem como produzir um fluxo constante de moléculas no estado gasoso; Fonte de ionização – região onde essas moléculas são transformadas em íons em fase gasosa; Analisador de massa – separa os íons com base na razão massa/carga; Detector – quantificam os íons analisados; e Sistema de dados – coleta as informações e produz o espectro de massa (YADAV, 2013).

Nessa técnica analítica, a etapa que pode variar é a fonte de ionização. Existem vários métodos para converter o fluxo de moléculas gasosas em íons. O mais comum é a ionização por elétrons, quando ocorrer a colisão de um feixe de elétrons com a corrente gasosa produzindo fragmentos de moléculas carregadas positivamente. Nesse processo pode ocorrer a formação de moléculas carregadas negativamente pela absorção de elétrons, porém elas são absorvidas por placas repelentes. Em alguns equipamentos é possível inverter a polaridade e analisar os íons negativos ao invés dos positivos. O excesso de energia cinética transmitida à amostra leva a fragmentação dos íons moleculares de forma sistemática e reproduzível, o que permite a construção de bibliotecas espectrais de milhares de compostos essenciais na identificação rápida de moléculas. Além da ionização por elétrons também é bastante comum nos equipamentos a formação de íons através da ionização química. Nesse caso, o fluxo de moléculas da amostra encontra um fluxo de gás reagente ionizado, normalmente metano. Quando eles se encontram ocorre a formação de íons de várias maneiras, com transferências de prótons, transferência de elétrons e formação de íons secundários (PAVIA et al., 2010; YADAV, 2013).

1.6. CÁLCULOS COMPUTACIONAIS

1.6.1. Teoria do Funcional da Densidade (DFT)

A Teoria do Funcional da Densidade (DFT) surgiu como alternativa aos tradicionais métodos *ab initio* e semi-empíricos no estudo de propriedades no estado fundamental de grandes sistemas moleculares orgânicos, inorgânicos, metálicos e semicondutores. Por essa razão, muitas áreas têm empregado essa técnica no desenvolvimento de fármacos, agroquímicos, polímeros, complexos organometálicos, aglomerados para catálise, bem como no estudo de superfície no estado sólido, eletroquímica e microeletrônica. Domínios estes que até então eram inacessíveis aos métodos de natureza *ab initio* convencionais. Os princípios da DFT estão atrelados à teoria da função de onda (Ψ) e à equação de Schrödinger (MORGON; CUSTODIO, 1995).

Qualquer problema envolvendo a estrutura eletrônica da matéria pode ser abordado pela equação de Schrödinger. Para um sistema atômico ou molecular contendo N elétrons no estado estacionário tem-se a equação 3.

$$\hat{H}\Psi = E\Psi \quad (3)$$

E é a energia eletrônica, Ψ é a função de onda e \hat{H} é o operador Hamiltoniano, que é representado na equação

$$\hat{H} = \sum_{i=1}^N \left(-\frac{1}{2} \nabla_i^2\right) + \sum_{i=1}^N v(r_i) + \sum_{i<j}^N \frac{1}{r_{ij}} \quad (4)$$

em que

$$v(r_i) = - \sum_{\alpha} \frac{Z_{\alpha}}{r_{i\alpha}} \quad (5)$$

é o potencial de atuação "externo" no elétron i , sendo Z_{α} o potencial devido aos núcleos de cargas e r_i as coordenadas espaciais do elétron i . A função de onda (Ψ) contém todas as informações para descrever um sistema quanticamente, porém não possui significado físico em si, já $|\Psi|^2$ é interpretada como sendo proporcional a probabilidade de encontrar o sistema numa determinada posição em relação a um sistema de coordenadas. Cada elétron pode ser descrito por três coordenadas cartesianas $r_k = (x_k, y_k, z_k)$ e uma coordenada spin denominada σ_k . Então se numa molécula há N elétrons, sua função de onda será descrita em $4N$ coordenadas. Dessa forma $|\Psi|^2$ pode ser melhor descrito como $|\Psi(r^N, \sigma^N)|^2 dr^N$ que significa a probabilidade de encontrar o sistema numa posição entre as coordenadas r^N e $r^N + dr^N$ e com coordenadas spin σ^N . A função $\Psi(r^N, \sigma^N)$ pode ser simplificada para três dimensões ($3N$ coordenadas) pela equação 6

$$P(r) = \int_a^b \Psi^* \Psi dr^N \quad (6)$$

em que Ψ^* é o complexo conjugado de Ψ e $\int dr^N$ significa a integração sobre as $3N$ coordenadas espaciais e somando sobre as N coordenadas de spin. Assim tem-se uma função resultante conhecida como função densidade de probabilidade $P(r)$. Se a densidade de probabilidade for multiplicada pelo número de elétrons N , tem-se a densidade eletrônica $\rho(r)$, que descreve a distribuição de carga de um sistema multieletrônico. O diferencial da DFT é justamente trabalhar com a densidade eletrônica ao invés das funções de ondas totais como fazem os outros métodos de química quântica quando da resolução da equação de Schrödinger, bem como ela pode ser comparada com a densidade obtida experimentalmente (FIRME, 2007; PARR; YANG, 1989).

Os primeiros a descrever a energia de um átomo em função da densidade eletrônica foram Thomas e Fermi em 1927, mais tarde Slater criou uma expressão, originalmente derivada das equações de Hartree-Fock, que representava um funcional de densidade para a energia de troca. Para uma melhor aproximação foi acrescentado um parâmetro empírico α na equação que ficou conhecida como Hartree-Fock-Slater. Em 1964, Hohenberg e Kohn desenvolveram as bases para a DFT quando postularam seu primeiro teorema que prova que a energia do estado fundamental de qualquer sistema eletrônico é determinada por um funcional da densidade do elétron. Isto quer dizer que, em princípio, só precisamos saber a densidade de elétrons no espaço tridimensional e não a função de onda completa, a fim de calcular qualquer propriedade no estado fundamental tal como a energia. Já o segundo teorema estabelece que o funcional de Hohenberg-Kohn (F_{HK}) fornece a energia mais baixa (estado fundamental), se e somente se, a densidade de entrada é a densidade do estado fundamental, ρ_0 . Contudo, existe um problema pois esses teoremas não fornecem nenhuma pista quanto à natureza do funcional de densidade nem como buscá-lo. O artigo publicado em 1965 por Kohn e Sham fornecem uma solução prática para esse problema com uma modificação nas equações de Hartree-Fock (HINCHLIFFE, 2008).

Sendo assim a energia de um sistema multieletrônico sob um determinado potencial externo $v(r)$ pode ser escrito por

$$E_v[\rho(r)] = \int v(r) \rho(r) dr + F[\rho] \quad (7)$$

em que F é o funcional universal de ρ , que independe do potencial $v(r)$. Caso a função densidade exata seja substituída por uma função aproximada $\tilde{\rho}$, a energia aumentará em relação a energia E_v , como mostra a equação 8 (MORGON; CUSTODIO, 1995).

$$\tilde{E} = \tilde{E}[\tilde{\rho}] = \int v(r) \tilde{\rho}(r) dr + F[\tilde{\rho}] \geq E_0 = E[\rho] \quad (8)$$

A DFT inicia com a energia sendo escrita como funcional da densidade eletrônica total para uma dada posição dos núcleos atômicos, $E[\rho]$. Essa energia total é decomposta em termos de energia cinética, energia eletrostática (Coulomb) e um termo que combina todos os efeitos de troca e correlação, $E[\rho] = T[\rho] + U[\rho] + E_{XC}[\rho]$. A densidade eletrônica total é decomposta em densidades de partículas únicas que se originam de funções de onda de uma partícula livre, $\rho(r) = \sum_{occ} |\Psi(r)|^2$. Então a DFT requer que mediante variação da densidade total de elétrons, a energia total assuma um mínimo, $\frac{\partial E}{\partial \rho} = 0$. Isso leva a condições para formar

uma função de onda na forma da equação de Kohn-Sham de uma partícula efetiva, $\left[-\frac{1}{2}\nabla^2 + V_c(r) + u_{xc}(r)\right]\Psi_i = \varepsilon_i\Psi_i$. Essa equação deve ser resolvida por um método de campo autoconsistente iterativo (Figura 14) similar ao usado na teoria de Hartree-Fock (WIMMER, 2012).

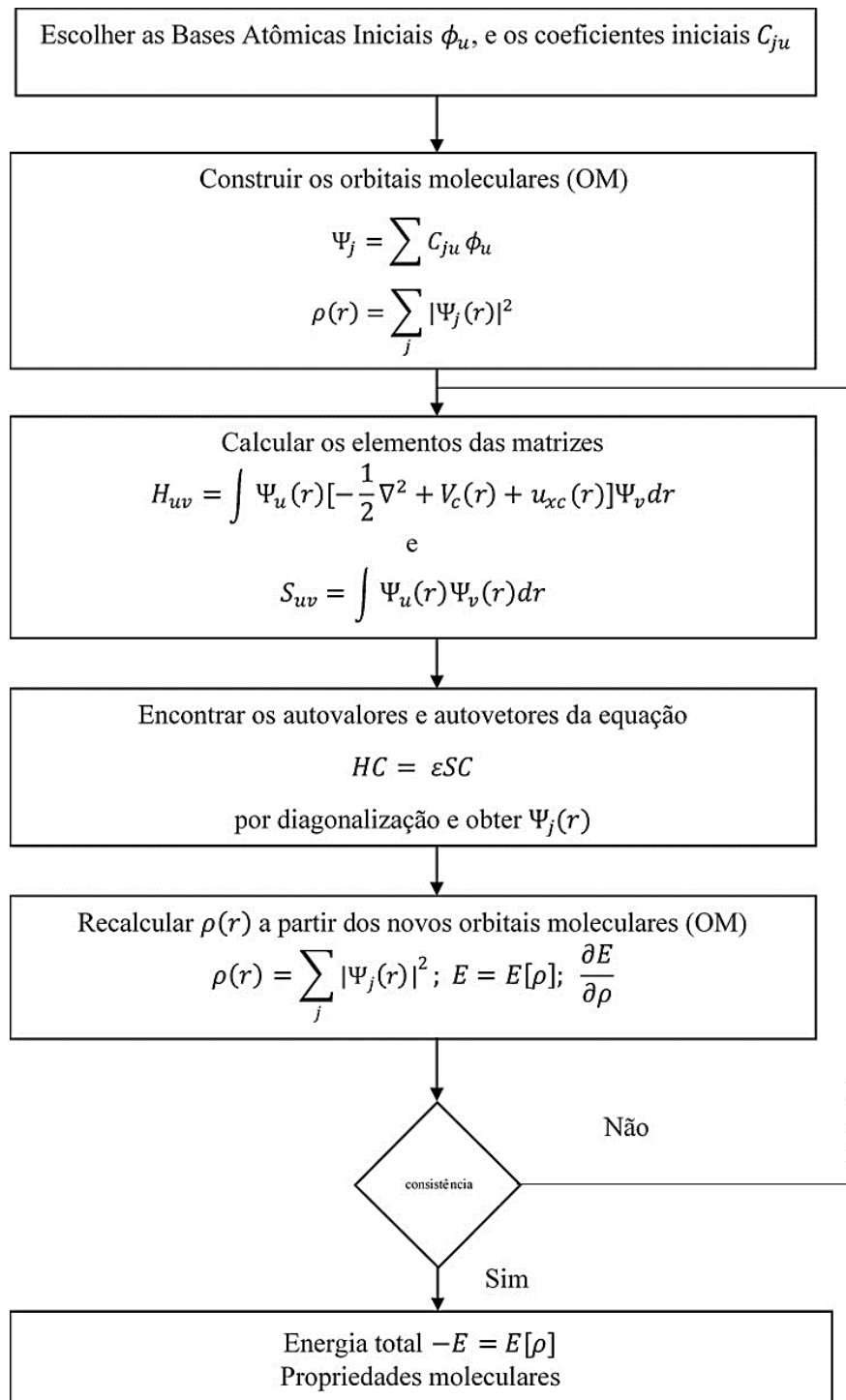


Figura 14. Método de campo autoconsistente iterativo para determinação da energia e propriedades de um sistema eletrônico. Adaptado de Morgon e Custodio (1995).

1.6.2. Docagem molecular

As interações enzima-substrato são essenciais para os processos biológicos bem como para a atuação de muitos fármacos. Atualmente o conceito da interação proteína-substrato pelo modelo “chave e fechadura” foi remodelado em função da flexibilidade conformacional das proteínas, e é mais comum hoje encontrar denominações como sítio ativo, sítio de ligação, substrato ou ligante. Já se sabe que os sítios ativos das proteínas não são estáticos, ao contrário, eles são remodelados durante a interação com os substratos. Esse entendimento pode explicar por que alguns ligantes não são substratos de algumas proteínas embora satisfaçam os requisitos específicos para se ligarem no sítio ativo. As interações proteína-substrato são dinâmicas e mais complexas do que a abordagem básica “chave e fechadura”, esse efeito de “ajuste” induzido pelo substrato torna o estudo dos encaixes moleculares mais sofisticados em muitos aspectos, e necessitam não raramente, de uma abordagem química computacional (TRIPATHI; BANKAITIS, 2018).

A Docagem Molecular é uma técnica *in silico* que analisa as possíveis conformações e orientações de uma molécula num sítio de ligação de uma macromolécula, ranqueados por funções de pontuação, utilizando algoritmos específicos. Essa técnica é amplamente utilizada na previsão dos complexos proteína-substrato, em análises de estrutura/função, bem como na descoberta de novas drogas pelo desenvolvimento de métodos de encaixe mais precisos (PINZI; RASTELLI, 2019). Existem diversos *softwares* que realizam a docagem molecular, dentre eles destaca-se o *AutoDock4* (MORRIS et al., 1998), o *AutoDock Vina* (TROTT; OLSON, 2010), o *GOLD* (JONES et al., 1997), o *FlexX* (RAREY et al., 1996) e o *Molegro Virtual Docker* (THOMSEN; CHRISTENSEN, 2006). Também é possível encontrar diversos serviços online para simulação de docagem molecular (DA SILVEIRA et al., 2019).

Os processos de docagem possuem dois pilares básicos, um algoritmo eficiente para procura das posições do ligante, e um sistema robusto de ranqueamento dessas posições. Os algoritmos de busca podem ser classificados como sistemáticos, estocásticos ou determinísticos. Os sistemáticos realizam a pesquisa incrementando os graus de liberdade do ligante de forma regular, os estocásticos realizam a busca mudando aleatoriamente os graus de liberdade do ligante e os determinísticos modificam a orientação do ligante em função do estado obtido na interação anterior, o novo estado tem valor de energia igual ou inferior ao anterior (TORRES et al., 2019). Um dos algoritmos mais utilizados é o Algoritmo Genético (AG), que imita as principais características da evolução darwiniana e aplica a genética Mendeliana. Na docagem molecular, o arranjo particular de um ligante e uma proteína pode ser definido por um

conjunto de valores que descrevem a tradução, orientação e conformação do ligante em relação à proteína. Estas são as variáveis de estado do ligante e, no AG, cada variável de estado corresponde a um gene. O estado do ligante corresponde ao genótipo, enquanto suas coordenadas atômicas correspondem ao fenótipo. Então a energia de interação proteína-ligante é avaliada usando uma função de pontuação. Pares aleatórios de indivíduos são acasalados usando um processo de cruzamento, no qual novos indivíduos herdam genes de um dos pais. Além disso, alguns descendentes sofrem mutações aleatórias, na qual um gene muda de forma randômica. A seleção dos filhos da geração atual ocorre com base na aptidão do indivíduo. Assim as soluções mais adequadas ao seu ambiente se reproduzem, enquanto as menos adequadas morrem. Existe uma variação desse algoritmo que é chamado Algoritmo Genético Lamarckiano (AGL), nele ocorre uma minimização de energia local, cuja busca não é aleatória como no AG. Se essa configuração for melhor que a configuração anterior, essa configuração é passada para uma nova geração (MORRIS et al., 1998). Outro algoritmo também bastante utilizado, implementado no *AutoDock Vina*, é o *Local Search global optimizer*, nele uma sucessão de etapas consistindo em uma mutação e uma otimização local são realizadas, sendo cada etapa aceita de acordo com o critério de Metropolis. Esse algoritmo usa o método Broyden-Fletcher-Goldfarb-Shanno para a otimização local, que é um método quasi-Newton bastante eficiente (TROTT; OLSON, 2010).

As funções de pontuação são utilizadas para determinar o modo de ligação e o local de ligação de um ligante a uma proteína, prever a afinidade de ligação entre a proteína e o ligante, e para triagem virtual, que identifica potenciais drogas para um determinado alvo pesquisando em um vasto banco de dados de ligantes. Essas funções de pontuação são classificadas atualmente como sendo: baseadas na física, empíricas/semi-empíricas, baseada no conhecimento e baseadas em aprendizado de máquina (LI; FU; ZHANG, 2019).

Dentre as funções baseadas na física podemos destacar aquelas baseadas em campos de força, cujos parâmetros contém termos para forças intramoleculares (ligação, ângulo e termos diédricos) entre átomos ligados, mais termos de energia das forças intermoleculares entre átomos não ligados (TRIPATHI; BANKAITIS, 2018). Contudo ela desconsidera efeitos entrópicos e do solvente, portanto, pode-se incorporar à essas funções, termos de entropia de torção do ligante bem como termos do efeito da solvatação de forma implícita. Mais recentemente foram desenvolvidas funções de pontuação baseadas na mecânica quântica, que embora resolva problemas relacionadas às interações covalentes, polarização e transferência de carga no acoplamento, possuem um custo computacional maior. Por essa razão surgiram as

funções híbridas, que agregam aspectos da mecânica molecular e da mecânica quântica (LI; FU; ZHANG, 2019). As funções empíricas estimam a energia livre da ligação não covalente proteína-ligante como sendo a soma de uma série de interações localizadas. Dentre os principais termos que contribuem para a função tem-se as ligações de hidrogênio, interações hidrofóbicas, efeitos entrópicos etc. Esses termos são ponderados por fatores empíricos obtidos através de regressões feitas com dados de interações já conhecidos. Quando se incorporam termos energéticos empíricos com termos baseados em campo de força em uma função de pontuação, diz-se que ele é semi-empírica. Em contraste com as funções de pontuação baseadas no campo de força, os termos de pontuação semi-empíricos estimam com mais precisão as energias de ligação, contabilizando os efeitos entrópicos e de solvatação que reconhecidamente afetam as interações biológicas em meio aquoso (TRIPATHI; BANKAITIS, 2018).

O *Autodock4*, um dos *softwares* mais utilizados, possui uma função de pontuação que usa um campo de força de energia livre semi-empírico para prever energias livres de ligação proteína-substrato. Os termos da equação incluem avaliações por pares de átomos para interações de Van der Waals, ligação de hidrogênio, interações eletrostáticas e solvatação (HUEY et al., 2007). Abaixo são mostradas as equações utilizadas para ranquear as conformações do ligante do *Autodock4*.

$$\Delta G = (V_{ligado}^{L-L} - V_{n\grave{a}o-ligado}^{L-L}) + (V_{ligado}^{P-P} - V_{n\grave{a}o-ligado}^{P-P}) + (V_{ligado}^{P-L} - V_{n\grave{a}o-ligado}^{P-L} + \Delta S_{conf}) \quad (9)$$

$$V = W_{vdw} \sum_{ij} \left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^6} \right) + W_{hb} \sum_{ij} E(t) \left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}} \right) + W_{elec} \sum_{ij} \frac{q_i q_j}{\epsilon(r_{ij}) r_{ij}} + W_{sol} \sum_{ij} (S_i V_j + S_j V_i) e^{\left(\frac{-r_{ij}^2}{2\sigma^2} \right)} \quad (10)$$

Onde: Na equação 9, ΔG se refere a variação da energia livre de Gibbs na formação do complexo proteína-substrato, V é o potencial elétrico, L se refere ao ligante(substrato), P se refere à proteína e ΔS_{conf} é a entropia conformacional perdida na formação do complexo. Na equação 10, W são os parâmetros de ponderação empíricos, 1º termo, chamado de (6/12), é típico para interações de Van der Waals em que A e B são parâmetros do campo de força Amber, o 2º termo, baseado num potencial (10/12), é o termo de ligação de hidrogênio direcional em que os parâmetros C e D são usados para limitar o comprimento da ligação e E(t) é o termo que restringe o ângulo para formação da ligação de hidrogênio, o 3º termo é puramente para avaliar interações eletrostáticas e o 4º termo se refere ao potencial de solvatação.

Já o *AutoDock Vina*, utiliza uma função de pontuação que leva em conta as seguintes contribuições: Interações estéricas (contendo os termos $gauss_1$, $gauss_2$ e repulsão), hidrofobicidade, ligações de hidrogênio e número de ângulos de torção de rotação livre dos

ligantes (termo N_{rot}). Os valores dos pesos que cada termo contém na função de pontuação são -0,0356; -0,00516; 0,840; -0,0351; -0,587; 0,0585 respectivamente (TROTT; OLSON, 2010).

As funções de pontuação baseadas no conhecimento derivam os potenciais de pares de elétrons de estruturas tridimensionais baseado no princípio estatístico inverso de Boltzmann, já as funções baseadas em aprendizado de máquina empregam uma variedade de algoritmos de aprendizado de máquina tais como máquina de vetor de suporte (*Support Vector Machine* - SVM), floresta aleatória (*Random Forest* - RF), rede neural artificial (*Artificial Neural Network* - ANN) e aprendizado profundo (*Deep Learning* - DL) (LI; FU; ZHANG, 2019).

Isto posto, o processo geral para realização dos cálculos de docagem molecular se resumem em escolher o alvo (proteína) e o ligante. Suas estruturas tridimensionais podem ser obtidas em serviços de bancos de dados. Em seguida realiza-se a preparação dessas estruturas, ajusta-se o estado de protonação e os conflitos estéricos da proteína, e adiciona-se a carga parcial em cada átomo tanto da proteína quanto do ligante (TORRES et al., 2019). Existem diversos serviços online que adicionam os hidrogênios ajustando o estado de protonação dos aminoácidos (WILLIAMS et al., 2017), bem como solucionam conflitos estéricos (LASKOWSKI et al., 1993) dos átomos da proteína. O *Autodock4* permite adicionar cargas parciais à proteína pelos métodos de Kollman e Gaisteger. Ao ligante só é possível adicionar cargas pelo método de Gaisteger. Por fim, realiza-se a docagem e obtém-se as conformações do ligante ranqueadas por pontuação. Com essas posições, interpretam-se os resultados obtidos (MORRIS et al., 1998; TORRES et al., 2019).

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CAPÍTULO 2

Spectroscopic Characterization and Efflux Pump Modulation of a Thiophene Curcumin Derivative

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Abstract

Curcumin, along with its derivatives, form a large class of natural and synthetic compounds with notable biological activity. However, their highly reactive β -diketone moiety renders this type of compounds unstable at pH above 6.5. The substitution of this group for a mono-carbonyl solves this problem, while improving antibacterial and anti-inflammatory activities. A thiophene curcuminoid, (1*E*,4*E*)-1,5-Di(thiophen-2-yl)penta-1,4-dien-3-one (DB thiophene), has been synthesized and its molecular and spectroscopic characterization is reported, as well as a complete vibrational assignment. An efflux pump inhibition assay determined that DB thiophene exhibits a remarkable NorA and MepA efflux pump inhibition activity. Molecular docking studies were carried out in order to understand this inhibition mechanism.

Keywords: Curcumin; Chalcone; Efflux pump; DFT; Spectroscopy; Docking.

1. Introduction

Curcumin is a polyphenol (Scheme 1) and an active principle of the *Curcuma longa* herb, also known as turmeric. Its derivatives, called curcuminoids, form a large class of natural and synthetic compounds with notable biological activity [1]. Curcumin interacts with numerous targets, such as transcription and growth factors and their receptors, as well as genes that regulate cell proliferation and apoptosis [2]. It has thus been widely used as a chemopreventive agent [2] and it is a prime candidate for cancer treatment. In fact, it can suppress the tumorigenic activity of a variety of carcinogens in the colon, duodenum, oesophagus, stomach, breast, and prostate. Curcumin and its derivatives are also known to suppress the growth of bacteria of several genera, such as *Streptococcus*, *Staphylococcus*, *Lactobacillus* and *Helicobacter*[3]. Additionally, it exhibits antiviral activity, including anti-HIV through inhibition of the HIV-1 integrase, an enzyme needed for viral replication[4].

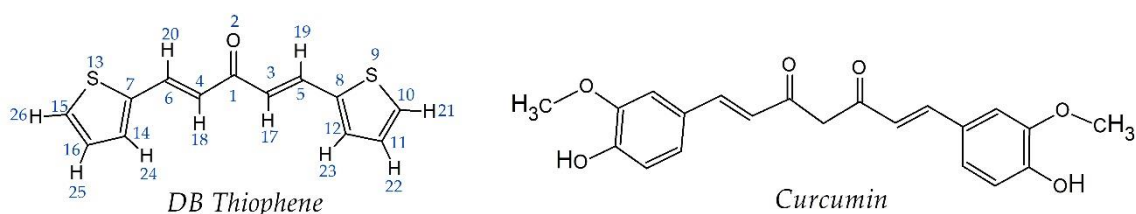
The potential of curcumin is only hampered by its poor bioavailability and pharmacokinetic profile, as its highly reactive diketone moiety makes it unstable *in vitro* at pH above 6.5 [5]. Several studies have focused on finding synthetic derivatives with improved pharmacokinetic profiles [6,7] by substitution of the β -diketone moiety for a mono-carbonyl one. It has been shown that this substitution not only improves stability, but also leads to a better antibacterial and anti-inflammatory activities [8,9]. Likewise, modifications of the aromatic rings substitution also seems to improve the biological activity of curcumin derivatives [10].

The use of new substances in the fight against bacterial infections is often accompanied by increased resistance of these organisms. There are several mechanisms of resistance to antibiotics, one of them being known as efflux pump. This active efflux is able to extrude different compounds from the cell and is therefore seen as a key element in antimicrobial resistance. Currently, more than ten multidrug efflux pump types have been identified for *S. aureus* [11], namely NorA and MepA.

NorA is a fluoroquinolone-resistant efflux pump that expels a set of different antibiotics and other substances, such as norfloxacin, ciprofloxacin, ethidium bromide and quaternary ammonium compounds [12–14]. MepA belongs to the multidrug and toxin extrusion (MATE) efflux pump family. First described by Kaatz *et al.*[15], it is also found in *S. aureus* mutant strains. Inhibition of the efflux pumps can be achieved by reducing the pump gene expression, interrupting pump assembly, reducing substrate

binding by competitive or non-competitive means and disrupting the pump's required energy source. Compounds that are capable of performing such inhibition are called efflux pump inhibitors (EPI) [16].

Since chalcone derivatives with an heterocyclic thiophene ring have an increased antibacterial activity [17], a mono-carbonyl curcumin derivative with thiophene instead the usual aromatic rings was synthesised - the (1*E*,4*E*)-1,5-Di(thiophen-2-yl)penta-1,4-dien-3-one, henceforth denominated DB thiophene (Scheme 1). This curcuminoid was fully characterized by NMR, Raman and IR spectroscopies, with a complete assignment of bands based on frequency calculations using the DFT approach. In order to test the ability of DB thiophene as an EPI, inhibition tests focused on *S. aureus* strains were performed. The inhibition mechanism of the NorA efflux pump was then studied by means of molecular docking using a NorA protein homolog model.



Scheme 1. DB thiophene structure and atom numbering along with Curcumin structure.

2. Materials and Methods

2.1 Materials

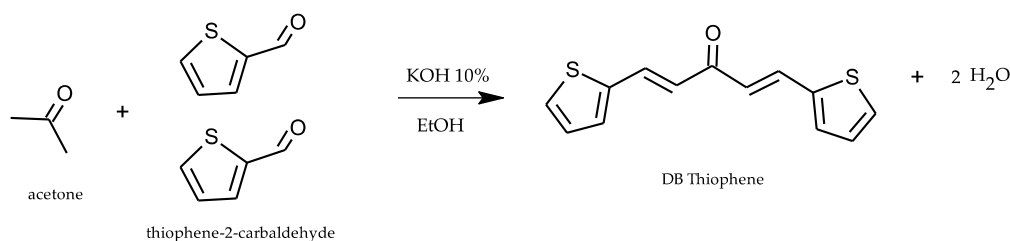
Potassium hydroxide was purchased from Dinâmica Química Contemporânea Ltd. (Indaiatuba, Brazil). Ethanol and acetone were obtained from Vetec Química Fina Ltd (Duque de Caxias, Brazil). Chlorpromazine (CPZ), ciprofloxacin, norfloxacin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), thiophene-2-carbaldehyde and ethidium bromide (EtBr) were acquired from Sigma Aldrich Brasil Ltd. (São Paulo, Brazil). Brain heart infusion (BHI) was purchased from KASVI company (São José do Pinhais, Brazil).

2.2 Synthesis

The (1*E*,4*E*)-1,5-Di(thiophen-2-yl)penta-1,4-dien-3-one was synthesised by the Claisen–Schmidt condensation method [18,19], with thiophene-2-carbaldehyde instead of benzaldehyde. In an Erlenmeyer flask containing 50 mL of KOH 10% (*m/v*) and 40

mL of ethanol, 7 mL of thiophene-2-carbaldehyde:acetone (5:2) was slowly added, under agitation, and the temperature was kept at 0 °C. After 30 minutes, the mixture was filtered: a precipitate was obtained and washed with small portions of distilled water. The compound was redissolved in hot ethanol, filtered and allowed to cool in an ice bath. The formed crystals were washed with cold ethanol and dried at room temperature. Scheme 2 shows the DB thiophene synthesis reaction.

(¹H-NMR (CDCl₃, 500 MHz): δ=7.48 (2H, d, J = 5.1 Hz, H21, H25), 7.15 (2H, m, H22, H26), 7.40 (2H,d, J = 3.6 Hz, H23,H24), 6.89 (2H, d, J = 15.6 Hz, H17, H18), 7.92 (2H, d, J = 15.6 Hz, H19, H20) ¹³C-NMR (CDCl₃, 125 MHz): δ=140.3 (CH, C7, C8), 128.4 (CH, C10, C15), 128.8 (C11, C16), 135.6 (CH, C12 C14), 124.4 (CH, C3, C4), 131.8 (CH, C5, C6), 187.7 (C, C1)); EIMS m/z (M⁺. 246), calcd for: C₁₃H₁₀OS₂/246.



Scheme 2. DB thiophene synthesis reaction.

2.3 Vibrational Spectroscopy

The Raman spectrum was recorded using compacted powder in the sample holder of a Bruker RAM II FT-Raman module, equipped with a liquid nitrogen cooled high-sensitivity Ge detector, and coupled to a Bruker Optics VERTEX 70v FTIR spectrometer. The 1064 nm line of a Nd:YAG laser was used as the excitation radiation, with a nominal laser power of 150 mW. The spectrum was the sum of 60 scans, at a typical resolution of ca. 4 cm⁻¹.

The FTIR-ATR spectrum was measured at room temperature using a spectrometer Bruker Vertex 70v equipped with a RT-DLaTGS (Deuterated Lanthanum α -Alanine doped TriGlycine Sulphate) detector and a Wide Range MIR-FIR. The spectra were recorded in the region 130 to 4000 cm⁻¹ with a resolution of 2 cm⁻¹ and accumulating 32 scans *per* spectrum. A Blackman–Harris three-term apodization function and the Mertz search phase correction algorithm were used.

2.4 NMR and GC-MS

The chemical reagents were from Sigma-Aldrich. ^1H and ^{13}C NMR spectra were obtained using a Bruker Spectrometer, model Avance DPX - 300 and model Avance DRX-500 operating at a frequency of 300 MHz and 500 MHz for hydrogen, 75 MHz and 125 MHz for carbon respectively. The spectra were measured in CDCl_3 solvents at 27°C and chemical shifts are reported as δ values in parts per million (ppm) relative to tetramethylsilane (δ 0.00) as the internal standard. The mass spectra were obtained with a Shimadzu QP201 GC-MS (Gas Chromatography coupled to Mass Spectrometry) using RTX-5MS capillary column (30.0m x 0.25mm x 0.30mm) for compounds within the literature record.

2.5 Computational Details

Density Functional Theory (DFT) calculations [20] were performed using the Gaussian 09 program [21]. Initial coordinates were generated according to the NMR results. The B3LYP exchange-correlation functional [22] was applied, combined with the 6-31G* basis set. All geometries were fully optimised within the Berny algorithm, using redundant internal coordinates and considering the Gaussian default convergence criteria. Vibrational wavenumber calculations were carried out for the optimised geometries, at the same theory level, to verify convergence to a real minimum within the potential energy surface (no negative eigenvalues) and to assist in the vibrational mode description.

For an accurate comparison between the calculated and experimental vibrational wavenumbers, the former were corrected for anharmonicity and incomplete electron correlation treatment, using the scaling factors of 0.960 [23] and 0.942 for predicted values below and above 2000 cm^{-1} , respectively. Vibrational modes were analysed, in terms of their Potential Energy Distribution (PED), using the VEDA [24] program with default optimisation options.

2.6 Evaluation of Efflux Pump Inhibition by MIC Reduction

The evaluation of the efflux pump inhibition was performed according to Tintino *et al.*[12]. The two strains of *S. aureus* used were: SA-1199B, which overexpresses the *norA* gene encoding the NorA efflux protein and the multi-drug resistant (MDR) mutant strain SA-K2068 which presents the MepA efflux pump. The strains were maintained on blood agar base slants and, prior to use, the cells were grown overnight at 37°C in Heart Infusion Agar slants.

Chlorpromazine (CPZ), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and ethidium bromide (EtBr) were used for this essay. Norfloxacin and Ciprofloxacin were the antibiotics used for NorA and MepA efflux inhibition respectively. The antibiotics and DB thiophene were dissolved in dimethyl sulfoxide aqueous solution (5.1%, v/v). The CPZ and EtBr were dissolved in sterile water and CCCP in methanol/water (1:1, v/v). All the compounds were stored at -20 °C, at a final concentration of 4162.60 µM for DB thiophene, 5004.50 µM for CCCP, 3211.44 µM for CPZ, 2597.00 µM for EtBr, 3206.71 µM for norfloxacin and 3090.48 µM for ciprofloxacin.

The Minimal Inhibitory Concentration (MIC) is defined as the lowest concentration in which no growth can be observed [25]. The MIC's of CPZ, CCCP, EtBr and DB thiophene were obtained in a microdilution assay using 150 µL of each strain suspended in saline solution, corresponding to a final concentration of 10⁵ colony-forming units/mL, followed by addition of 1350 µL of brain heart infusion (BHI) broth. Aliquots of 100 µL were transferred to a 96-well multiplate with two-fold serial dilutions by adding 100 µL of each compound solutions with final concentrations ranging from 2.03 to 2081.00 µM for DB thiophene, 2.44 to 2502.25 µM for CCCP, 1.57 to 1605.72 µM for CPZ and 1.27 to 1298.50 µM for EtBr. The trays were incubated at 37 °C for 24 h and bacterial growth was revealed by staining with resazurin.

In order to evaluate the potential inhibition of the efflux pump by DB thiophene, a comparative study was made assessing its ability to decrease the MIC of antibiotics and EtBr (substrate for MDR pumps including NorA [11,13,26,27] and MepA [15,28]), by comparing with CPZ and CCCP which are standard EPI's [29–32]. Eppendorf's were prepared with the EPI's and DB thiophene at a sub-inhibitory concentration (MIC/8), which corresponds to 520.32 µM for DB thiophene, 625.56 µM for CCCP and 401.43 µM for CPZ. Then 100 µL of the Eppendorf's content were transferred to a 96-well microtiter tray with two-fold serial dilution by adding 100 µL of antibiotics and EtBr with a final concentration ranging from 1.27 to 1298.50 µM for EtBr, 1.56 to 1603.36 µM for norfloxacin and 1.50 to 1545.24 µM for ciprofloxacin. The trays were incubated for 24 h at 37 °C and bacterial growth was revealed by staining with resazurin.

2.7 Statistical Analysis of the Microbiological Results

Antibacterial assays were performed in triplicate and results were expressed as an average of the replicates. The results of the tests are expressed as the geometric mean.

Statistical hypothesis analysis was applied using a Two-Way ANOVA followed by the Bonferroni post hoc test (using the GraphPad Prism 5.0 [33] software).

2.8 Docking

The NorA sequence of *S. aureus* 1199 strain (Entry Q03325) was retrieved from the Universal Protein Resource database (Uniprot). The web-based SWISS-MODEL [34] service was used to build a homology model of NorA. The HHblits-based automated mode was used, resulting in 50 different templates, the best one being based on the structure of the *E. coli* YajR transporter (PDB-ID: 3wdo).

The grid box for the docking procedure was defined as a 80Åx80Åx80Å box around the geometrical center of the NorA model. Partial Gasteiger charges were added to ligand atoms while non-polar hydrogen atoms were mixed and rotational bonds determined. Docking studies were carried out using the Lamarckian genetic algorithm in Autodock 4.0 [35]. All other parameters were kept at their default values. The ten best results were chosen by least binding energy.

3. Results and Discussion

3.1 Structural and Conformational Analysis

Figures 1a and 1b display the ¹H NMR and ¹³C NMR spectra of DB thiophene, respectively. The NMR profile (Figure 1a) shows two doublets, one at 6.89 ppm, assigned to H17 and H18 (J=15.6 Hz), and another at 7.92 ppm, due to H19 and H20 (J=15.6 Hz). Their coupling constants confirm the stereochemistry E of the double bond. The signals at 7.48 (d, J=5.1 Hz), 7.40 (d, J=3.6 Hz) and 7.15 ppm (m) are ascribed to the hydrogens of the thiophene ring. In the ¹³C spectra the signal from the unsaturated carbonyl is at 187.7 ppm, while the ketone peak is seen at 203.8 ppm. However, the presence of unsaturation causes a shift of the latter to high field. The charge delocalisation is either caused by the aromatic ring (thiophene) or by the double bond, rendering the carbonylic carbon less electron deficient. The olefinic carbons C3 and C4 are observed at 124.4 ppm, while C5 and C6 appear at 131.8 ppm. The carbon atoms belonging to the thiophene rings are at 140.3, 135.6, 128.8 and 128.4 ppm. The chromatogram (Figure S1) confirms the presence of only one compound, thereby indicating the analytical purity of the compound. ESIMS and a proposed fragmentation scheme are provided as supplementary material as Figure S2 and Scheme S1, respectively.

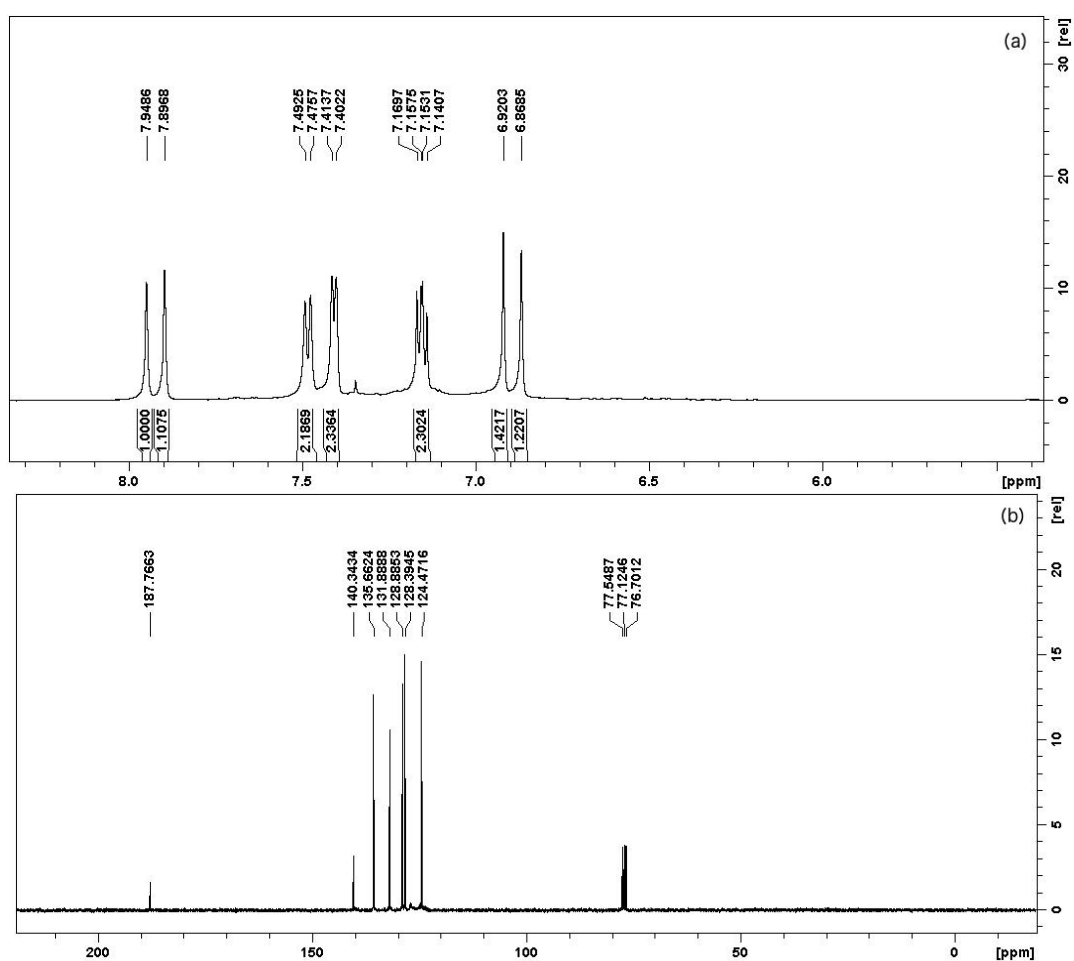


Figure 1. (a) ¹H-NMR Spectrum of DB thiophene (b) ¹³C-NMR Spectrum of DB thiophene.

Rotations around the single bonds of the enone groups lead to three possible conformational isomers, namely: *cis-cis*, *cis-trans* and *trans-trans* isomers, henceforth denominated C0, C1 and C2, respectively (Figure 2). DFT calculations performed on the isolated molecule show that the *trans-trans* conformation, *i.e.* the C2 conformer, is the preferred geometry (at least *in vacuo*). The calculated SCF (self-consistent field method) energies for the C0 and C1 isomers are 10.0 and 5.0 kJ mol⁻¹ higher, respectively. This is consistent with the crystal structure reported by Murugavel *et al.* [36], in which the asymmetric unit of the compound was found to be in *trans-trans* conformation.

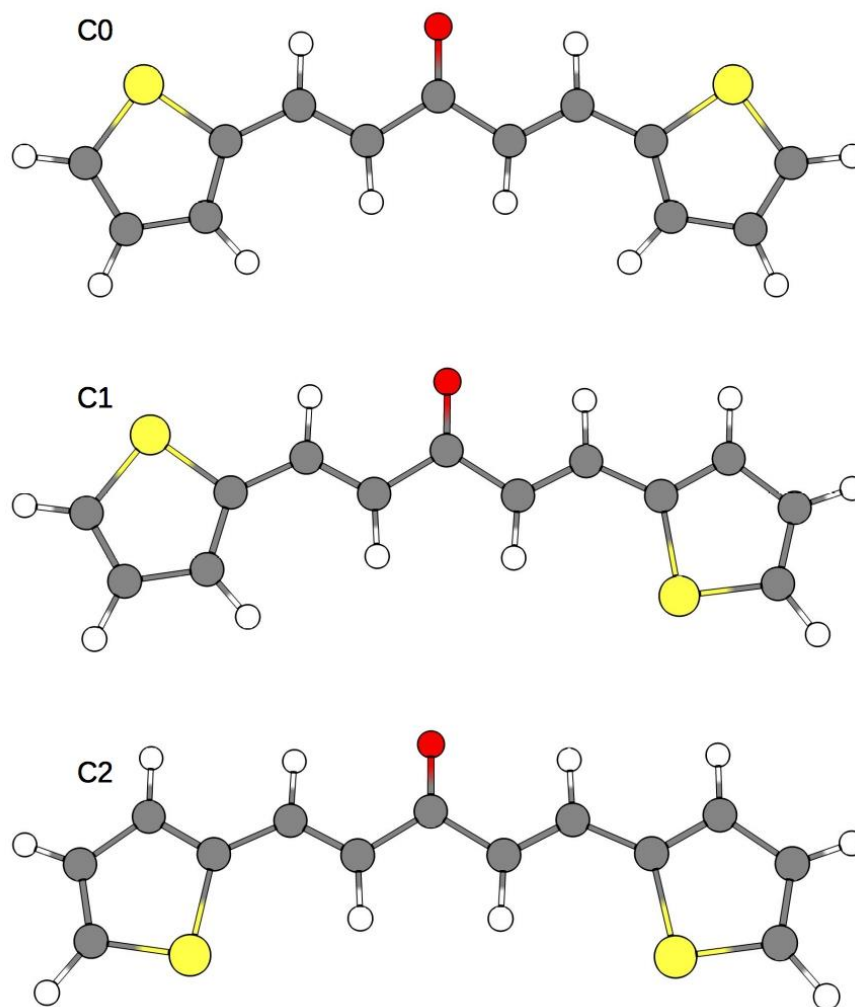


Figure 2. Representation of the three conformers of DB thiophene.

3.2 Vibrational Analysis

The DB thiophene molecule contains 26 atoms, yielding 72 vibrational modes. The Raman and IR spectra of the compound, as well as the calculated spectra for the considered conformers are shown in Figures 3a and 3b. Comparison of spectra for wavenumbers higher than 2000 cm^{-1} are given in Figures S3 and S4 (Supplementary information).

The calculated Raman profile of C1 is the one which matches more closely the experimental data, although the three conformers of the compound exhibit almost the same modes at roughly the same frequencies, within 10 cm^{-1} of each other, which hardly affects the overall assignment. These differences will be discussed below. A conformation consistent with C1 model, shown by calculated Raman profile, is

corroborated by the fact that the experimental dipole moment measured previously[37] (3.19D) is closer to the theoretical calculated C1 value (3.11D) than C0 (4.15D) and C2 (1.98D).

Table 1 comprises the Raman and IR data for DB thiophene, as well as the corresponding assignments. The calculated vibrational wavenumbers and Potential Energy Distribution (PED) analysis, for all three conformers of the compound, are comprised in Tables S1, S2 and S3 (Supplementary information).

Table 1. Observed (Raman and Infrared) and calculated vibrational wavenumbers (cm^{-1}) for DB thiophene (C1).

ω_{IR}	ω_{Raman}	ω_{Calc}	Assignment
3088	3088	3081	$\nu(\text{CH})_{\text{ring}}$
3071	3074	3079	$\nu(\text{CH})_{\text{ring}}$
3044		3047	$\nu(\text{CH})_{\text{ring}}$
3013	3012	3034	$\nu(\text{CH})_{\text{ring}}$
	2990	2900	$\nu(\text{CH})_{\text{enone}}$
1662	1658	1673	$\nu(\text{OC})_{\text{enone}} + \nu(\text{CC})_{\text{enone}}$
1602	1604	1599	$\nu(\text{CC})_{\text{enone}}$
1562	1567	1573	$\nu(\text{OC})_{\text{enone}} + \nu(\text{CC})_{\text{enone}}$
1512	1512	1517	$\nu(\text{CC})_{\text{ring}} + \delta(\text{HCC})_{\text{ring}}$
	1425	1426	$\nu(\text{CC})_{\text{ring}} + \delta(\text{HCC})_{\text{ring}}$
1419	1420	1417	$\nu(\text{CC})_{\text{ring}} + \delta(\text{HCC})_{\text{ring}}$
1376	1376	1355	$\nu(\text{CC})_{\text{ring}} + \delta(\text{HCS})_{\text{ring}} + \delta(\text{HCC})_{\text{ring}}$
1359	1359	1351	$\nu(\text{CC})_{\text{ring}} + \delta(\text{HCS})_{\text{ring}}$
1319		1307	$\nu(\text{CC})_{\text{enone}} + \delta(\text{HCC})_{\text{enone}}$
1307	1309	1299	$\nu(\text{CC})_{\text{enone}} + \delta(\text{HCC})_{\text{enone}}$
1270	1271	1258	$\delta(\text{HCC})_{\text{enone}} + \delta(\text{HCC})_{\text{ring}}$
1224	1225	1232	$\delta(\text{HCS})_{\text{ring}} + \delta(\text{HCC})_{\text{ring}}$
1199	1199	1203	$\nu(\text{CC})_{\text{enone}} + \delta(\text{CCC})_{\text{ring}}$
1169		1185	$\nu(\text{CC})_{\text{ring}} + \delta(\text{HCC})_{\text{enone}} + \delta(\text{HCC})_{\text{ring}}$
1106	1095	1115	$\nu(\text{CC})_{\text{ring}} + \nu(\text{CC})_{\text{enone}} + \delta(\text{CCC})_{\text{ring}}$
1095	1088	1079	$\nu(\text{CC})_{\text{enone}} + \delta(\text{HCS})_{\text{ring}} + \delta(\text{HCC})_{\text{ring}}$
1045	1047	1034	$\nu(\text{CC})_{\text{ring}} + \delta(\text{HCC})_{\text{ring}}$
1038	1041	1025	$\nu(\text{CC})_{\text{ring}} + \delta(\text{CCC})_{\text{ring}} + \delta(\text{HCC})_{\text{ring}}$

967		977	$\tau(\text{HC}_{\text{enone}}\text{C}_{\text{enone}}\text{C}_{\text{ring}}) + \tau(\text{HC}_{\text{enone}}\text{C}_{\text{ring}}\text{C}_{\text{ring}})$
959	963	950	$\nu(\text{CC})_{\text{enone}} + \delta(\text{CCC})_{\text{enone}}$
873		881	$\tau(\text{HCSC})_{\text{ring}} + \tau(\text{HCCC})_{\text{ring}} + \tau(\text{CCCC})_{\text{ring}}$
856		867	$\tau(\text{HCCC})_{\text{enone}} + \gamma(\text{OCCC})_{\text{enone}}$
836		837	$\nu(\text{SC})_{\text{ring}} + \delta(\text{CCC})_{\text{ring}}$
814	815	825	$\tau(\text{HCCC})_{\text{enone}} + \tau(\text{HCCC})_{\text{ring}}$
	788	801	$\delta(\text{OCC})_{\text{enone}} + \delta(\text{CCC})_{\text{enone}}$
727		727	$\nu(\text{SC})_{\text{ring}} + \delta(\text{CCC})_{\text{ring}}$
711	705	716	$\nu(\text{SC})_{\text{ring}} + \delta(\text{CCC})_{\text{ring}}$
681	680	679	$\delta(\text{OCC})_{\text{enone}} + \delta(\text{CCC})_{\text{ring}} + \delta(\text{SCC})_{\text{ring}}$
666		675	$\tau(\text{HCSC})_{\text{ring}} + \gamma(\text{OCCC})_{\text{enone}}$
579		584	$\nu(\text{SC})_{\text{ring}} + \delta(\text{CCC})_{\text{ring}} + \delta(\text{SCC})_{\text{ring}}$
564		559	$\tau(\text{CCCC})_{\text{ring}} + \tau(\text{SCCC})_{\text{ring}}$
503	504	480	$\delta(\text{CCC})_{\text{enone}}$
477		478	$\tau(\text{CCCC})_{\text{ring}} + \tau(\text{C}_{\text{ring}}\text{C}_{\text{ring}}\text{C}_{\text{ring}}\text{C}_{\text{enone}}) + \tau(\text{SCCC})_{\text{ring}}$
397		375	$\delta(\text{CCC})_{\text{enone}} + \delta(\text{C}_{\text{ring}}\text{C}_{\text{ring}}\text{C}_{\text{enone}}) + \delta(\text{C}_{\text{enone}}\text{C}_{\text{ring}}\text{S})$
314		303	$\tau(\text{C}_{\text{ring}}\text{C}_{\text{ring}}\text{C}_{\text{ring}}\text{C}_{\text{enone}}) + \tau(\text{CCCC})_{\text{enone}} + \tau(\text{SCCC})_{\text{ring}}$
264		263	$\delta(\text{OCC})_{\text{enone}} + \delta(\text{CCC})_{\text{enone}} + \delta(\text{C}_{\text{enone}}\text{C}_{\text{ring}}\text{S})$
191	197	196	$\tau(\text{CCCC})_{\text{enone}}$
	71	88	lattice

The IR absorptions in the 3020-3100 cm^{-1} region arise from the CH stretching vibrations. In this region the Raman spectrum shows two bands, at 3088 and 3074 cm^{-1} , both assigned to CH stretching modes of the thiophene rings. Two other Raman bands at 3012 and 2990 cm^{-1} are due to the antisymmetric and symmetric olefinic CH stretching modes, respectively.

In the fingerprint region, there are two weak Raman bands at 1658 and 1604 cm^{-1} . The former was assigned as a combination of stretching modes of the carbonyl group and of the C=C bonds, while the latter was attributed to stretching of CC bonds. A very strong Raman band at 1567 cm^{-1} was assigned to a combination of the carbonyl and C=C stretchings. For curcumin, the intense band related to the diketone stretching mode is recorded at 1626 cm^{-1} [38]. On the IR spectrum, said bands appear at 1662, 1602 and 1562 cm^{-1} , respectively.

A strong Raman band at 1419 cm^{-1} was assigned to a combination of $\nu(\text{CC})$ and $\delta(\text{HCC})$ bands of the thiophene rings. Accordingly, this mode appears at 1425 cm^{-1} in thiophene substituted chalcone [39]. In the IR spectrum, the same mode is seen as a medium intensity absorption at 1419 cm^{-1} .

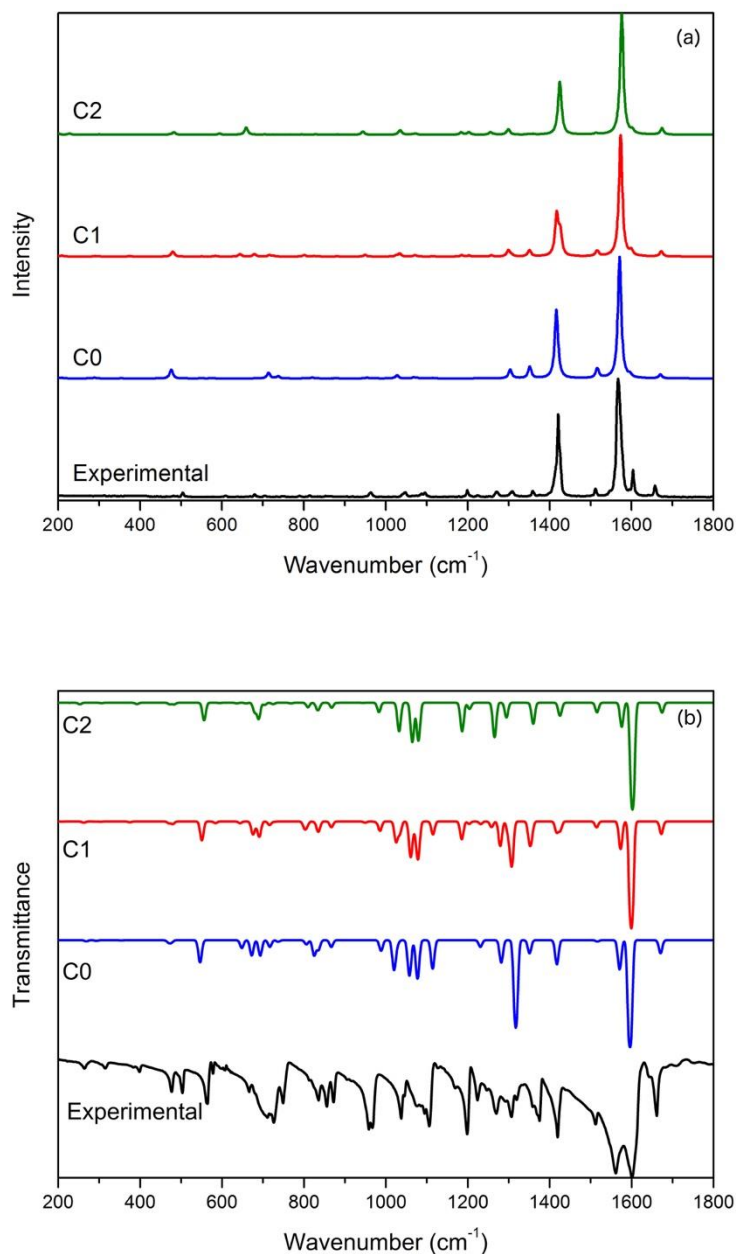


Figure 3. Raman (a) and IR (b) spectra of DB thiophene.

In the Raman spectrum, two very weak modes occur at 1359 and 1309 cm^{-1} , that were assigned as a combination of $\nu(\text{CC})$ and $\delta(\text{HCS})$ of the thiophene rings, and as $\nu(\text{CC})+\delta(\text{HCC})$ of the enone spacers, respectively. The three very weak bands at 1271 ,

1225 and 1199 cm^{-1} are not visible for the C0 conformer, appearing as very weak bands for the other two. The first one was assigned as HCC bending modes of both enone and thiophene rings, while the second is a combination of $\delta(\text{HCS})$ and $\delta(\text{HCC})$ of the thiophene atoms. The third band was assigned as $\nu(\text{CC})$ of the enone spacer and $\delta(\text{CCC})$ of the rings. Three medium intensity features appear at 873, 856 and 836 cm^{-1} : the first two are torsional modes from the rings and the enones, while the last was assigned to a S-C stretching and a C-C-C bending modes. Finally, an isolated weak Raman band at 504 cm^{-1} was ascribed to the bending of the enone group. The equivalent IR band is a medium absorption at 503 cm^{-1} .

3.3 Efflux Pump Modulation

Unlike the standard inhibitors, DB thiophene did not reveal clinically relevant antibacterial activity, with a MIC $\geq 4162.60 \mu\text{M}$. However, when used in association with norfloxacin against SA-1199B, significant synergistic effects were noted - DB thiophene was able to reduce the MIC of norfloxacin by 30%. This modulating effect against NorA efflux pump is due to the inhibition of the efflux pump mechanism. That could be confirmed by examining the association of this curcumin derivative with EtBr, since an identical behaviour was observed.

Similar compounds containing the thiophene rings were already tested as inhibitors of the SA-1199B NorA efflux pump and showed a significant enhancement in the activity of ciprofloxacin, with an increase in the intracellular level of the antibiotic, confirming the mode of action as an EPI [40–42]. In the present experiment, DB thiophene led to an effective reduction of norfloxacin MIC compared to CCCP. When used in conjunction with EtBr, DB thiophene was effective as CPZ, while there was no statistical difference compared to CCCP. To sum up, the essays indicated that DB thiophene seems to be an inhibitor of NorA when co-administered with norfloxacin, and it is more effective than CCCP (Figure 4a).

The mutant SA-K2068 is recognized as having a multi-drug resistance that is mediated by MepA instead of NorA. The essays show the ability of DB thiophene to inhibit the MepA efflux pump, with a two-fold reduction in MIC of ciprofloxacin. This is a remarkable result since DB thiophene performed better than CPZ, although it was not as effective as CCCP (Figure 4b). We can't make direct comparisons with other curcumin derivatives, since, to the best of our knowledge, there are no studies on NorA or MepA efflux pump inhibition involving other curcuminoids. The mechanism by which DB

thiophene inhibits the NorA or MepA efflux pumps can be explored by molecular docking (to be discussed further on).

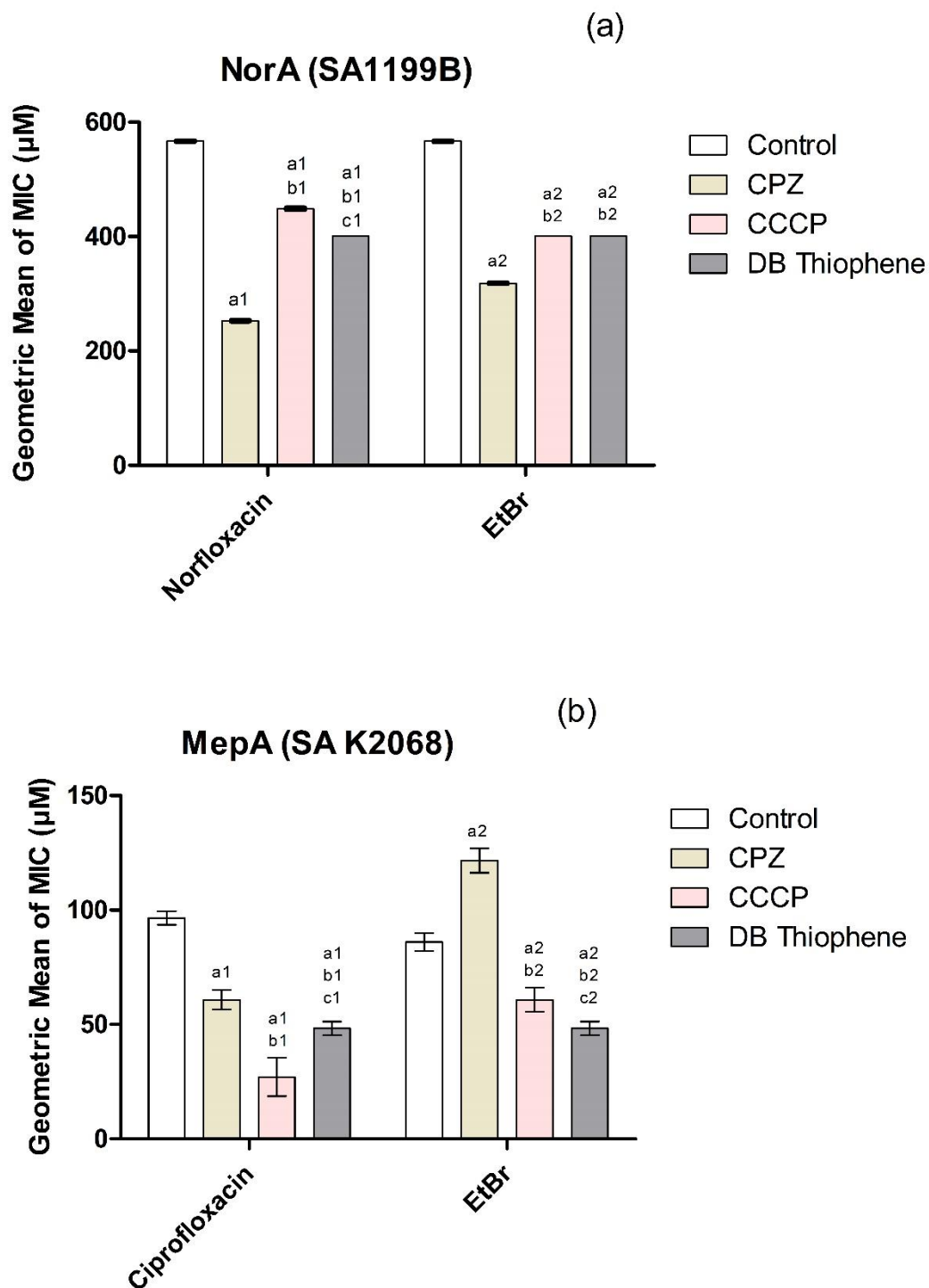


Figure 4. (a) MIC of Norfloxacin and EtBr, in association with the standard inhibitors and in association with DB thiophene against the strain *S. aureus* 1199B. ^{a1,a2} $p < 0.0001$ vs control, ^{b1,b2} $p < 0.0001$ vs CPZ, ^{c1,c2} $p < 0.0001$ vs CCCP.

(b) MIC of Ciprofloxacin and EtBr, in association with the standard inhibitors and in association with DB thiophene against the strain *S. aureus* K2068. ^{a1,a2} $p < 0.0001$ vs control, ^{b1,b2} $p < 0.0001$ vs CPZ, ^{c1,c2} $p < 0.0001$ vs CCCP (bottom).

3.4 Docking Results

Figure 5a shows the five best poses of DB thiophene, inserted in the binding site of the NorA model, with energies ranging from -26.527 to -26.276 kJ/mol. For comparison, Li *et al.* [43] found that the affinity of tetracycline to the same protein was -28.033 kJ/mol. In our simulations, norfloxacin best pose energy was -32.677 kJ/mol. Docking calculations showed that there are two preferred regions in the binding site of the NorA model, labeled regions 1 and 2 (Figure 5a).

Figure 5b displays a 2D ligand-protein interaction diagram, with the contact distances in green. The best pose of DB thiophene is in region 1. In region 2, the C=O group is favorably oriented to form a hydrogen bond with Arg310 (Figure 5b). Norfloxacin best pose overlaps with poses of DB thiophene in both regions, as can be seen in Figure 5a. Additionally, and confirming these results, Dantas *et al.* [44] described the binding site of norfloxacin of a NorA model identical to the one reported here, with interactions with residues 340, 310, 51 and 16. It can thus be argued that DB thiophene hinders the binding of norfloxacin to NorA, being expelled from the bacteria in place of the antibiotic.

All these results suggest that DB thiophene is likely to function as a competitive inhibitor of NorA efflux protein.

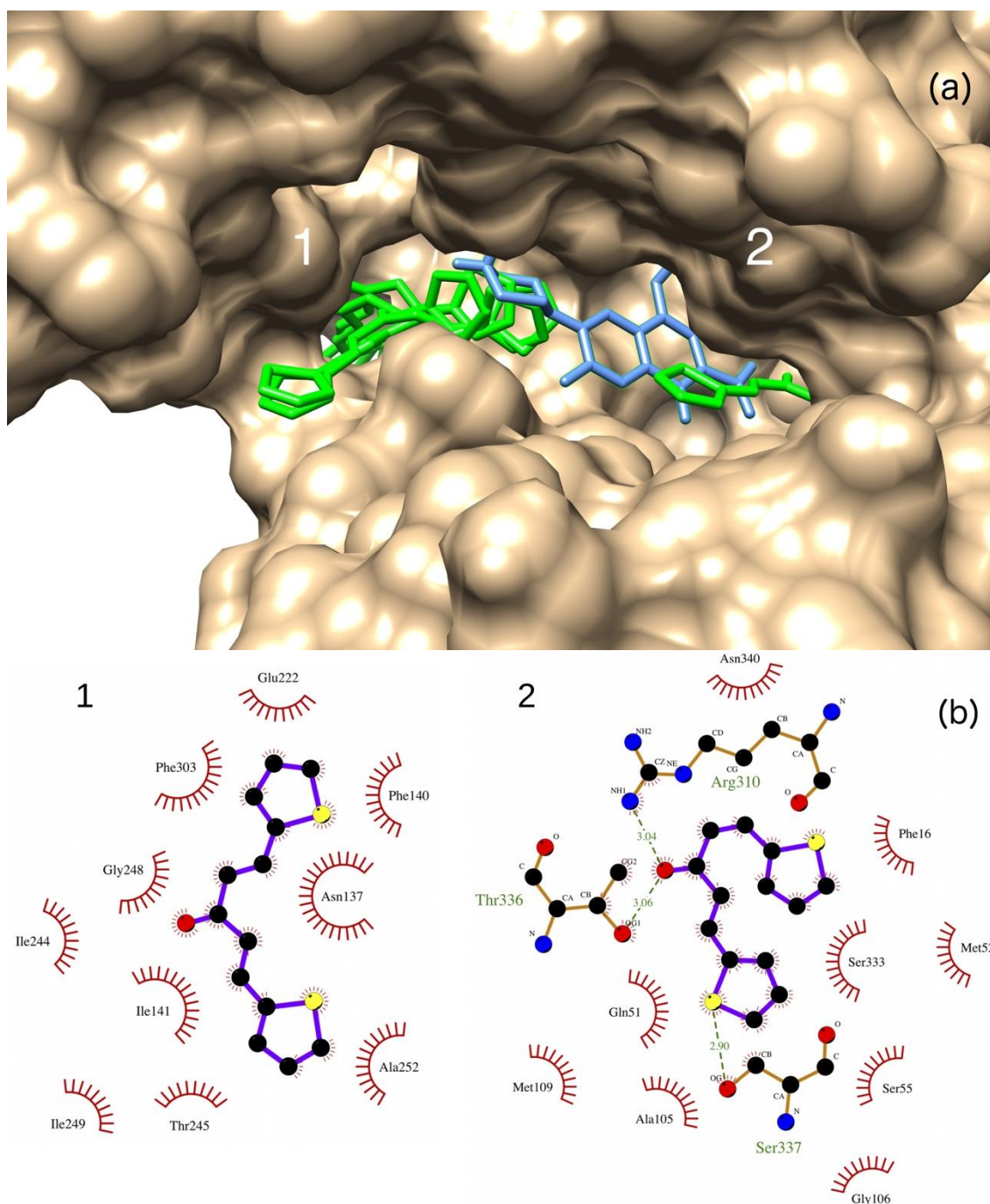


Figure 5. (a) The best five poses of DB thiophene (green) and the best pose of norfloxacin (blue) on the binding site of the NorA model. Regions on the binding site are marked 1 and 2. (b) 2D ligand-protein interaction diagram of DB thiophene and the NorA model on regions 1 and 2.

4. Conclusions

The compound (1*E*,4*E*)-1,5-Di(thiophen-2-yl)penta-1,4-dien-3-one was synthesised and fully characterised. A study on its vibrational properties was performed using FTIR and FT-Raman spectroscopies as well as quantum chemical calculations at the DFT level

(for the three possible conformers of the compound). The experimental and calculated (scaled) vibrational spectra were compared and showed a very good correspondence. These results and the description of the normal modes according to the PED, were used to elucidate the vibrational wavenumber assignments of this synthetic substance. It was shown that this curcuminoid not only inhibits the NorA efflux pump, but also the MepA, thus being a potential new EPI. DB thiophene interacts with the binding site of efflux pump models and it's likely that DB thiophene acts as a competitive inhibitor of efflux pumps.

Author's Contribution

Mauro M. Oliveira – investigation, Writing, original draft, Formal analysis, Helcio S. Santos – investigation, conducted the spectroscopic analysis by NMR, Henrique D. M. Coutinho – conceptualization, conceived the experimental design of the microbiological studies, Paulo N. Bandeira – investigation, performed the synthesis of the compound, Priscila T. da Silva – investigation, performed the synthesis of the compound, Thiago S. Freitas – investigation, conducted the efflux pump inhibition assays, Janaina E. Rocha – investigation, conducted the efflux pump inhibition assays, Jayze C. Xavier – investigation, carried out the antibacterial assays, Fabia F. Campina – investigation, carried out the antibacterial assays, Cristina R. S. Barbosa – investigation, carried out the antibacterial assays, José B. Araújo Neto – investigation, carried out the antibacterial assays, Raimundo L. S. Pereira – investigation, performed modulatory antimicrobial activity assays, Maria M. C. Silva – investigation, performed modulatory antimicrobial activity assays, Débora F. Muniz – investigation, performed modulatory antimicrobial activity assays, Alexandre M.R. Teixeira – investigation, carried out the Raman spectroscopy studies, Vanessa M. Frota – investigation, conducted the analysis by CG-MS, Tigressa H. S. Rodrigues – investigation, conducted the analysis by CG-MS, Ana M. Amado – writing and investigation, reviewed the manuscript and conducted the Raman analysis, Maria P. M. Marques – overviewed the writing, reviewed the manuscript and aided on the conceptualization, Luis A. E. Batista de Carvalho – supervised the experiments, reviewed manuscript, Carlos E. S. Nogueira – investigation, carried out the DFT calculations, supervised and wrote the original draft. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Material

Spectroscopic Characterization and Efflux Pump Modulation of a Thiophene Curcumin Derivative

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Table S1. Calculated vibrational wavenumbers (in cm^{-1}) unscaled and scaled by the scale factor 0.9600 ($\omega < 2000$) and 0.942 ($\omega \geq 2000$), experimental Raman and infrared bands positions in units of cm^{-1} and assignment of vibrational modes for DB thiophene C0.

ω_{calc}	ω_{scal}	ω_{Raman}	ω_{IR}	Assignment of the molecular vibrations with PED (%)
3272	3082	3088vw	3088w	$\nu(\text{C15H25})(95)$
3272	3082	3074vw	3071w	$\nu(\text{C10H21})(95)$
3236	3048		3044vw	$\nu(\text{C11H22})(23) + \nu(\text{C12H23})(28) + \nu(\text{C14H24})(25) + \nu(\text{C16H26})(20)$
3236	3048			$\nu(\text{C11H22})-(20) + \nu(\text{C12H23})-(25) + \nu(\text{C14H24})(28) + \nu(\text{C16H26})(23)$
3222	3035			$\nu(\text{C11H22})(29) + \nu(\text{C12H23})-(26) + \nu(\text{C14H24})-(20) + \nu(\text{C16H26})(22)$
3222	3035	3012vw	3013vw	$\nu(\text{C11H22})-(22) + \nu(\text{C12H23})(20) + \nu(\text{C14H24})-(26) + \nu(\text{C16H26})(29)$
3187	3002			$\nu(\text{C3H17})(47) + \nu(\text{C4H18})(47)$
3178	2994			$\nu(\text{C3H17})-(34) + \nu(\text{C4H18})(34) + \nu(\text{C5H19})(16) + \nu(\text{C6H20})-(16)$
3175	2991	2990vw		$\nu(\text{C5H19})(47) + \nu(\text{C6H20})(46)$
3173	2989			$\nu(\text{C3H17})(15) + \nu(\text{C4H18})-(15) + \nu(\text{C5H19})(34) + \nu(\text{C6H20})-(35)$
1740	1671	1658w	1662s	$\nu(\text{O2C1})(56) + \nu(\text{C4C6})-(11) + \nu(\text{C5C3})-(11)$
1663	1596	1604m	1602vs	$\nu(\text{C4C6})-(30) + \nu(\text{C5C3})(30)$
1636	1571	1567vs	1562vs	$\nu(\text{O2C1})-(31) + \nu(\text{C4C6})-(21) + \nu(\text{C5C3})-(21)$
1580	1517	1512w	1512s	$\nu(\text{C10C11})(11) + \nu(\text{C15C16})-(12) + \nu(\text{C7C14})(20) + \nu(\text{C12C8})-(19)$
1579	1516			$\nu(\text{C10C11})-(12) + \nu(\text{C15C16})-(11) + \nu(\text{C7C14})(19) + \nu(\text{C12C8})(21)$
1477	1418	1425s		$\nu(\text{C10C11})(17) + \nu(\text{C15C16})-(17)$
1475	1416	1420vs	1419s	$\nu(\text{C10C11})(17) + \nu(\text{C15C16})(17)$
1408	1351	1376vw	1376w	$\delta(\text{H21C10S9})-(13) + \delta(\text{H25C15S13})-(12)$
1407	1351	1359w	1359w	$\nu(\text{C11C12})-(10) + \nu(\text{C16C14})(10) + \delta(\text{H21C10S9})-(12)$
				$\delta(\text{H25C15S13})(12)$
1372	1317		1319w	$\nu(\text{C1C4})-(12) + \nu(\text{C3C1})(12) + \delta(\text{H17C3C5})(17) + \delta(\text{H18C4C6})-(17)$
1358	1304	1309w	1307w	$\nu(\text{C4C6})-(11) + \nu(\text{C5C3})-(11) + \delta(\text{H17C3C5})-(12) + \delta(\text{H18C4C6})-(12)$
				$\delta(\text{H19C5C8})(20) + \delta(\text{H20C6C7})(20)$
1335	1282			$\nu(\text{C4C6})-(13) + \nu(\text{C5C3})(13) + \delta(\text{H19C5C8})-(22) + \delta(\text{H20C6C7})(22)$
1323	1270	1271w	1270w	$\delta(\text{H17C3C5})(27) + \delta(\text{H18C4C6})(27) + \delta(\text{H19C5C8})(11) +$
				$\delta(\text{H20C6C7})(11)$
1283	1231	1225vw	1224w	$\delta(\text{H22C11C12})-(11) + \delta(\text{H23C12C11})(18) + \delta(\text{H24C14C16})(18) +$
				$\delta(\text{H26C16C15})(12)$
1282	1231	1199w	1199w	$\delta(\text{H22C11C12})(11) + \delta(\text{H23C12C11})-(17) + \delta(\text{H24C14C16})(18) +$
				$\delta(\text{H26C16C15})(12)$
1161	1115		1169w	$\delta(\text{H24C14C7}) + \delta(\text{H23C12C8}) + \nu(\text{C6C7}) + \delta(\text{C15C16C14}) + \nu(\text{C5C8}) +$
				$\delta(\text{C8C12C11})$
1161	1114	1095w	1106w	$\delta(\text{H24C14C7}) + \delta(\text{H23C12C8}) + \nu(\text{C6C7}) + \delta(\text{C15C16C14}) + \nu(\text{C5C8}) +$
				$\delta(\text{C8C12C11})$
1122	1077	1088w	1095w	$\nu(\text{C1C4})(11) + \nu(\text{C3C1})-(11) + \delta(\text{H21C10S9})(14) + \delta(\text{H22C11C12})(10) +$
				$\delta(\text{H25C15S13})-(14) + \delta(\text{H26C16C15})(10)$
1113	1068			$\nu(\text{C10C11})-(10) + \nu(\text{C15C16})-(10) + \delta(\text{H21C10S9})(24) +$
				$\delta(\text{H25C15S13})(24)$
1102	1058			$\nu(\text{C11C12})-(10) + \nu(\text{C16C14})(10) + \delta(\text{H21C10S9})(11) + \delta(\text{H25C15S13})-($
				$11)$
1071	1028	1047w	1045w	$\nu(\text{C11C12})(13) + \nu(\text{C16C14})(13) + \delta(\text{H23C12C11})(13) +$
				$\delta(\text{H24C14C16})(13)$
1062	1020	1041w	1038w	$\delta(\text{C7C14C16})(10) + \delta(\text{H23C12C11})(10) + \delta(\text{H24C14C16})-(10)$
1030	988			$\tau(\text{H19C5C8C12})-(35) + \tau(\text{H20C6C7C14})(35)$
1021	980		967w	$\tau(\text{H19C5C8C12})(35) + \tau(\text{H20C6C7C14})(35)$
994	954	963w	959w	$\nu(\text{C1C4})(21) + \nu(\text{C3C1})(21) + \delta(\text{C5C3C1})-(10) + \delta(\text{C1C4C6})-(10)$
917	881			$\tau(\text{H22C11C12C8})(25) + \tau(\text{H26C16C15S13})(29)$
917	880		873w	$\tau(\text{H22C11C12C8})(27) + \tau(\text{H26C16C15S13})-(26)$
903	867		856w	$\tau(\text{H17C3C5C8})(33) + \tau(\text{H18C4C6C7})-(33) + \gamma(\text{O2C3C4C1})(15)$
871	836		836w	$\nu(\text{S9C10})(12) + \nu(\text{S13C15})-(12) + \delta(\text{S9C10C11})-(12) + \delta(\text{S13C15C16})(12)$
869	834			$\nu(\text{S9C10})(22) + \nu(\text{S13C15})(22) + \delta(\text{C10C11C12})(12) + \delta(\text{C15C16C14})(14)$
859	825			$\delta(\text{C10C11C12})-(10) + \delta(\text{C15C16C14})(11)$
855	820	815vw	814w	$\tau(\text{H17C3C5C8})(28) + \tau(\text{H18C4C6C7})(28)$
840	806			$\tau(\text{H23C12C11C10})-(30) + \tau(\text{H24C14C16C15})(30)$
833	800	788vw		$\tau(\text{H17C3C5C8})-(10) + \tau(\text{H18C4C6C7})-(10) + \tau(\text{H23C12C11C10})-(24) +$
				$\tau(\text{H24C14C16C15})-(24)$
768	737			$\delta(\text{C7C14C16})(11)$
747	717		727w	$\nu(\text{S9C10})(19) + \nu(\text{S13C15})-(18) + \delta(\text{C10C11C12})-(23) +$
				$\delta(\text{C15C16C14})(21)$
743	714	705vw	711w	$\nu(\text{S9C10})-(10) + \nu(\text{S13C15})-(10) + \delta(\text{C10C11C12})(27) +$
				$\delta(\text{C15C16C14})(26)$

722	693			$\tau(\text{H21C10S9C8})(19) + \tau(\text{H25C15S13C7})-(19) + \gamma(\text{O2C3C4C1})-(26)$
713	684	680vw	681w	$\tau(\text{H21C10S9C8})(36) + \tau(\text{H22C11C12C8})-(11) + \tau(\text{H25C15S13C7})(36) + \tau(\text{H26C16C15S13})(12)$
701	673		666vw	$\tau(\text{H21C10S9C8})(17) + \tau(\text{H25C15S13C7})-(17) + \gamma(\text{O2C3C4C1})(35)$
675	648			$\delta(\text{O2C1C4})(32) + \delta(\text{S9C10C11})(15) + \delta(\text{S13C15C16})-(15)$
600	576		579w	$\delta(\text{S9C10C11})(22) + \delta(\text{S13C15C16})(22)$
580	557		564w	$\tau(\text{C10C11C12C8})(27) + \tau(\text{C15C16C14C7})(29)$
573	550			$\tau(\text{C10C11C12C8})-(26) + \tau(\text{C15C16C14C7})(30)$
569	546			$\delta(\text{O2C1C4})-(19) + \delta(\text{S9C10C11})(17) + \delta(\text{S13C15C16})-(17)$
496	476	504vw	503w	$\delta(\text{C8C5C3})-(12) + \delta(\text{C4C6C7})-(12) + \delta(\text{C3C1C4})(38)$
495	475		477w	$\tau(\text{S9C10C11C12})-(35) + \tau(\text{S13C15C16C14})-(24)$
489	469			$\tau(\text{S9C10C11C12})-(34) + \tau(\text{S13C15C16C14})(23)$
369	354		397vw	$\delta(\text{C8C5C3})(12) + \delta(\text{C4C6C7})-(12) + \delta(\text{C12C8C5})-(12) + \delta(\text{C11C12C8})(12) + \delta(\text{C6C7S13})-(18)$
305	293		314vw	$\tau(\text{C15C16C14C7})(10) + \tau(\text{C11C12C8C5})(12) + \tau(\text{C8C5C3C1})(13) + \tau(\text{C1C4C6C7})-(13)$
300	288			$\tau(\text{C15C16C14C7})-(11) + \tau(\text{C11C12C8C5})(14) + \tau(\text{C8C5C3C1})(15) + \tau(\text{C1C4C6C7})(15) + \tau(\text{S13C15C16C14})-(10)$
280	269		264vw	$\delta(\text{O2C1C4})(20) + \delta(\text{C5C3C1})-(22) + \delta(\text{C1C4C6})(22)$
218	209			$\delta(\text{C12C8C5})-(27) + \delta(\text{C6C7S13})(27) + \delta(\text{C3C1C4})(21)$
200	192	197vw	191vw	$\tau(\text{C5C3C1C4})(38) + \tau(\text{C3C1C4C6})(38)$
175	168			$\nu(\text{C1C4})(11) \nu(\text{C3C1})(11)$
124	119			$\tau(\text{C5C3C1C4})(15) + \tau(\text{C11C12C8C5})-(24) + \tau(\text{C3C1C4C6})-(15) + \gamma(\text{C6C14S13C7})(21)$
107	103			$\delta(\text{C8C5C3})-(20) + \delta(\text{C4C6C7})(20) + \delta(\text{C5C3C1})(10) + \delta(\text{C12C8C5})-(18) + \delta(\text{C6C7S13})-(18) + \delta(\text{C1C4C6})-(10)$
93	90	71w		$\tau(\text{C11C12C8C5})(26) + \tau(\text{C8C5C3C1})-(21) + \tau(\text{C1C4C6C7})-(21) + \gamma(\text{C6C14S13C7})(24)$
79	76			$\tau(\text{C4C6C7C14})(19) + \tau(\text{C12C8C5C3})-(19) + \tau(\text{C8C5C3C1})-(23) + \tau(\text{C1C4C6C7})(23)$
39	37			$\delta(\text{C8C5C3})-(16) + \delta(\text{C4C6C7})-(16) + \delta(\text{C5C3C1})(19) + \delta(\text{C1C4C6})(19) + \delta(\text{C3C1C4})-(19)$
24	23			$\tau(\text{C4C6C7C14})(32) + \tau(\text{C5C3C1C4})(12) + \tau(\text{C12C8C5C3})(33) + \tau(\text{C3C1C4C6})(11)$
22	21			$\tau(\text{C4C6C7C14})-(12) + \tau(\text{C5C3C1C4})(29) + \tau(\text{C12C8C5C3})(12) + \tau(\text{C3C1C4C6})-(29)$

Abbreviation: ν , stretching; δ , bending; τ , torsion; γ , out-of-plane; vw, very weak; w, weak; m, medium; s, strong; vs, very strong.

Table S2. Calculated vibrational wavenumbers (in cm^{-1}) unscaled and scaled by the scale factor 0.9600 ($\omega < 2000$) and 0.942 ($\omega \geq 2000$), experimental Raman and infrared bands positions in units of cm^{-1} and assignment of vibrational modes for DB thiophene C1.

ω_{calc}	ω_{scal}	ω_{Raman}	ω_{IR}	Assignment of the molecular vibrations with PED (%)
3271	3081	3088vw	3088w	$\nu(\text{C10H21})(95)$
3269	3079	3074vw	3071w	$\nu(\text{C15H25})(94)$
3235	3047			$\nu(\text{C11H22})(42) + \nu(\text{C12H23})(53)$
3235	3047		3044vw	$\nu(\text{C14H24})(32) + \nu(\text{C16H26})(62)$
3221	3034			$\nu(\text{C11H22})(53) + \nu(\text{C12H23})-(45)$
3220	3034	3012vw	3013vw	$\nu(\text{C14H24})(67) + \nu(\text{C16H26})-(32)$
3185	3000			$\nu(\text{C3H17})(76) + \nu(\text{C4H18})(16)$
3175	2990	2990vw		$\nu(\text{C4H18})(17) + \nu(\text{C5H19})(76)$
3173	2989			$\nu(\text{C3H17})(16) + \nu(\text{C4H18})-(21) + \nu(\text{C5H19})(15) + \nu(\text{C6H20})(48)$
3170	2986			$\nu(\text{C4H18})(45) + \nu(\text{C6H20})(45)$
1742	1673	1658w	1662s	$\nu(\text{O2C1})(54) + \nu(\text{C3C5})-(10) + \nu(\text{C6C4})-(12)$
1666	1599	1604m	1602vs	$\nu(\text{C3C5})-(26) + \nu(\text{C6C4})(32)$
1639	1573	1567vs	1562vs	$\nu(\text{O2C1})-(32) + \nu(\text{C3C5})-(26) + \nu(\text{C6C4})-(15)$
1580	1517	1512w	1512s	$\nu(\text{C10C11})-(21) + \nu(\text{C8C12})(37) + \delta(\text{H23C12C11})(13)$
1578	1514			$\nu(\text{C15C16})-(21) + \nu(\text{C14C7})(35) + \delta(\text{H24C14C16})(14)$
1485	1426	1425s		$\nu(\text{C15C16})(39) + \nu(\text{C14C7})(14) + \nu(\text{C16C14})-(12) + \delta(\text{H26C16C15})-(12)$
1476	1417	1420vs	1419s	$\nu(\text{C10C11})(33) + \nu(\text{C8C12})(13) + \nu(\text{C12C11})-(16) + \delta(\text{H22C11C12})(14)$
1412	1355	1376vw	1376w	$\nu(\text{C16C14})(18) + \delta(\text{H25C15S13})(16) + \delta(\text{H26C16C15})(11)$
1407	1351	1359w	1359w	$\nu(\text{C10C11})(12) + \nu(\text{C12C11})(15) + \delta(\text{H21C10S9})(23)$
1362	1307		1319w	$\nu(\text{C6C4})(10) + \delta(\text{H18C4C6})(17) + \delta(\text{H19C5C8})-(24)$
1353	1299	1309w	1307w	$\nu(\text{C3C5})(15) + \delta(\text{H17C3C5}) (46)$

1333	1279			$v(C6C4)(11) + \delta(H18C4C6)(24) + \delta(H19C5C8)(30) + \delta(H20C6C7)-(11)$
1311	1258	1271w	1270w	$\delta(H17C3C5)(12) + \delta(H20C6C7)(31) + \delta(H24C14C16)-(11) + \delta(H26C16C15)-(11)$
1283	1232	1225vw	1224w	$\delta(H21C10S9)(14) + \delta(H22C11C12)-(23) + \delta(H23C12C11)(36)$
1254	1203	1199w	1199w	$v(C7C6)(35) + \delta(C16C14C7)-(15)$
1235	1185		1169w	$v(C14C7)-(17) + \delta(H20C6C7)(17) + \delta(H24C14C16)(30)$
1162	1115	1095w	1106w	$v(C8C12)-(12) + v(C12C11)(11) + v(C5C8)-(12) + \delta(C8C12C11)(17)$
1123	1079	1088w	1095w	$v(C1C3)(10) + v(C4C1)-(11) + \delta(H21C10S9)-(11) + \delta(H25C15S13)(16) + \delta(H26C16C15)-(13)$
1116	1071			$v(C15C16)-(12) + \delta(H21C10S9)(18) + \delta(H25C15S13)(27) + \delta(H26C16C15)-(14)$
1105	1061			$v(C10C11)(10) + v(C12C11)(12) + v(C4C1)(12) + \delta(H21C10S9)-(20)$
1077	1034	1047w	1045w	$v(C16C14)-(38) + \delta(H24C14C16)-(30) + \delta(H26C16C15)(15)$
1068	1025	1041w	1038w	$v(C12C11)(19) + \delta(C8C12C11)-(16) + \delta(H22C11C12)(16) + \delta(H23C12C11)(23)$
1027	986			$\tau(H17C3C5C8)-(12) + \tau(H19C5C8C12)(52) + \tau(H20C6C7C14)-(19)$
1018	977		967w	$\tau(H18C4C6C7)(10) + \tau(H19C5C8C12)-(19) + \tau(H20C6C7C14)-(57)$
989	950	963w	959w	$v(C1C3)(27) + v(C4C1)(20) + \delta(C1C3C5)-(11)$
919	882			$\tau(H24C14C16C15)-(21) + \tau(H25C15S13C7)-(10) + \tau(H26C16C15S13)(50) + \tau(S13C15C16C14)-(13)$
917	881		873w	$\tau(H21C10S9C8)(11) + \tau(H22C11C12C8)(53) + \tau(H23C12C11C10)(19) + \tau(C10C11C12C8)-(15)$
903	867		856w	$\tau(H17C3C5C8)-(32) + \tau(H18C4C6C7)(32) + \gamma(O2C3C4C1)-(15)$
872	837		836w	$v(S13C15)(24) + \delta(C15C16C14)(23)$
868	833			$v(S9C10)(38) + \delta(C10C11C12)(21) + \delta(S9C10C11)-(11)$
859	825	815vw	814w	$\tau(H17C3C5C8)-(26) + \tau(H18C4C6C7)-(23) + \tau(H24C14C16C15)-(16)$
841	807			$\tau(H21C10S9C8)-(10) + \tau(H22C11C12C8)-(12) + \tau(H23C12C11C10)(39) + \tau(H24C14C16C15)(18)$
835	801	788vw		$\delta(O2C1C4)(13) + \delta(C3C5C8)(10)$
830	797			$\tau(H17C3C5C8)-(14) + \tau(H18C4C6C7)-(15) + \tau(H23C12C11C10)-(18) + \tau(H24C14C16C15)(24)$
757	727		727w	$v(S13C15)(31) + \delta(C15C16C14)-(13) + \delta(C16C14C7)(13)$
746	716	705vw	711w	$v(S9C10)-(27) + \delta(C10C11C12)(42)$
720	691			$\tau(H21C10S9C8)-(27) + \tau(H25C15S13C7)-(10) + \gamma(O2C3C4C1)(25)$
714	686			$\tau(H21C10S9C8)-(15) + \tau(H25C15S13C7)(55) + \tau(H26C16C15S13)(18)$
708	679	680w	681vw	$\delta(O2C1C4)(14) + \delta(C15C16C14)(24) + \delta(S9C10C11)(12)$
703	675		666vw	$\tau(H21C10S9C8)(29) + \gamma(O2C3C4C1)(34)$
671	644			$\delta(S13C15C16)(50)$
608	584		579w	$v(S13C15)-(10) + \delta(C16C14C7)(15) + \delta(S9C10C11)-(20) + \delta(S13C15C16)-(13)$
583	559		564w	$\tau(C15C16C14C7)(52) + \tau(S13C15C16C14)-(12)$
575	552			$\tau(H23C12C11C10)(12) + \tau(C10C11C12C8)(40) + \tau(C15C16C14C7)(14)$
574	551			$\delta(O2C1C4)-(24) + \delta(C16C14C7)(10) + \delta(S9C10C11)(23)$
500	480	504w	503w	$\delta(C7C6C4)-(14) + \delta(C3C5C8)-(11) + \delta(C4C1C3)(33)$
497	478		477w	$\tau(C15C16C14C7)-(10) + \tau(C16C14C7C6)(11) + \tau(S9C10C11C12)-(17) + \tau(S13C15C16C14)(39)$
490	470			$\tau(S9C10C11C12)-(46) + \tau(S13C15C16C14)-(11) + \gamma(C5C12S9C8)-(14)$
391	375		397vw	$\delta(C7C6C4)-(10) + \delta(C3C5C8)(12) + \delta(C14C7C6)-(18) + \delta(C5C8S9)(16)$
316	303		314vw	$\tau(C16C14C7C6)(33) + \tau(C7C6C4C1)-(22) + \tau(S13C15C16C14)-(17)$
302	290			$\tau(C10C11C12C8)-(18) + \tau(C1C3C5C8)(28) + \tau(S9C10C11C12)-(20) + \gamma(C5C12S9C8)(13)$
273	263		264vw	$\delta(O2C1C4)-(18) + \delta(C6C4C1)-(19) + \delta(C5C8S9)-(10) + \delta(C1C3C5)(25)$
219	210			$\delta(C14C7C6)(25) + \delta(C5C8S9)(26) + \delta(C4C1C3)(22)$
204	196	197vw	191vw	$\tau(C6C4C1C3)-(32) + \tau(C4C1C3C5)-(34)$
182	174			$v(C1C3)(10) + \delta(C3C5C8)(12) + \delta(C6C4C1)(19)$
123	118			$\tau(C6C4C1C3)(13) + \tau(C14C7C6C4)-(11) + \tau(C16C14C7C6)(14) + \tau(C4C1C3C5)-(17) + \gamma(C5C12S9C8)(23)$
103	99			$\delta(C7C6C4)-(23) + \delta(C3C5C8)(18) + \delta(C14C7C6)(18) + \delta(C5C8S9)-(18) + \delta(C1C3C5)-(12)$
92	88	71w		$\tau(C16C14C7C6)(22) + \tau(C7C6C4C1)(21) + \tau(C1C3C5C8)(22) + \gamma(C5C12S9C8)-(23)$
86	82			$\tau(C3C5C8C12)-(15) + \tau(C14C7C6C4)(21) + \tau(C7C6C4C1)(23) + \tau(C1C3C5C8)-(19)$
39	38			$\delta(C7C6C4)(18) + \delta(C3C5C8)(15) + \delta(C6C4C1)-(18) + \delta(C1C3C5)-(17) + \delta(C4C1C3)(19)$
28	27			$\tau(C3C5C8C12)(14) + \tau(C6C4C1C3)(34) + \tau(C14C7C6C4)(36) + \tau(C1C3C5C8)(10)$
23	22			$\tau(C3C5C8C12)(31) + \tau(C6C4C1C3)-(11) + \tau(C4C1C3C5)(42)$

Abbreviation: v, stretching; δ , bending; τ , torsion; γ , out-of-plane; vw, very weak; w, weak; m, medium; s, strong; vs, very strong.

Table S3. Calculated vibrational wavenumbers (in cm^{-1}) unscaled and scaled by the scale factor 0.9600 ($\omega < 2000$) and 0.942 ($\omega \geq 2000$), experimental Raman and infrared bands positions in units of cm^{-1} and assignment of vibrational modes for DB thiophene C2.

ω_{calc}	ω_{scal}	ω_{Raman}	ω_{IR}	Assignment of the molecular vibrations with PED (%)
3269	3079	3088vw	3088w	$\nu(\text{C15H25})(94)$
3269	3079	3074vw	3071w	$\nu(\text{C10H21})(94)$
3235	3047			$\nu(\text{C11H22})(55) + \nu(\text{C12H23})(28)$
3235	3047		3044vw	$\nu(\text{C14H24})(29) + \nu(\text{C16H26})(55)$
3220	3033			$\nu(\text{C11H22})-(29) + \nu(\text{C12H23})(62)$
3220	3033	3012vw	3013vw	$\nu(\text{C14H24})(62) + \nu(\text{C16H26})-(30)$
3180	2995			$\nu(\text{C3H17})(45) + \nu(\text{C4H18})(44)$
3173	2989			$\nu(\text{C3H17})(16) + \nu(\text{C4H18})-(15) + \nu(\text{C5H19})-(32) + \nu(\text{C6H20})(37)$
3171	2987	2990vw		$\nu(\text{C5H19})(47) + \nu(\text{C6H20})(42)$
3168	2985			$\nu(\text{C3H17})-(34) + \nu(\text{C4H18})(34) + \nu(\text{C5H19})-(16) + \nu(\text{C6H20})(15)$
1744	1674	1658w	1662s	$\nu(\text{O2C1})(52) + \nu(\text{C4C6})-(12) + \nu(\text{C5C3})-(12)$
1669	1602	1604m	1602vs	$\nu(\text{C4C6})-(29) + \nu(\text{C5C3})(29)$
1642	1576	1567vs	1562vs	$\nu(\text{O2C1})-(34) + \nu(\text{C4C6})-(19) + \nu(\text{C5C3})-(19)$
1579	1516			$\nu(\text{C10C11})-(11) + \nu(\text{C15C16})(10) + \nu(\text{C7C14})-(18) + \nu(\text{C12C8})(20)$
1576	1513	1512w	1512s	$\nu(\text{C10C11})-(11) + \nu(\text{C15C16})-(12) + \nu(\text{C7C14})(19) + \nu(\text{C12C8})(18)$
1487	1427	1425s		$\nu(\text{C10C11})-(20) + \nu(\text{C15C16})(20)$
1484	1424	1420vs	1419s	$\nu(\text{C10C11})-(19) + \nu(\text{C15C16})-(19)$
1416	1360	1376vw	1376w	$\nu(\text{C11C12})(11) + \nu(\text{C16C14})-(10)$
1404	1348	1359w	1359w	$\delta(\text{H21C10S9})-(11) + \delta(\text{H25C15S13})-(11)$
1354	1299		1319w	$\nu(\text{C4C6})(10) + \delta(\text{H17C3C5})(23) + \delta(\text{H18C4C6})(24) + \delta(\text{H19C5C8})-(10) + \delta(\text{H20C6C7})-(10)$
1348	1294	1309w	1307w	$\delta(\text{H17C3C5})-(18) + \delta(\text{H18C4C6})(18)$
1318	1265			$\delta(\text{H19C5C8})-(25) + \delta(\text{H20C6C7})(24)$
1308	1256	1271w	1270w	$\delta(\text{H19C5C8})-(14) + \delta(\text{H20C6C7})-(14)$
1256	1205			$\nu(\text{C6C7})(15) + \nu(\text{C8C5})-(13) + \delta(\text{C7C14C16})-(11)$
1253	1203	1225vw	1224w	$\nu(\text{C6C7})(18) + \nu(\text{C8C5})(20)$
1236	1186	1199w	1199w	$\delta(\text{H23C12C11})(16) + \delta(\text{H24C14C16})-(16)$
1234	1184		1169w	$\delta(\text{H23C12C11})(14) + \delta(\text{H24C14C16})(14)$
1124	1079	1095w	1106w	$\nu(\text{C1C4})(11) + \nu(\text{C3C1})-(11) + \delta(\text{H21C10S9})(13) + \delta(\text{H22C11C12})(11) + \delta(\text{H25C15S13})-(12) + \delta(\text{H26C16C15})(11)$
1118	1073	1088w	1095w	$\nu(\text{C10C11})(10) + \nu(\text{C15C16})(10) + \delta(\text{H21C10S9})-(24) + \delta(\text{H22C11C12})-(13) + \delta(\text{H25C15S13})-(24) + \delta(\text{H26C16C15})(14)$
1109	1065			$\nu(\text{C1C4})(13) + \nu(\text{C3C1})-(13) + \delta(\text{H21C10S9})-(12) + \delta(\text{H25C15S13})(12)$
1079	1036	1047w	1045w	$\nu(\text{C11C12})(20) + \nu(\text{C16C14})(21) + \delta(\text{H23C12C11})(15) + \delta(\text{H24C14C16})(16)$
1075	1032	1041w	1038w	$\nu(\text{C11C12})(18) + \nu(\text{C16C14})-(17) + \delta(\text{H23C12C11})(16) + \delta(\text{H24C14C16})-(15)$
1024	983			$\tau(\text{H19C5C8C12})-(38) + \tau(\text{H20C6C7C14})(38)$
1016	975		967w	$\tau(\text{H19C5C8C12})-(39) + \tau(\text{H20C6C7C14})-(39)$
984	944	963w	959w	$\nu(\text{C1C4})(26) + \nu(\text{C3C1})(26)$
919	883		873w	$\tau(\text{H22C11C12C8})(21) + \tau(\text{H23C12C11C10})(10) + \tau(\text{H24C14C16C15})-(11) + \tau(\text{H26C16C15S13})(25)$
919	882			$\tau(\text{H22C11C12C8})(25) + \tau(\text{H23C12C11C10})(11) + \tau(\text{H24C14C16C15})(10) + \tau(\text{H26C16C15S13})-(25)$
904	868		856w	$\tau(\text{H17C3C5C8})-(30) + \tau(\text{H18C4C6C7})(30) + \gamma(\text{O2C3C4C1})-(14)$
872	837		836w	$\nu(\text{S9C10})(15) + \nu(\text{S13C15})-(15) + \delta(\text{C10C11C12})(13) + \delta(\text{C15C16C14})-(14)$
867	832			$\nu(\text{S9C10})-(18) + \nu(\text{S13C15})-(18) + \delta(\text{C10C11C12})-(15) + \delta(\text{C15C16C14})-(17)$
862	828	815vw	814w	$\tau(\text{H17C3C5C8})-(24) + \tau(\text{H18C4C6C7})-(24) + \tau(\text{H23C12C11C10})-(10) + \tau(\text{H24C14C16C15})-(10)$
843	810			$\tau(\text{H23C12C11C10})-(28) + \tau(\text{H24C14C16C15})(28)$
828	795	788vw		$\tau(\text{H17C3C5C8})(15) + \tau(\text{H18C4C6C7})(15) + \tau(\text{H23C12C11C10})-(20) + \tau(\text{H24C14C16C15})-(20)$
800	768			$\delta(\text{O2C1C4})-(19) + \delta(\text{C5C3C1})-(10) + \delta(\text{C1C4C6})(10)$
755	725		727w	$\nu(\text{S9C10})(23) + \nu(\text{S13C15})(23) + \delta(\text{C10C11C12})-(12) + \delta(\text{C15C16C14})-(10)$
733	704	705vw	711w	$\nu(\text{S9C10})(14) + \nu(\text{S13C15})-(14) + \delta(\text{C10C11C12})-(20) + \delta(\text{C15C16C14})(19)$
718	690			$\tau(\text{H21C10S9C8})-(21) + \tau(\text{H25C15S13C7})(21) + \gamma(\text{O2C3C4C1})-(20)$
715	686			$\tau(\text{H21C10S9C8})(35) + \tau(\text{H22C11C12C8})-(11) + \tau(\text{H25C15S13C7})(35) + \tau(\text{H26C16C15S13})(12)$
708	680	680vw	681vw	$\tau(\text{H21C10S9C8})-(14) + \tau(\text{H25C15S13C7})(14) + \gamma(\text{O2C3C4C1})(40)$
687	659		666vw	$\delta(\text{C10C11C12})-(13) + \delta(\text{C15C16C14})-(13) + \delta(\text{S9C10C11})-(17) + \delta(\text{S13C15C16})-(18)$

663	636			$\delta(\text{S9C10C11})-(33) + \delta(\text{S13C15C16})(32)$
619	594		579w	$\delta(\text{C11C12C8})-(11) + \delta(\text{S9C10C11})(13) + \delta(\text{S13C15C16})(13)$
584	561		564w	$\tau(\text{C10C11C12C8})(27) + \tau(\text{C15C16C14C7})(32)$
579	556			$\delta(\text{C7C14C16})-(13) + \delta(\text{O2C1C4})(32) + \delta(\text{C11C12C8})(15)$
579	556			$\tau(\text{C10C11C12C8})(27) + \tau(\text{C15C16C14C7})-(34)$
503	483	504vw	503w	$\delta(\text{C8C5C3})-(12) + \delta(\text{C4C6C7})-(12) + \delta(\text{C3C1C4})(35)$
499	479		477w	$\tau(\text{S9C10C11C12})(34) + \tau(\text{S13C15C16C14})(20) + \gamma(\text{C6C14S13C7})(12)$
492	473			$\tau(\text{S9C10C11C12})-(32) + \tau(\text{S13C15C16C14})(20) + \gamma(\text{C6C14S13C7})(13)$
409	392		397vw	$\delta(\text{C8C5C3})-(11) + \delta(\text{C4C6C7})(11) + \delta(\text{C12C8C5})-(17) + \delta(\text{C6C7S13})-(12)$
319	306		314vw	$\tau(\text{C11C12C8C5})(17) + \tau(\text{C8C5C3C1})-(11) + \tau(\text{C1C4C6C7})(11) +$ $\tau(\text{S9C10C11C12})-(10) + \tau(\text{S13C15C16C14})(15)$
313	300			$\tau(\text{C11C12C8C5})(19) + \tau(\text{C8C5C3C1})-(13) + \tau(\text{C1C4C6C7})-(13) +$ $\tau(\text{S9C10C11C12})-(10) + \tau(\text{S13C15C16C14})-(15) + \gamma(\text{C6C14S13C7})(10)$
264	253		264vw	$\delta(\text{O2C1C4})(19) + \delta(\text{C5C3C1})-(24) + \delta(\text{C1C4C6})(24)$
237	228			$\delta(\text{C12C8C5})(20) + \delta(\text{C6C7S13})-(17) + \delta(\text{C3C1C4})(27)$
209	201	197vw	191vw	$\tau(\text{C5C3C1C4})(30) + \tau(\text{C3C1C4C6})(30)$
174	167			$\delta(\text{C5C3C1})-(14) + \delta(\text{C6C7S13})-(10) + \delta(\text{C1C4C6})-(14)$
122	117			$\tau(\text{C4C6C7C14})(15) + \tau(\text{C5C3C1C4})(14) + \tau(\text{C12C8C5C3})-(15) +$ $\tau(\text{C11C12C8C5})(14) + \tau(\text{C3C1C4C6})-(14) + \gamma(\text{C6C14S13C7})-(11)$
102	98			$\delta(\text{C8C5C3})-(21) + \delta(\text{C4C6C7})(21) + \delta(\text{C12C8C5})(18) + \delta(\text{C6C7S13})(19)$
93	90			$\tau(\text{C4C6C7C14})(15) + \tau(\text{C12C8C5C3})-(15) + \tau(\text{C8C5C3C1})-(21) +$ $\tau(\text{C1C4C6C7})(21)$
91	87	71w		$\tau(\text{C11C12C8C5})(21) + \tau(\text{C8C5C3C1})(22) + \tau(\text{C1C4C6C7})(22) +$ $\gamma(\text{C6C14S13C7})(20)$
38	36			$\delta(\text{C8C5C3})-(17) + \delta(\text{C4C6C7})-(17) + \delta(\text{C5C3C1})(16) + \delta(\text{C1C4C6})(17) +$ $\delta(\text{C3C1C4})-(19)$
29	28			$\tau(\text{C4C6C7C14})(30) + \tau(\text{C5C3C1C4})(15) + \tau(\text{C12C8C5C3})(29) +$ $\tau(\text{C3C1C4C6})(16)$
25	24			$\tau(\text{C4C6C7C14})-(11) + \tau(\text{C5C3C1C4})(31) + \tau(\text{C12C8C5C3})(12) +$ $\tau(\text{C3C1C4C6})-(30)$

Abreviation: v, stretching; δ , bending; τ , torsion; γ , out-of-plane; vw, very weak; w, weak; m, medium; s, strong; vs, very strong.

Figure S1. Chromatogram of DB thiophene.

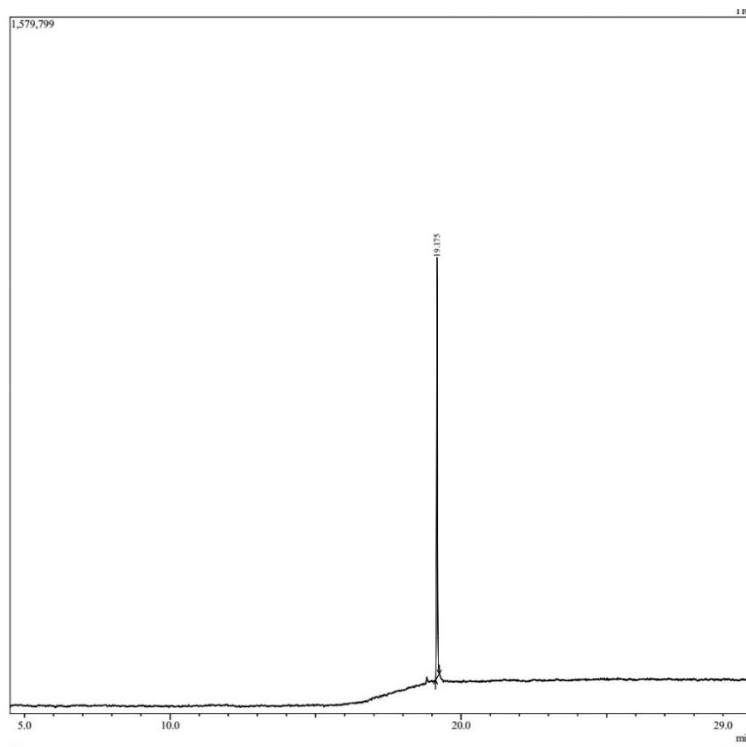


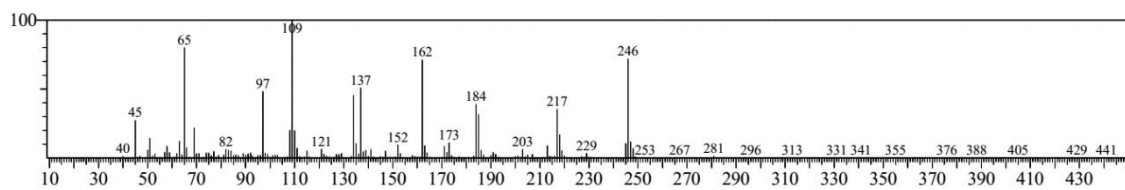
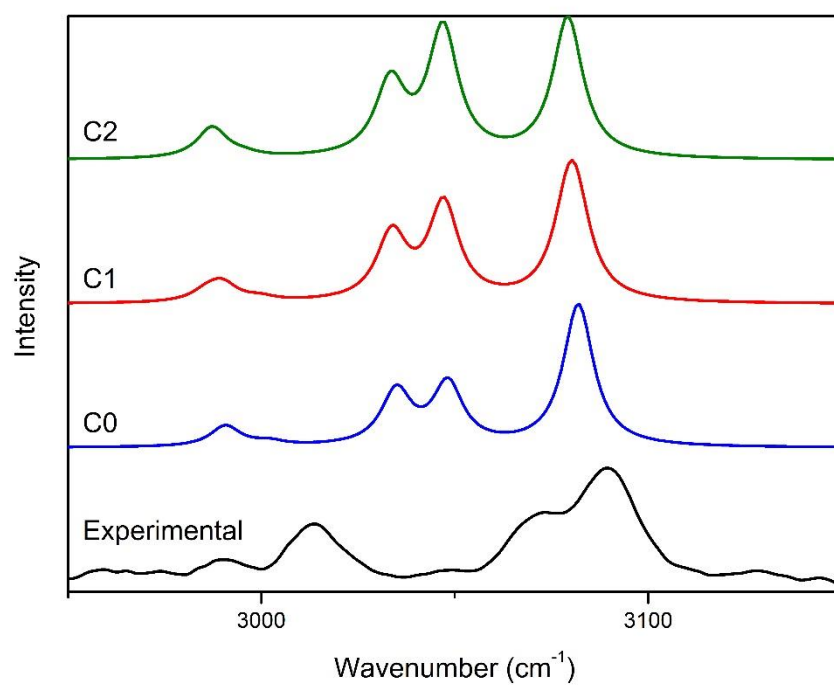
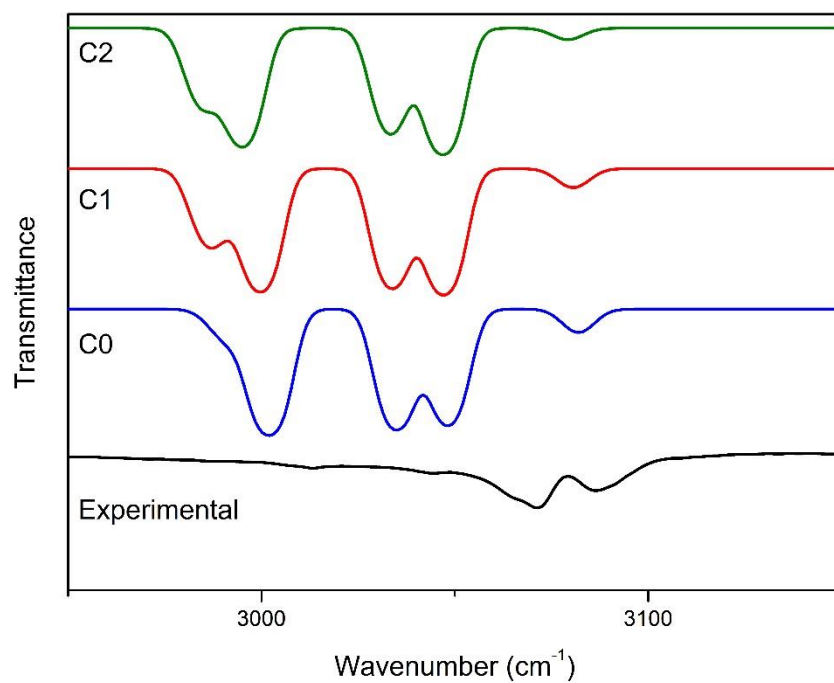
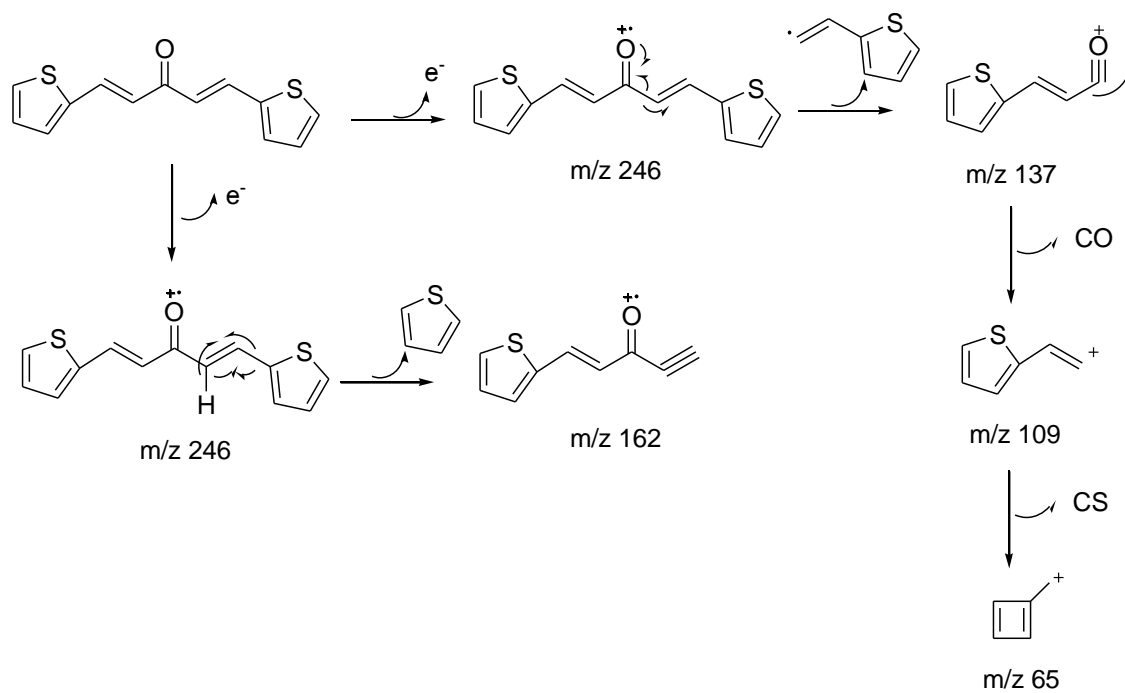
Figure S2. ESIMS of DB thiophene.**Figure S3.** Raman spectra of DB thiophene higher than 2000 cm^{-1} .

Figure S4. IR spectra of DB thiophene higher than 2000 cm^{-1} .**Scheme S1.** Proposed fragmentation of DB thiophene.

CAPÍTULO 3

Full Spectroscopic Characterization and Cytotoxicity Activity of Synthetic Dibenzalacetone Derivatives.

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Abstract

Dibenzalacetone derivatives are organic compounds formed by two α, α' active sites that provide a large relocation of π -electrons. They have great polarizability that make them excellent chromophore and draw attention for their biological properties, mainly for their activity against nasopharyngeal, oral, colon, prostate and cervical cancer. Two dibenzalacetone derivatives, (1*E*,4*E*)-1,5-bis(4-ethoxyphenyl)penta-1,4-dien-3-one and (1*E*,4*E*)-1,5-bis(4-chlorophenyl)penta-1,4-dien-3-one, were synthesized. Spectroscopic characterizations as well as vibrational assignments were predicted using density Functional Theory (DFT) calculations with B3LYP exchange-correlation functional. Cytotoxicity assays of the synthesized compounds were performed against HCT-116, SNB and PC3 cells, showing promising results against cancer cells HCT-116.

Keywords: Dibenzalacetone derivatives; Cytotoxicity; DFT; Spectroscopy, Cancer cells.

1. Introduction

A double condensation reaction of a ketone that has two α, α' - active sites with two equivalents of aromatic aldehyde yields a molecule with an extensive delocalization of π -electrons, which favors an easier polarizability, that is achieved by the presence of strong electron donor (D) and strong acceptor (A) groups with a π -electron bridge joining D and A. These compounds are closely related to the class of organic chromophores known as “bis-chalcones” which have several multifunctional characteristics (pharmacological and biological activities)[1]. In literature, dibenzalacetone derivatives as the ones reported here, are sometimes referred to as bis-chalcones[2–4] or simply chalcone derivatives[5].

Chalcones are a class of naturally occurring or readily synthesized compounds that have a 1,3-diaryl-2-propen-1-one basic framework. They are an open-chain precursor of flavonoids and may exist as *trans* and *cis* isomers, although the *trans* isomer is thermodynamically more stable. Natural chalcones are commonly found in fruits, vegetables, petals, bark, leaves and roots of various trees or plants[6–9]. The chalcone class and its derivatives have attracted a lot of interest, as they have a wide range of bioactivities, e.g.: anti-inflammatory[10], antimicrobial[11], anti-oxidant[12], anticancer[13], antifungal[14], antimalarial[15], anti-protozoal[16], etc. This broad spectrum of biological activity is probably related to its small structure and its characteristics as Michael acceptors, which make them tolerant to different biological molecules and allow them to bind quickly or reactively to them[9].

Several bis-chalcones have shown remarkable anticancer activity against nasopharyngeal, oral, colon, prostate and cervical cancer[13,17]. In a recent study, nine bis-chalcones were tested in vitro for cytotoxic activity against cancer cell lines of lung (A549), prostate (DU-145), nasopharyngeal carcinoma (KB) and vincristine-resistant KB subline (KB-VIN). Best results were obtained in non-substituted phenyl B rings or in *para*-methoxyphenyl B-rings[18]. Other bis-chalcones with methoxy groups inserted at *para* position of phenyl rings have displayed encouraging results by inhibiting tumor-initiating cells in the brain[19]. Lima and collaborators obtained significant antiproliferative effects against SF-295 (central nervous system), HCT-116 (colon cancer) and OVCAR-8 (breast cancer) cell lines using halogenated and nitro bis-chalcone *ortho/para* substituted[20]. Furthermore, heterocyclic rings have exhibited more

cytotoxic potential, especially thiophene-chalcones derivatives. They are known as central structures in the design of new scaffolds with potential pharmacological interest. *Duddukuri et al.* for example synthesized a thiophene-chalcone also containing a *para*-chlorine phenyl ring that inhibited the growth of A549 cell line by inducing apoptosis[21]. Enone bearing enamines derivatives also seem to display anticancer activity[22].

Thus, due to the above, we were motivated to synthesize two dibenzalacetone derivatives, (1*E*,4*E*)-1,5-bis(4-ethoxyphenyl)penta-1,4-dien-3-one and (1*E*,4*E*)-1,5-bis(4-chlorophenyl)penta-1,4-dien-3-one, hereinafter referred to DB ethoxy and DB chlorine, respectively. We conducted a cytotoxicity assay in three human cancer cell line as well as a druglikeness test. In addition, these compounds were fully characterized by NMR, Raman, IR and mass spectroscopies, and all quantum chemical calculations were performed using the Density Functional Theory (DFT) approach. The comparison of the normal modes obtained by theoretical calculations with the experimental spectra allowed us to assign all the normal modes of these molecules. Although these compounds are known and already have their crystal structures solved[23,24], there is not, as far as we know, a complete IR and Raman vibrational spectroscopy characterization or cytotoxic evaluation studies against the strains mentioned.

2. Materials and Methods

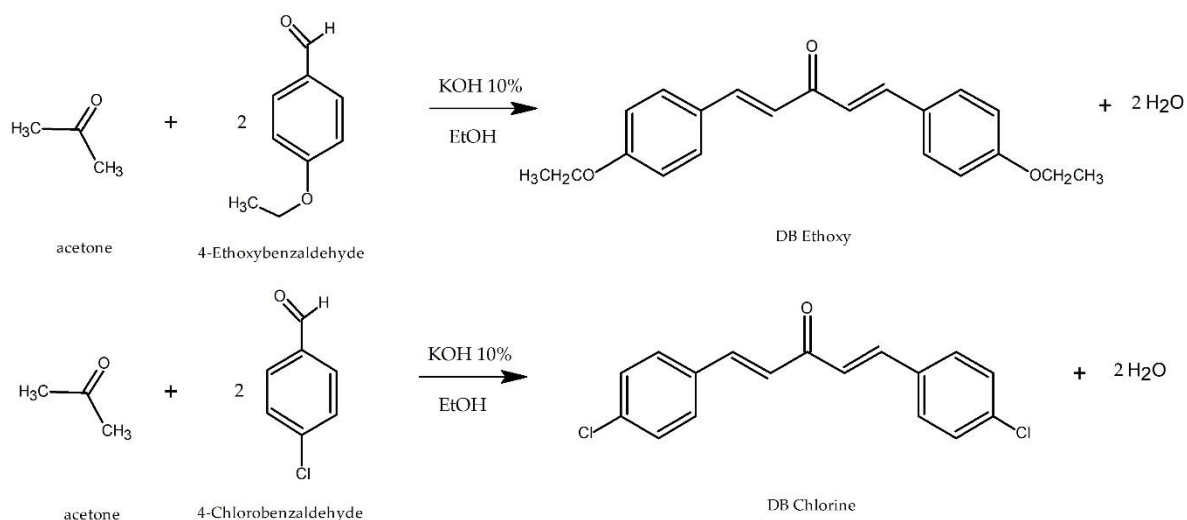
2.1. Materials

The reagents 4-ethoxybenzaldehyde, 4-chlorobenzaldehyde, Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and RPMI-1640 Medium were purchased from Sigma-Aldrich Inc. Etanol, Isopropanol and acetone were obtained from Vetec Química Fina Ltd., and potassium hydroxide (KOH) and hydrochloric acid (HCl) from Dinâmica Química Contemporânea Ltd. Fetal Bovine Serum (FBS) and Phosphate Buffered Saline (PBS) were from Gibco™. All materials were analytical grade.

2.2. Synthesis

DB ethoxy and DB chlorine were obtained by controlled addition of 7mL of 4-ethoxybenzaldehyde:acetone (5:2) and 4-chlorobenzaldehyde:acetona (5:2), respectively, in a mixture of 50mL KOH 10%(w/v) and 40mL of ethanol. These reactions were kept under mechanical stirring at the temperature of 0°C. After 30 minutes the mixtures were

filtered and the precipitates washed with distilled water. Then the compounds were recrystallized using ethanol as solvent. The syntheses were based on Claisen-Schmidt condensation[25,26]. Scheme 1 shows the compounds synthesis reaction.



Scheme 1. DB ethoxy and DB chlorine synthesis reaction.

2.3. Vibrational Spectroscopy

The Raman scattering measurements of DB ethoxy and DB chlorine were recorded in powder form in the wavenumber range from 40 to 4000cm⁻¹ on a Bruker RAM II FT-Raman module coupled to the VERTEX FT-IR spectrometer as well as a liquid nitrogen cooled high-sensitivity Ge detector. The light output (1064 nm) on Nd:YAG laser was used to excite the sample. The resolution of ~ 4 cm⁻¹ with accumulation of 60 scans per spectra and a nominal laser power of 150 mW were used.

FTIR spectra of the compounds were recorded in the region of 600 to 4000 cm⁻¹ due to Attenuated Total Reflectance (ATR) accessory working with a germanium (Ge) crystal with a resolution of 4 cm⁻¹ and accumulating 32 scans per spectrum. The module used was an Agilent Cary 660 FTIR spectrometer. The measures were made in the room temperature.

2.4. NMR and CG/MS

NMR experiments were performed on a Bruker Avance DRX-500 spectrometer (¹H 500 MHz, ¹³C 125 MHz) equipped with a 5mm inverse detection z-gradient probe. ¹H and ¹³C NMR spectra were measured at 27°C using CDCl₃ as the solvent.

Tetramethylsilane (δ 0.00) was used as the internal standard. The GC/MS analyses were conducted on an Agilent instrument model GC-7890B/MSD-5977A with a quadrupole mass analyzer of electron impact at 70 eV and a RTX5-MS (30 m \times 0.25 mm \times 0.25 μ m) capillary column. The mass spectrometer was adjusted for acquisition in the range of 40–660 m/z. The injector temperature was kept constant at 230 °C with a helium flow rate of 1.00 mL min⁻¹ (carrier gas). The temperatures of the source and transfer lines were set at 230 °C and 280 °C, respectively. The following program was used in the chromatographic oven: initial temperature of 40 °C, with a heating ramp from 3 °C min⁻¹ to 280 °C for 5 min at the end of the run.

2.5. Computational details

The quantum calculations were performed at the Gaussian 09 package[27] using Density Functional Theory[28] to optimize geometry with B3LYP exchange-correlation functional[29] and 6-31G(d,p) basis set in the gas phase. Initial coordinates were generated according to the NMR results. Harmonic vibrational frequencies were dual scaled as proposed by Halls *et al.*[30]. Two scaling factors were obtained by mean least squares for each model in the regions above and below 1800cm⁻¹. The scaling factors for DB chlorine, DB ethoxy, DB ethoxy monohydrated and DB ethoxy trihydrated models were 0.9712 and 0.9476, 0.9716 and 0.9449, 0.9702 and 0.9440, 0.9694 and 0.9430 respectively (Figure S16). All of these values are in agreement with NIST - Standard Reference Database[31]. The fundamental normal modes were assigned and the Potential energy distribution (PED) calculations were carried out by VEDA (Vibrational Energy Distribution Analysis) with default optimization options[32]. Next, the ¹H and ¹³C NMR calculations were done using the GIAO method[33–36] available in Gaussian 09[27] at B3LYP/6-311++G(d,p) level of theory with chloroform as an implicit solvent to compute the magnetic isotopic shielding for the hydrogen (σ_H) and carbon (σ_C) atoms. To determine the theoretical chemical shift for the hydrogen (δ_H) and for the carbon (δ_C) atoms, the tetramethylsilane (TMS) reference molecule was optimized at B3LYP/6-311++G(d,p) level of theory and the NMR magnetic isotopic shielding for the hydrogen ($\sigma_{H(TMS)}$) and carbon ($\sigma_{C(TMS)}$) atoms were determined at the same level of theory. Hence, the theoretical chemical shift were calculated using the relations as follows: $\delta_H = \sigma_{H(TMS)} - \sigma_H$ and $\delta_C = \sigma_{C(TMS)} - \sigma_C$.

2.6. Cell lines

The three human cancer cell lines used in cytotoxicity assay were: HCT-116 (colon carcinoma), SNB (glioblastoma) and PC3(prostate). They were provided by National Cancer Institute (USA). The cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics, in 5% CO₂ atmosphere at 37°C.

2.7. Cytotoxicity assay

The dibenzalacetone derivatives were initially dissolved in DMSO with a concentration of 2mM, maintained at -20°C until use. The Cytotoxicity of the samples were evaluated by Colorimetric MTT assay[37]. MTT was dissolved in PBS at 5 mg/mL and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. Before the test itself, a calibration curve was prepared with cells plated in 96-well trays in double dilutions starting with 10⁵ cells/well. MTT solution stock (0.01mL) was added to all wells and the plate was incubated at 37°C for 3 h. Acid isopropanol (0.1mL of HCl 0.04 M in isopropanol) was added to each well and mixed. After a few minutes at room temperature to ensure dissolution of all dark blue crystals, optical density (absorbance) was read at wavelength of 595 nm. Regression calculations were fulfilled with all points in triplicate in order to obtain the best line that represents the direct proportionalide that exists between the number of living cells and the absorbance of the medium. For the cytotoxicity assay 0.1mL of neoplastic cells growth medium were plated in a 96-well tray (0.7 x 10⁵ cells/mL) and then the compounds were added after 24 hours with a final concentration of 10µM. The plates were then incubated at 37°C in 5% CO₂ atmosphere. The colorimetric MTT procedure was performed after 72 hours, the optical density was recorded at the same wavelength and the reduction in cell viability was calculated with the aid of the calibration curve. Doxorubicin (Dox, 1 µM) was used as the positive control.

2.8. Statistical analysis

Each compound was tested in triplicate from two independent experiments and the results were expressed as reduction in cell viability (% RCV) ± standard error of the mean (SEM). Statistical hypothesis analysis was applied using a Two-Way ANOVA followed by the Bonferroni post hoc test using the GraphPad Prism 5.0[38].

2.9. *In silico* drug-likeness evaluation

In order to predict the potential of said compounds to become a medicine, we driven an in silico evaluation of physicochemical properties, pharmacokinetics, drug-likeness and medicinal chemistry friendliness tests on SwissADME[39], a free-on-line platform that gives free access to a pool of predictive models.

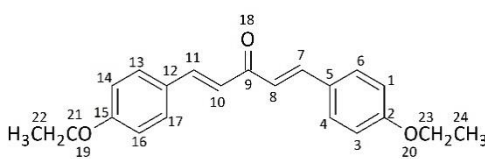
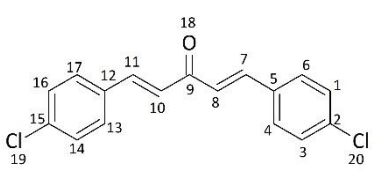
3. Results and Discussion

3.1. Structural and Conformational Analysis

NMR spectral data for DB ethoxy and DB chlorine are shown in Table 1 and the ^1H NMR (CDCl_3 , 300MHz) and ^{13}C NMR (CDCl_3 , 125MHz) spectra are presented in the Supplementary Material on figures ranging from S1 to S8. In the ^1H NMR spectrum, it is possible to observe a couple of doublets for both dibenzalacetone derivatives. At 6.94 (d, $J = 15.3 \text{ Hz}$) and 7.69 ppm (d, $J = 15.8 \text{ Hz}$) for DB ethoxy and 7.04 (d, $J = 8.0 \text{ Hz}$) and 7.69 ppm (d, $J = 15.9 \text{ Hz}$) for DB chlorine. These signals are attributed to the unsaturated olefinic carbon hydrogens, assigned as H29/H30/H31/H32 and H25/H26/H27/H28 respectively. Their coupling constants confirm the stereochemistry E of the double bonds. The aromatic hydrogens of DB ethoxy H25/H26/H34/H35 and H27/H28/H33/H36 are ascribed to the signals at 7.54 (d, $J = 8.6 \text{ Hz}$) and 6.89 ppm (s). In DB chlorine, the signals related to these sorts of hydrogens, here numbered as H23/H24/H29/H32 and H21/H22/H30/H31, are at 7.40 (d, $J = 8.0 \text{ Hz}$) and 7.55 ppm (d, $J = 8.0 \text{ Hz}$). The dibenzalacetone derivative DB ethoxy has also two additional signals, at 4.05 (q, $J = 6.9 \text{ Hz}$) and 1.42 ppm (t, $J = 6.9 \text{ Hz}$) that reveal the hydrogens of the ethoxy groups. The ^{13}C NMR spectra displayed peaks in both compounds at 188.9 and 188.3 ppm respectively, that were related to unsaturated carbonyl group (C9). These peaks should be seen at 203.8 ppm. The presence of α,β unsaturation causes a displacement to high field and the probable cause is the displacement of charge by the benzene ring or by the double bond that makes carbonyl carbon less electron deficient. The olefinic carbons are observed at 127.5 and 142.8 ppm for DB ethoxy and 125.7 and 142.0 ppm for DB chlorine, here named C8/C10 and C7/C11, while the aromatic ones appear at 161.1, 130.2, 123.5 and 115.0 ppm, and 136.5, 133.2, 129.5 and 129.3 ppm for DB ethoxy and DB chlorine respectively. The 63.7 and 14.9 ppm signals in the ^{13}C NMR spectrum of DB ethoxy were ascribed to ethoxy carbons. The indicative numbering of the carbons can be seen in Table 1.

The results of the quantum chemical calculations for the theoretical chemical shift for the DB ethoxy and the DB chlorine molecules are also shown in Table 1. To compare the experimental results of the NMR spectroscopic data with the theoretical chemical shift computed at B3LYP/6-311++G(d,p) computational level, a linear fitting was made and the results are shown in the Figure 1. The DB ethoxy molecule showed a coefficient of determination (R-squared) of 0.98835 with the linear equation $y = 1.04802x + 0.77567$ and the DB chlorine molecule exhibited a R-squared of 0.99745 and the linear equation $y = 1.05799x + 0.27575$. Therefore, the excellent agreement between the experimental and the theoretical data confirms the proposed structures for the title dibenzalacetone derivatives.

Table 1. NMR spectroscopic experimental and theoretical data for DB ethoxy and DB chlorine (^1H : 300MHz; ^{13}C : 125 MHz; in CDCl_3).

Molecular structure	C	δ_{C}	δ_{H}	$\delta_{\text{C}}(\text{Theo})$	$\delta_{\text{H}}(\text{Theo})$	
	C5/12	130.20		134.58		
	C4/17	115.00	6.89 (s)	133.66	8.21	
	C6/13	115.00	6.89 (s)	143.66	7.67	
	C1/14	123.50	7.54 (d, $J = 8.6$ Hz)	115.63	6.91	
	C3/16	123.50	7.54 (d, $J = 8.6$ Hz)	124.95	7.25	
	C2/15	161.10		171.25		
	C8/10	127.50	6.94 (d, $J = 15.3$ Hz)	128.96	7.08	
	C7/11	142.80	7.69 (d, $J = 15.8$ Hz)	152.18	8.07	
	C9	188.90		193.00		
	C21/23	63.70	4.05 (q, $J = 6.9$ Hz)	69.06	4.04	
	C22/24	14.90	1.42 (t, $J = 6.9$ Hz)	17.19	1.52	
		C5/12	133.20		140.89	
		C4/13	129.30	7.40 (d, $J = 8.0$ Hz)	133.15	8.24
C6/17		129.30	7.40 (d, $J = 8.0$ Hz)	142.54	7.70	
C1/16		129.50	7.55 (d, $J = 8.0$ Hz)	136.48	7.64	
C3/14		129.50	7.55 (d, $J = 8.0$ Hz)	136.07	7.65	
C2/15		136.50		153.69		
C8/10		125.70	7.04 (d, $J = 15.9$ Hz)	131.75	7.30	
C7/11		142.00	7.69 (d, $J = 15.9$ Hz)	152.10	8.13	
C9		188.30		193.42		

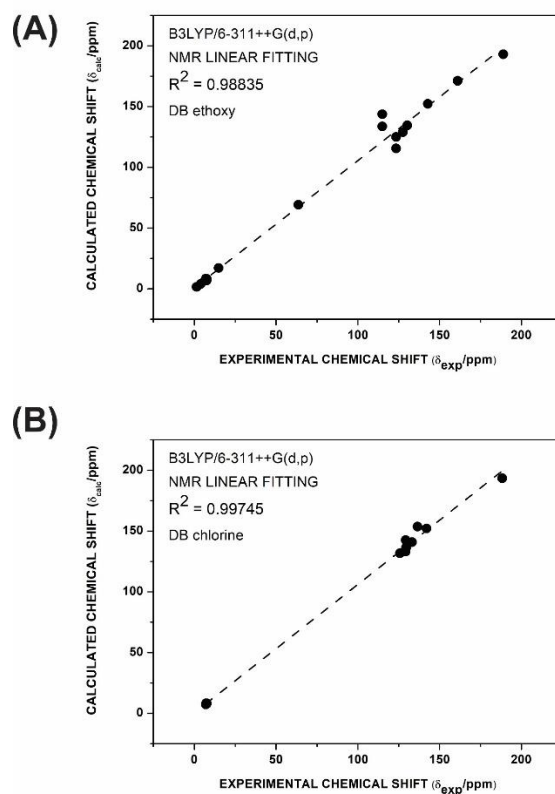
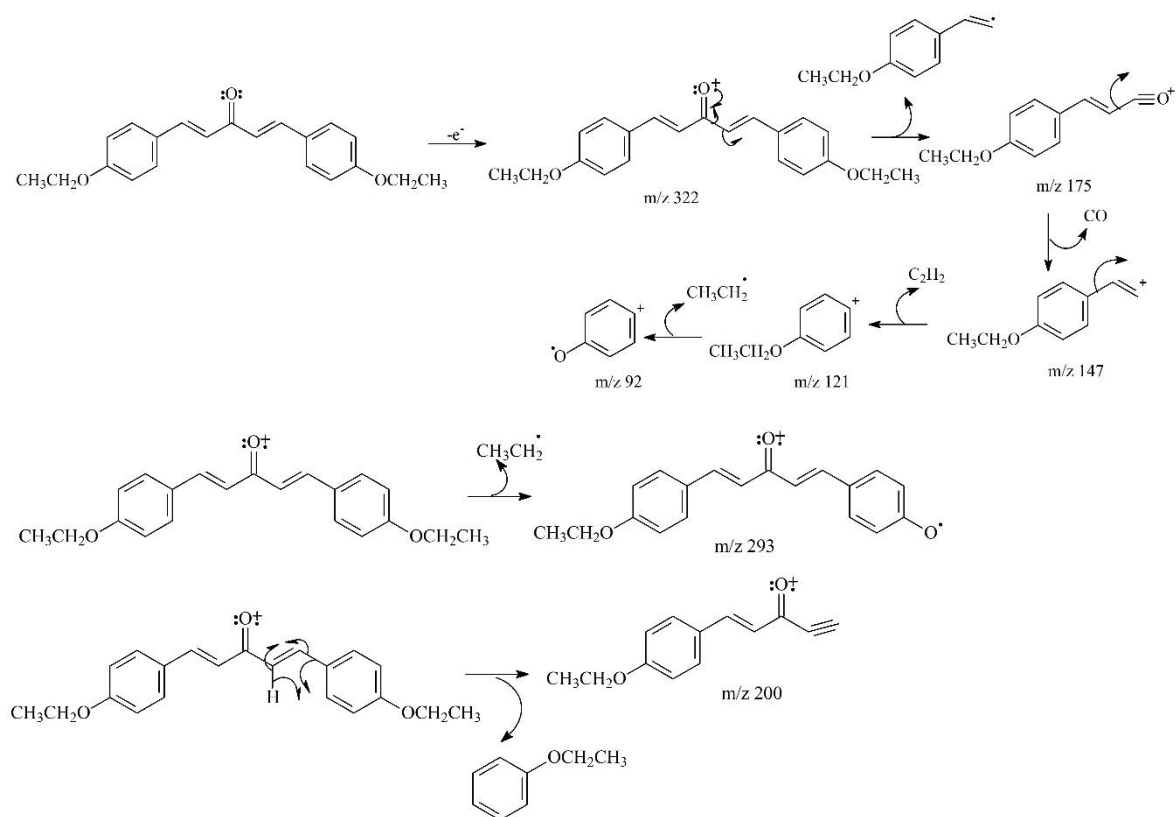
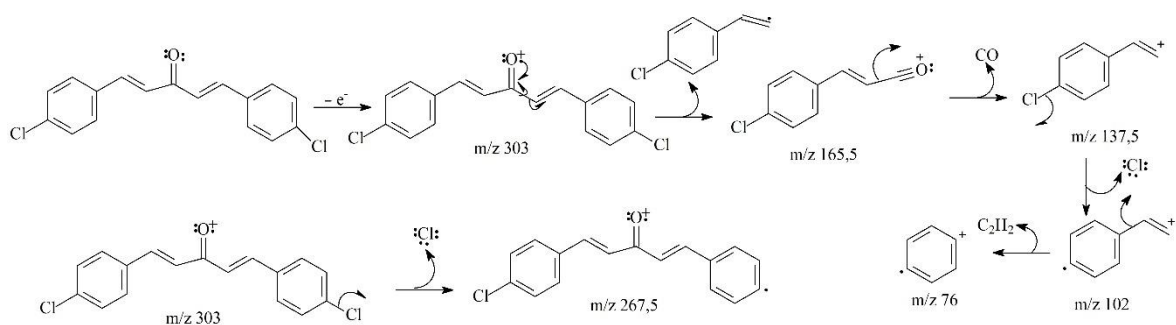


Figure 1. Linear fitting for the NMR spectroscopic experimental and theoretical data for the (a) DB ethoxy and (b) DB chlorine molecules.

The structures of the synthesized compounds were also confirmed through the analysis of the mass spectra in a fragmentation proposal, whose ions formed come from an α segmentation. The mass spectra revealed peaks of the M^+ molecular ion of the synthesized compounds DB ethoxy ($m/z = 322$) and DB chlorine ($m/z = 303$), in addition to the base peak characteristic of the general process of fragmentation of the compounds (Schemes 2 and 3).



Scheme 2. Proposed fragmentation of dibenzalacetone derivative DB ethoxy.



Scheme 3. Proposed fragmentation of dibenzalacetone derivative DB chlorine.

3.2. Vibrational Analysis

As evidenced by the structural NMR determination, DB ethoxy and DB chlorine have 46 and 32 atoms respectively. Therefore, they have 132 and 90 theoretical vibrational modes. The experimental IR and Raman spectra of both compounds are presented in Figures 2a and 2b. Concerning IR spectra, we initially noted that DB ethoxy exhibited a very wide band at 3300 cm⁻¹, that is: DB ethoxy might have precipitated in hydrated crystal form. The sample was heated and the dehydrated infrared spectrum of the compound, shown in Figure S9, confirmed the disappearance of broadband related to

hydration water without any change to the other modes. Other aspect that is worth mentioning is a weak signal of around 1.6 ppm in the ^1H NMR spectrum (Figure S1) which denotes a water signal in the CDCl_3 solvent. Thus, we developed three models for DB ethoxy to assist in spectroscopic band assignments. An anhydrous model, with an isolated molecule, a monohydrated one, consisting of a water molecule positioned close to the carbonyl group, and finally, a trihydrate model with three molecules of water placed next to carbonyl and ethoxy groups. The calculated Raman of said models and DB ethoxy were compared (Figures S10 and S12) and we found that the shape of the experimental spectrum in the region between 1500 cm^{-1} and 1700 cm^{-1} is much more similar to the mono and trihydrated models than to the anhydrous, especially in relation to the experimental modes 1569 , 1585 , 1603 and 1620 cm^{-1} . The experimental modes 578 , 566 and 554 cm^{-1} , could not be assigned over the calculated spectrum for the anhydrous and monohydrate models since it is related to the bending mode of the water molecule and the oxygen atom of the ethoxy group. In addition, modes 279 and 300 cm^{-1} could not be justified in the anhydrous model since these vibrational modes are related to the torsion of the water molecule with the oxygen atoms of the ethoxy groups in the trihydrated model and the bending of the water molecule and the oxygen atom of the carbonyl group in the monohydrate model. Therefore, the Raman profile for trihydrated model captured much more the features of experimental data. This does not mean, however, that we are proposing a crystal with three water molecules per unit cell, since depending on the organization of the crystal, the same water molecule can influence more than one molecule of DB ethoxy.

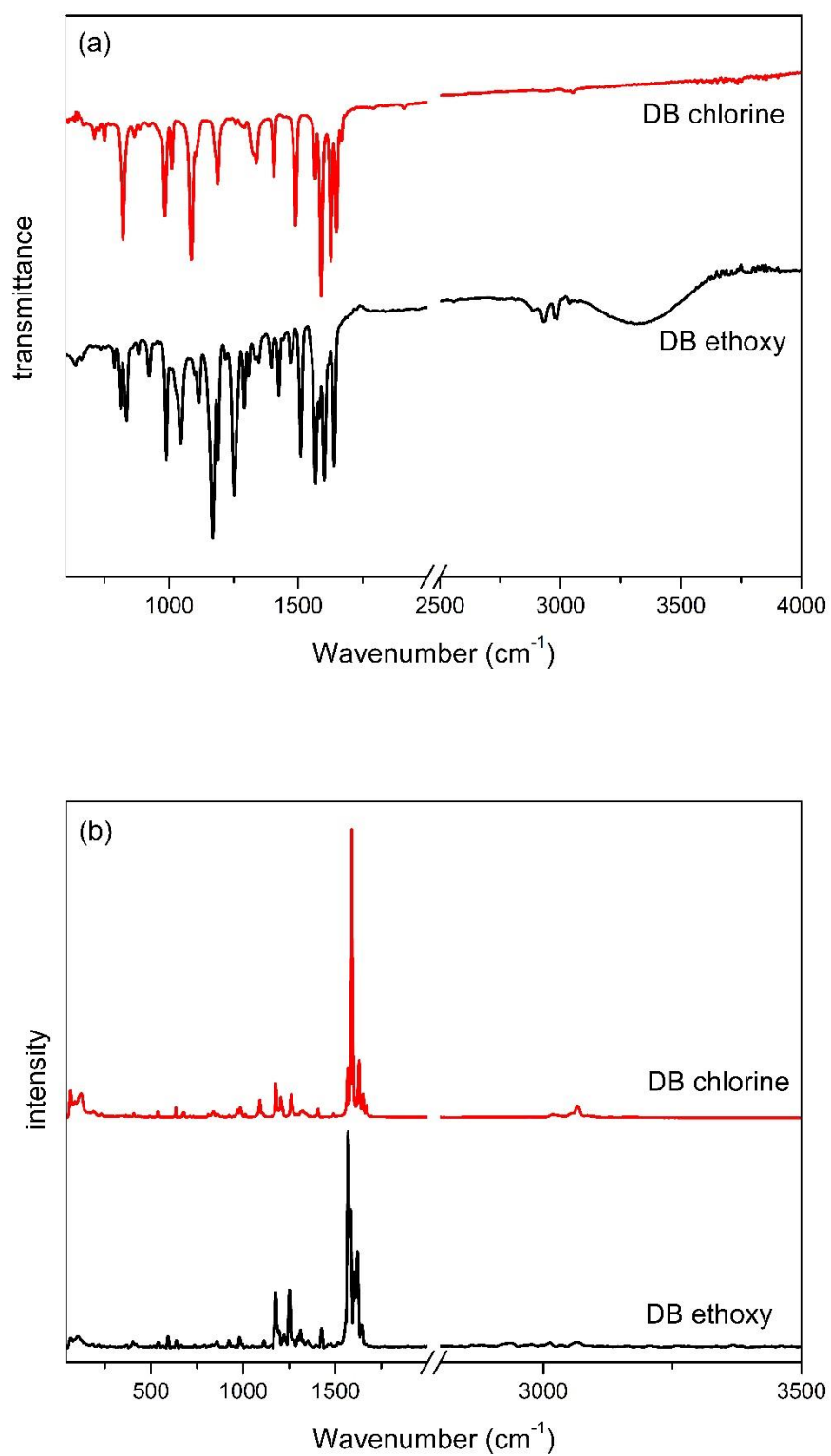


Figure 2. Experimental IR (a) and Raman (b) spectra of DB ethoxy and DB chlorine.

DB chlorine spectrum proved to be quite compatible with the theoretical model proposed. All tables containing the PED analysis of the three DB ethoxy and DB chlorine models as well as the comparison of the Raman and IR models along with experimental spectrum are provided in Supplementary Material (Tables S1 – S4).

Tables 2 and 3 summarize all the assignments of the Raman and IR experimental modes with the calculated values (scaled) of the trihydrated model of DB ethoxy and DB chlorine, respectively. The optimized structures (in vacuum) with the nomenclature used in the PED analysis of the dibenzalacetone derivative DB ethoxy trihydrated model and DB chlorine are exposed in Figures 3a and 3b.

Table 2. Observed Raman and IR band positions (cm^{-1}), calculated (scaled) vibrational wavenumbers (cm^{-1}) and assignments for the DB ethoxy trihydrated model.

ω_{Raman}	ω_{IR}	ω_{scal}	Assignment
3063		3045	$\nu(\text{CH})_{\text{ring}}$
	3037	3044	$\nu(\text{CH})_{\text{ring}}$
3034		3026	$\nu(\text{CH})_{\text{ring}}$
3012		3013	$\nu(\text{CH})_{\text{ring}}$
2977	2985	2980	$\nu(\text{CH})_{\text{enone}}$
	2976	2962	$\nu(\text{CH})_{\text{ethoxy}}$
2936	2935	2957	$\nu(\text{CH})_{\text{ethoxy}}$
	2885	2885	$\nu(\text{CH})_{\text{ethoxy}}$
2881		2885	$\nu(\text{CH})_{\text{ethoxy}}$
2862		2850	$\nu(\text{CH})_{\text{ethoxy}}$
1643	1640	1650	$\delta(\text{OH}_{\text{water}}\text{OC}_{\text{carbonyl}}) + \tau(\text{OH}_{\text{water}}\text{OC}_{\text{carbonyl}}) + \tau(\text{H}_{\text{water}}\text{OC}_{\text{carbonyl}}\text{C}_{\text{enone}})$
	1620	1627	$\delta(\text{HOH})_{\text{water}}$
1620		1615	$\nu(\text{CC})_{\text{ring}}$
1603	1601	1604	$\nu(\text{CC})_{\text{ring}}$
1584	1583	1578	$\nu(\text{CO})_{\text{carbonyl}}$
1569	1568	1566	$\nu(\text{CC})_{\text{ring}}$
1556		1564	$\nu(\text{CC})_{\text{ring}}$
1515	1510	1512	$\delta(\text{HCC})_{\text{ring}} + \delta(\text{HCH})_{\text{ethoxy}}$
1497		1489	$\delta(\text{HCH})_{\text{ethoxy}}$
	1477	1488	$\delta(\text{HCH})_{\text{ethoxy}}$
1475	1469	1466	$\delta(\text{HCH})_{\text{ethoxy}} + \tau(\text{HCCO})_{\text{ethoxy}}$
1459		1455	$\delta(\text{HCH})_{\text{ethoxy}} + \tau(\text{HCCO})_{\text{ethoxy}}$
1425	1425	1428	$\nu(\text{CC})_{\text{ring}} + \delta(\text{HCC})_{\text{ring}}$
1396	1396	1398	$\delta(\text{HCH})_{\text{ethoxy}} + \tau(\text{HCO})_{\text{ethoxy}}$
1351	1348	1357	$\delta(\text{HCC})_{\text{enone}}$
1325	1333	1337	$\nu(\text{CC})_{\text{enone}} + \nu(\text{CC})_{\text{ring}} + \delta(\text{HCC})_{\text{enone}} + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{ring}})$
1311		1321	$\delta(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
	1308	1313	$\delta(\text{HC}_{\text{enone}}\text{C}_{\text{ring}}) + \delta(\text{HCC})_{\text{ring}}$
1295		1308	$\delta(\text{HCC})_{\text{ring}} + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
	1292	1289	$\delta(\text{HCC})_{\text{enone}} + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{ring}})$
1272		1273	$\nu(\text{C}_{\text{ring}}\text{C}_{\text{enone}}) + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
1251	1252	1247	$\nu(\text{CC})_{\text{ring}} + \nu(\text{O}_{\text{ethoxy}}\text{C}_{\text{ring}})$
1220	1219	1218	$\nu(\text{CC})_{\text{ring}} + \nu(\text{C}_{\text{enone}}\text{C}_{\text{ring}}) + \delta(\text{HCC})_{\text{ring}}$
1196	1190	1204	$\nu(\text{CC})_{\text{ring}} + \nu(\text{C}_{\text{ring}}\text{C}_{\text{enone}}) + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{ring}}) + \delta(\text{HCC})_{\text{ring}}$
1176	1169	1176	$\delta(\text{HCC})_{\text{ring}}$
1158		1150	$\delta(\text{HCC})_{\text{ethoxy}} + \tau(\text{HCO}_{\text{ethoxy}}\text{C}_{\text{ring}})$
1127		1122	$\nu(\text{CC})_{\text{ring}} + \delta(\text{HCC})_{\text{ring}}$
1113	1115	1108	$\tau(\text{HCCO})_{\text{ethoxy}}$

1098	1097	1093	$v(\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}) + \delta(\text{HCC})_{\text{enone}}$
1049		1052	$\tau(\text{HO}_{\text{water}}\text{HC}_{\text{enone}}) + \tau(\text{HCC}_{\text{enone}}\text{C}_{\text{carbonyl}}) + \tau(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}})$
	1045	1038	$v(\text{CC})_{\text{ethoxy}} + v(\text{OC})_{\text{ethoxy}}$
	1025	1005	$\tau(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \tau(\text{HC}_{\text{enone}}\text{CC}_{\text{ring}})$
996	989	1000	$\delta(\text{CCC})_{\text{ring}}$
981		970	$v(\text{CO})_{\text{carbonyl}} + v(\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}})$
944		939	$\tau(\text{HCCC})_{\text{ring}} + \tau(\text{CCCC})_{\text{ring}}$
924		922	$\tau(\text{HCCC})_{\text{ring}} + \tau(\text{HCC}_{\text{ring}}\text{O}_{\text{ethoxy}}) + \tau(\text{CCCC})_{\text{ring}}$
909	922	912	$v(\text{CC})_{\text{ethoxy}} + v(\text{OC})_{\text{ethoxy}} + \tau(\text{HCCO})_{\text{ethoxy}}$
878	881	881	$\tau(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \gamma(\text{O}_{\text{carbonyl}}\text{CC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
	835	844	$\tau(\text{HCCC})_{\text{ring}}$
857		839	$\delta(\text{CCC})_{\text{ring}}$
830		832	$\tau(\text{HCCC})_{\text{ring}} + \tau(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}})$
813	812	818	$\tau(\text{HCCO})_{\text{ethoxy}}$
806	789	798	$\tau(\text{HCCC})_{\text{ring}}$
791		790	$\tau(\text{HCCC})_{\text{ring}} + \tau(\text{HCC}_{\text{ring}}\text{C}_{\text{enone}})$
776		766	$v(\text{OC})_{\text{ethoxy}}$
737		728	$\tau(\text{CCCC})_{\text{ring}} + \gamma(\text{O}_{\text{carbonyl}}\text{CC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
719		705	$\tau(\text{CCCC})_{\text{ring}}$
704		701	$\delta(\text{OC}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \delta(\text{CCC})_{\text{ring}}$
679		676	$\delta(\text{OH}_{\text{water}}\text{O}_{\text{carbonyl}}) + \delta(\text{H}_{\text{water}}\text{OC}_{\text{carbonyl}}) + \tau(\text{OH}_{\text{water}}\text{OC}_{\text{carbonyl}})$
664	661	672	$\tau(\text{CCCC})_{\text{ring}} + \gamma(\text{O}_{\text{carbonyl}}\text{CC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
638	638	634	$\delta(\text{CCC})_{\text{ring}}$
625		632	$\delta(\text{CCC})_{\text{ring}}$
593		581	$\delta(\text{CCC})_{\text{ring}}$
578		563	$\delta(\text{OH}_{\text{water}}\text{O}_{\text{ethoxy}}) + \delta(\text{CO}_{\text{ethoxy}}\text{H}_{\text{water}})$
566		557	$\delta(\text{OH}_{\text{water}}\text{O}_{\text{ethoxy}}) + \delta(\text{H}_{\text{water}}\text{O}_{\text{ethoxy}}\text{C}_{\text{ring}})$
554		553	$\delta(\text{OH}_{\text{water}}\text{O}_{\text{ethoxy}}) + \delta(\text{H}_{\text{water}}\text{O}_{\text{ethoxy}}\text{C}_{\text{ring}})$
539		518	$\delta(\text{C}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}})$
516		517	$\gamma(\text{O}_{\text{ethoxy}}\text{CCC}_{\text{ring}})$
438		433	$\delta(\text{CC}_{\text{ring}}\text{C}_{\text{enone}}) + \delta(\text{O}_{\text{ethoxy}}\text{CC}_{\text{ring}}) + \delta(\text{CCO})_{\text{ethoxy}}$
420		422	$\delta(\text{O}_{\text{ethoxy}}\text{CC}_{\text{ring}})$
414		415	$\tau(\text{HCCC})_{\text{ring}} + \tau(\text{CCCC})_{\text{ring}}$
403		414	$\tau(\text{HCCC})_{\text{ring}} + \tau(\text{CCCC})_{\text{ring}} + \tau(\text{CCC}_{\text{ring}}\text{C}_{\text{enone}})$
384		378	$\tau(\text{HO}_{\text{water}}\text{HC}_{\text{enone}}) + \tau(\text{CCCC})_{\text{ring}} + \gamma(\text{O}_{\text{ethoxy}}\text{CCC}_{\text{ring}}) + \gamma(\text{C}_{\text{enone}}\text{CCC}_{\text{ring}})$
368		372	$\tau(\text{CCCC})_{\text{ring}} + \tau(\text{CCC}_{\text{ring}}\text{C}_{\text{enone}}) + \tau(\text{C}_{\text{ring}}\text{CC}_{\text{enone}}\text{C}_{\text{carbonyl}}) + \gamma(\text{O}_{\text{ethoxy}}\text{CCC}_{\text{ring}})$
353		351	$\delta(\text{CCO})_{\text{ethoxy}}$
320		326	$\delta(\text{CCO})_{\text{ethoxy}} + \tau(\text{H}_{\text{water}}\text{OC}_{\text{carbonyl}}\text{C}_{\text{enone}})$
300		318	$\delta(\text{OH}_{\text{water}}\text{O}_{\text{carbonyl}}) + \tau(\text{HO}_{\text{water}}\text{HC}_{\text{enone}}) + \tau(\text{OH}_{\text{water}}\text{OC}_{\text{carbonyl}}) + \tau(\text{H}_{\text{water}}\text{OC}_{\text{carbonyl}}\text{C}_{\text{enone}})$
279		266	$\delta(\text{HO}_{\text{water}}\text{H}_{\text{enone}})$
271		262	$v(\text{O}_{\text{carbonyl}}\text{H}_{\text{water}}) + \delta(\text{CC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
256		253	$\tau(\text{HOH}_{\text{water}}\text{O}_{\text{ethoxy}}) + \tau(\text{OH}_{\text{water}}\text{O}_{\text{ethoxy}}\text{C}_{\text{ring}}) + \tau(\text{HCCO})_{\text{ethoxy}}$
249		251	$\tau(\text{HOH}_{\text{water}}\text{O}_{\text{ethoxy}}) + \tau(\text{OH}_{\text{water}}\text{O}_{\text{ethoxy}}\text{C}_{\text{ring}}) + \tau(\text{HCCO})_{\text{ethoxy}}$
236		237	$\tau(\text{HOH}_{\text{water}}\text{O}_{\text{ethoxy}}) + \tau(\text{CO}_{\text{ethoxy}}\text{HO}_{\text{water}})$
217		216	$\tau(\text{HOH}_{\text{water}}\text{O}_{\text{ethoxy}}) + \tau(\text{OH}_{\text{water}}\text{O}_{\text{ethoxy}}\text{C}_{\text{ring}})$
204		203	$\tau(\text{HOH}_{\text{water}}\text{O}_{\text{ethoxy}}) + \tau(\text{OH}_{\text{water}}\text{O}_{\text{ethoxy}}\text{C}_{\text{ring}}) + \tau(\text{CC}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}})$
184		186	Lattice
176		174	Lattice
153		159	Lattice
138		135	Lattice
122		127	Lattice
106		106	Lattice
103		99	Lattice
84		79	Lattice
65		66	Lattice
38		36	Lattice

Abbreviation: v, stretching; δ , bending; τ , torsion; γ , out-of-plane.

Table 3. Observed Raman and IR band positions (cm^{-1}), calculated (scaled) vibrational wavenumbers (cm^{-1}) and assignments for the DB chlorine.

ω_{Raman}	ω_{IR}	ω_{scal}	Assignment
3066	3050	3055	$\nu(\text{CH})_{\text{ring}}$
3054		3053	$\nu(\text{CH})_{\text{ring}}$
3018		3026	$\nu(\text{CH})_{\text{ring}}$
1670	1668	1701	$\nu(\text{CO})_{\text{carbonyl}} + \nu(\text{CC})_{\text{enone}}$
1649	1649	1632	$\nu(\text{CC})_{\text{enone}} + \nu(\text{CC})_{\text{enone}}$
1629	1626	1609	$\nu(\text{CO})_{\text{carbonyl}} + \nu(\text{CC})_{\text{enone}}$
	1589	1598	$\nu(\text{CC})_{\text{ring}}$
1590		1593	$\nu(\text{CO})_{\text{carbonyl}} + \nu(\text{CC})_{\text{ring}}$
1566	1566	1566	$\nu(\text{CC})_{\text{ring}}$
1490	1489	1489	$\delta(\text{HCC})_{\text{ring}}$
1407	1406	1407	$\nu(\text{CC})_{\text{ring}}$
1342	1338	1334	$\delta(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}) + \delta(\text{HCC})_{\text{enone}}$
1322	1327	1323	$\delta(\text{HCC})_{\text{enone}} + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{ring}})$
1312		1311	$\delta(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}) + \delta(\text{HCC})_{\text{enone}}$
1294		1292	$\nu(\text{CC})_{\text{ring}}$
1277		1292	$\delta(\text{HCC})_{\text{ring}}$
1267	1290	1283	$\delta(\text{HCC})_{\text{enone}}$
1259		1265	$\nu(\text{C}_{\text{ring}}\text{C}_{\text{enone}}) + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}) + \delta(\text{HCC})_{\text{enone}}$
1204	1188	1201	$\delta(\text{HCC})_{\text{enone}} + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{ring}})$
1189		1198	$\nu(\text{CC})_{\text{ring}} + \nu(\text{C}_{\text{ring}}\text{C}_{\text{enone}}) + \nu(\text{CC})_{\text{enone}} + \delta(\text{HCC})_{\text{enone}} + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{ring}}) + \delta(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
1176	1178	1173	$\delta(\text{HCC})_{\text{ring}}$
1104	1111	1105	$\nu(\text{CC})_{\text{ring}}$
	1103	1090	$\nu(\text{C}_{\text{enone}}\text{C}_{\text{carbonyl}})$
1092		1079	$\nu(\text{CC})_{\text{ring}}$
	1086	1069	$\nu(\text{C}_{\text{enone}}\text{C}_{\text{carbonyl}})$
1013	1011	1013	$\tau(\text{HCC}_{\text{enone}}\text{C}_{\text{carbonyl}}) + \tau(\text{HC}_{\text{enone}}\text{CC}_{\text{ring}})$
		1005	$\tau(\text{HCC}_{\text{enone}}\text{C}_{\text{carbonyl}}) + \tau(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \tau(\text{HC}_{\text{enone}}\text{CC}_{\text{ring}})$
985	984	998	$\delta(\text{CCC})_{\text{ring}}$
970		959	$\nu(\text{C}_{\text{enone}}\text{C}_{\text{carbonyl}})$
950		941	$\tau(\text{HCCC})_{\text{ring}} + \tau(\text{CCCC})_{\text{ring}}$
924		935	$\tau(\text{HCCC})_{\text{ring}}$
888	887	888	$\tau(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \tau(\text{HCC}_{\text{enone}}\text{C}_{\text{ring}}) + \gamma(\text{O}_{\text{carbonyl}}\text{CC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
866	866	853	$\delta(\text{CCC})_{\text{ring}}$
859		850	$\tau(\text{HC}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \tau(\text{HCC}_{\text{enone}}\text{C}_{\text{ring}})$
839		824	$\delta(\text{CCC})_{\text{ring}}$
832	822	819	$\tau(\text{HCCC})_{\text{ring}}$
811		810	$\tau(\text{HCCC})_{\text{ring}}$
774		801	$\tau(\text{HCC}_{\text{enone}}\text{C}_{\text{ring}}) + \tau(\text{HCCC})_{\text{ring}}$
729/751	750	739	$\delta(\text{OC}_{\text{carbonyl}}\text{C}_{\text{enone}})$
713	711	721	$\tau(\text{CCCC})_{\text{ring}} + \gamma(\text{O}_{\text{carbonyl}}\text{CC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
679		693	$\tau(\text{CCCC})_{\text{ring}}$
674		664	$\nu(\text{ClC})_{\text{ring}} + \delta(\text{CCC})_{\text{ring}}$
663		659	$\tau(\text{CCCC})_{\text{ring}} + \gamma(\text{O}_{\text{carbonyl}}\text{CC}_{\text{enone}}\text{C}_{\text{carbonyl}})$
635		629	$\delta(\text{CCC})_{\text{ring}}$
623		629	$\delta(\text{CCC})_{\text{ring}}$
611		617	$\nu(\text{ClC})_{\text{ring}} + \delta(\text{OC}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \delta(\text{CCC})_{\text{ring}}$
536		517	$\delta(\text{CC}_{\text{enone}}\text{C}_{\text{ring}}) + \delta(\text{C}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}})$
494		491	$\tau(\text{CCCC})_{\text{ring}} + \gamma(\text{ClCCCC})_{\text{ring}} + \gamma(\text{C}_{\text{enone}}\text{CCC}_{\text{ring}})$
460		462	$\delta(\text{OC}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \delta(\text{CC}_{\text{enone}}\text{C}_{\text{ring}}) + \delta(\text{CC}_{\text{ring}}\text{C}_{\text{enone}})$
429		419	$\nu(\text{ClC})_{\text{ring}}$
407		406	$\tau(\text{CCCC})_{\text{ring}}$
391		406	$\tau(\text{CCCC})_{\text{ring}}$
366		374	$\delta(\text{CC}_{\text{ring}}\text{C}_{\text{enone}})$
349		343	$\tau(\text{CCC}_{\text{ring}}\text{C}_{\text{enone}}) + \tau(\text{C}_{\text{carbonyl}}\text{CC}_{\text{enone}}\text{C}_{\text{ring}}) + \gamma(\text{ClCCCC})_{\text{ring}}$
334		343	$\tau(\text{CCC}_{\text{ring}}\text{C}_{\text{enone}}) + \gamma(\text{ClCCCC})_{\text{ring}}$

323	316	$\delta(\text{C}_{\text{enone}}\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \delta(\text{C}(\text{I}(\text{C}(\text{C}))_{\text{ring}})$
285	282	$\delta(\text{C}(\text{I}(\text{C}(\text{C}))_{\text{ring}})$
231	242	$\delta(\text{O}(\text{C}_{\text{carbonyl}}\text{C}_{\text{enone}}) + \delta(\text{C}(\text{C}_{\text{enone}}\text{C}_{\text{carbonyl}})$
190	192	Lattice
165	168	Lattice
122	148	Lattice
89	87	Lattice
64	67	Lattice

Abbreviation: ν , stretching; δ , bending; τ , torsion; γ , out-of-plane.

Both experimental spectra of dibenzalacetone derivatives depict many similarities as can be seen at Figures 2a and 2b. At the fingerprint region, a very strong Raman band at 1590 cm^{-1} , for DB chlorine, was attributed to the stretching of C=O in combination with CC stretching of aromatic ring. In the IR spectrum, a very strong C=O stretching mode appeared at 1626 cm^{-1} associated with enone's CC stretchings. For DB ethoxy the carbonyl stretching mode was identified at 1584 cm^{-1} e 1583 cm^{-1} for Raman and IR respectively, both with strong intensity. A very strong peak was noticed at 1640 cm^{-1} in the experimental IR data of DB ethoxy, which was related to bending and torsion modes between crystallization water and carbonyl group. The CC stretching modes of aromatic rings emerged in Raman spectrum at 1650 (strong), 1603 (medium), 1569 (very strong) and 1556 cm^{-1} (weak) for DB ethoxy while for DB chlorine were at 1566 (medium) and 1407 cm^{-1} (weak). These modes have also been identified in the IR spectrum at 1601 (very strong) and 1568 cm^{-1} (very strong) for DB ethoxy and at 1589 (very strong), 1566 (medium) and 1406 cm^{-1} (medium) for DB chlorine.

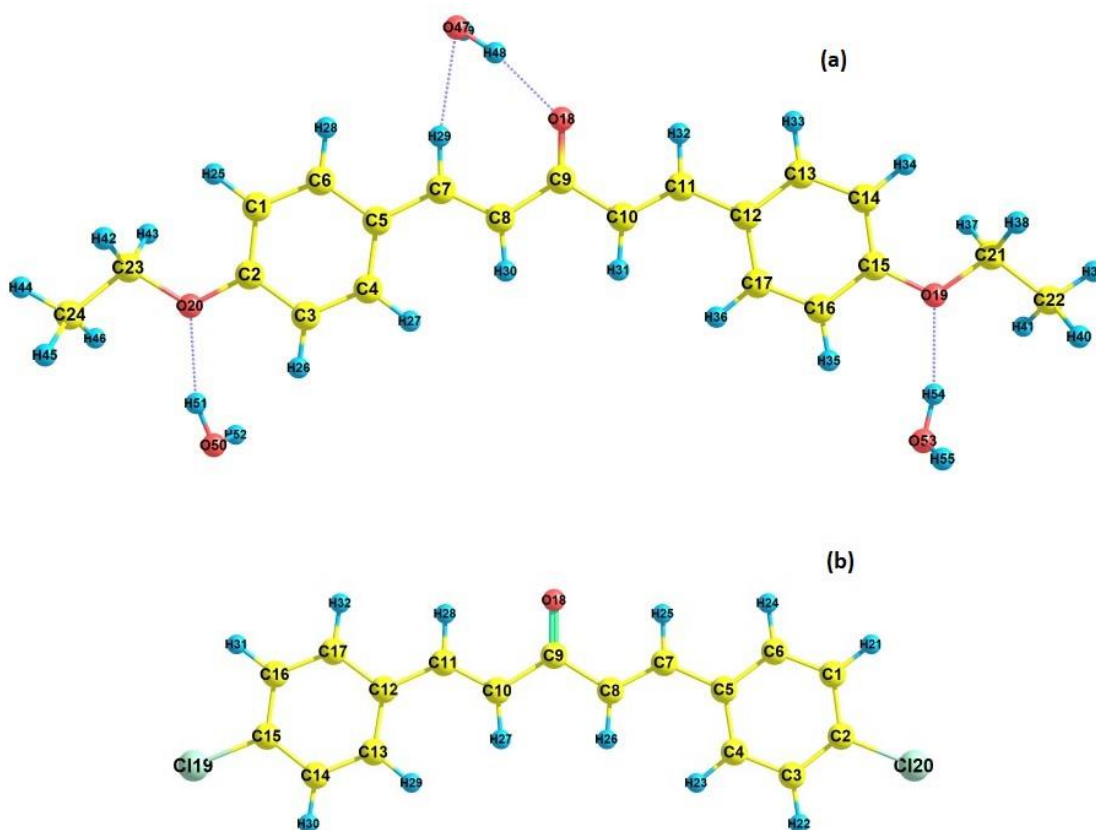


Figure 3. Optimized structure (in vacuum) of trihydrated model of DB ethoxy (a) and DB chlorine (b) with nomenclature used for the PED analysis.

Continuing down the fingerprint region, it was possible to visualize a decrease in the intensity of the bands for the two compounds mainly in the Raman spectrum. In the infrared, there were still some high intensity peaks. In this region, below 1500 cm^{-1} , the modes were mainly attributed to CC stretching, HCC, CCC and OCC bending, HCCC and CCCC torsion, OCCC and CCCC out-of-plane. Some specific modes of each compound can also be seen, mainly those due to the water molecules close to the ethoxy groups as well as some modes related to the chlorine atoms in DB chlorine.

In a spectral region comprised between 3066 and 3012 cm^{-1} , some very weak Raman peaks were attributed to CH stretching of the aromatic rings for both compounds. In addition, for DB ethoxy, from 2977 to 2862 cm^{-1} , we assigned modes to CH stretching due to ethoxy groups.

3.3. Cytotoxicity assay

The Figure 4 shows the % RCV of the investigated compounds whose data were used for a preliminary structure–activity relationship analysis. The results showed that

small modifications to the compound chemical structure were able to alter the biological activity. The analysis of the acquired cytotoxicity data revealed that the compound DB chlorine demonstrated the strongest activity against HCT-116 cells (% RCV = 85.03 ± 0.57). The strong cytotoxic effect of DB chlorine can be attributed to the combined effect of the presence of electron withdrawing chlorine atom at the rings. The presence of electron donor ethoxy groups at the rings reduces the cytotoxic effect demonstrated by the weak activity of DB ethoxy (% RCV = 8.21 ± 4.32). The compounds revealed little cytotoxicity activity against PC3 cells and the same behavior in relation to the substituent group, whereas for SNB cells they were not active.

Our work corroborates the study by Dias et al. [40], in which the halogen substituents on B-ring of chalcones improve cytotoxicity activity against HCT-116 cells compared to the hydroxylated, methylated or methoxylated analogues. In this same work, a structure-activity analysis also revealed an improvement in cell growth inhibition as the B-ring substituent goes from F to Cl and to Br, either in 3- or 4-position. However, chalcones with the halogen substituents at the 3-position of the aromatic B-ring seems to be more active than those substituted at the 4-position. This same improvement effect in cytotoxic activity provided by the chlorine substituted in the aromatic rings of chalcone derivatives at the *para* position was also observed against HepG2 liver cancer cells[41]. A different paper by Iftikhar et al. [42] revealed that chalcones with small substituents on B-ring display better anti-proliferative activity against HCT-116 human colon cancer cell line, which suggests that the linear extension of the structure may impose greater difficulty to fit inside of the p53 cavity, as well as adding an additional cost of interaction with the solvent for the aromatic ring.

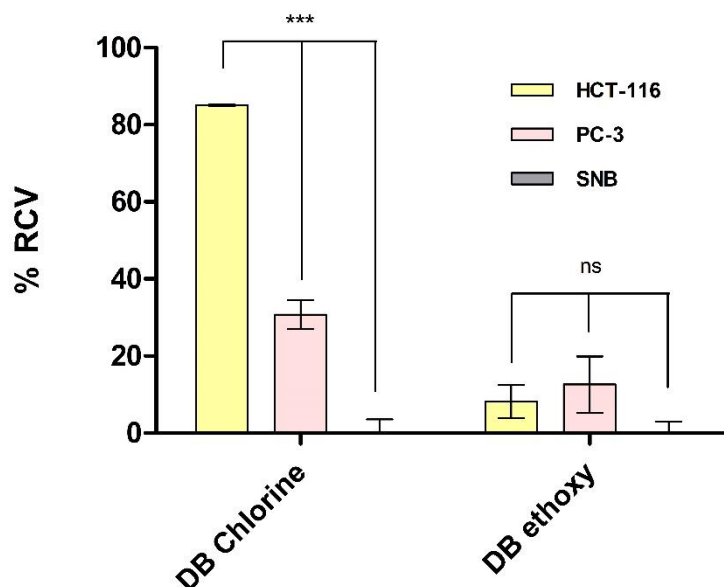


Figure 4. Cytotoxicity of the chalcones DB chlorine and DB ethoxy at a single concentration (10 μ M) against HCT-116 and PC3 cells after 72 h of incubation using MTT assay. *** $p < 0.001$, ^{ns} $p > 0.05$ (not significant).

3.4. In silico drug-likeness evaluation

The term "drug-likeness" is related how the medication is administrated. The R05 rules were created in order to predict whether a compound would have problems in orally activation. Table 4 shows the physicochemical properties, lipophilicity, drug-likeness and pharmacokinetics *in silico* of two said compounds. DB ethoxy and DB chlorine passed all Lipinski R05 rules, however DB chlorine displayed 1 violation (MLOGP > 4.15). Both were moderately soluble, had high gastrointestinal absorption and provided appropriate bioavailability. DB ethoxy compound appears to be an inhibitor of the cytochromes 450 enzyme family. However, DB chlorine did not inhibit the subfamilies CYP2D6 and CYP3A4. These enzymes play an important role in the metabolism of anticancer drugs, especially the subfamily CYP3A4 that catalyzes pro-drug activation by increasing the possibility of activation in tumor tissues. P-glycoprotein was not inhibited by any of the compounds.

Table 4. *In silico* Physicochemical, lipophilicity, Drug-likeness and pharmacokinetics profile of DB ethoxy and DB chlorine.

	Physicochemical Properties				Lipophilicity		Drug-likeness		Pharmacokinetics							
	mol. wt. g/mol	ROT B (n)	HBA (n)	HBD (n)	ESOL LogS	TPSA (Å)	CLogP (o/w)	Bioavailability Score	Lipinski	GIA	P-gp	CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4
DB ethoxy	322.4	8	3	0	-4.93 (MS)	35.53	4.55	0.55	Yes (0)	High	No	Yes	Yes	Yes	Yes	Yes
DB chlorine	303.2	4	1	0	-5.37 (MS)	17.07	4.92	0.55	Yes (1)	High	No	Yes	Yes	Yes	No	No
Ref.	<500 [43,44]	<10 [45]	≤10 [43,44]	≤5 [43,44]		<140 [43,44]	<5 [43,44]	0.55 [46]	-	-	-	-	-	-	-	-

Abbreviations: mol. wt., molecular weight; n, number; ROTB, rotatable bonds; HBA, hydrogen bond acceptors; HBD, hydrogen bond donors; ESOL[47], Estimated Solubility with classes in bracket (HS-highly soluble, VS-very soluble, MS-moderately soluble, and VS-vey soluble); TPSA, topological polar surface area; CLogP o/w, logarithm of compound partition coefficient between n-octanol and water, GIA, gastrointestinal absorption; P-gp, P-glycoprotein; CYP1A2, Cytochrome P450 1A2; CYP2C19, Cytochrome P450 2C19; CYP2C9, Cytochrome P450 2C9; CYP2D6, Cytochrome P450 2D6; CYP3A4, Cytochrome P450 3A4.

4. Conclusions

Dibenzalacetone derivatives, also called bis-chalcones by some authors, are substances with proven biological actions, especially against cancer cells. They can be synthesized by double condensation with two equivalents of aromatic aldehydes. The (1*E*,4*E*)-1,5-bis(4-ethoxyphenyl)penta-1,4-dien-3-one and (1*E*,4*E*)-1,5-bis(4-chlorophenyl)penta-1,4-dien-3-one were synthesized and characterised by NMR. The excellent agreement between theoretical and experimental NMR data confirmed the proposed structures of dibenzalacetone derivatives. It was shown that DB chlorine had a remarkable cytotoxic effect against HCT-116 colon cancer cells. Both compounds passed on drug-likeness test which may indicate good potential as oral medication, especially for DB chlorine that inhibited colon cancer cell growth. Also, a complete study on vibrational analysis was performed using the FT-IR and FT-Raman spectroscopy as well as quantum chemical calculations on B3LYP/6-31G(d,p) level. The description of normal modes according to the PED were used to elucidate the vibrational wave number assignments of

the synthetic dibenzalacetone derivatives, which led us to conclude that DB ethoxy was obtained in a hydrated form.

Author Contributions

Conceptualization, Mauro M. de Oliveira and Carlos E. S. Nogueira; methodology, Alexandre M. R. Teixeira; validation, Pedro de Lima-Neto; formal analysis, Manoel O. de Moraes, Claudia Pessoa, Francisco Washington A. Barros Nepomuceno; investigation, Mauro M. de Oliveira, Emmanuel S. Marinho; resources, Helcio S. Santos; data curation, Francisco Wagner Q. Almeida-Neto; writing—original draft preparation, Mauro M. de Oliveira; writing—review and editing, Mauro M. de Oliveira, Carlos E. S. Nogueira, Alexandre M. R. Teixeira; visualization, Mauro M. de Oliveira; supervision, Carlos E. S. Nogueira; project administration, Mauro M. de Oliveira. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

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Supplementary Material

Full Spectroscopic Characterization and Cytotoxicity Activity of Synthetic Dibenzalacetone Derivatives.

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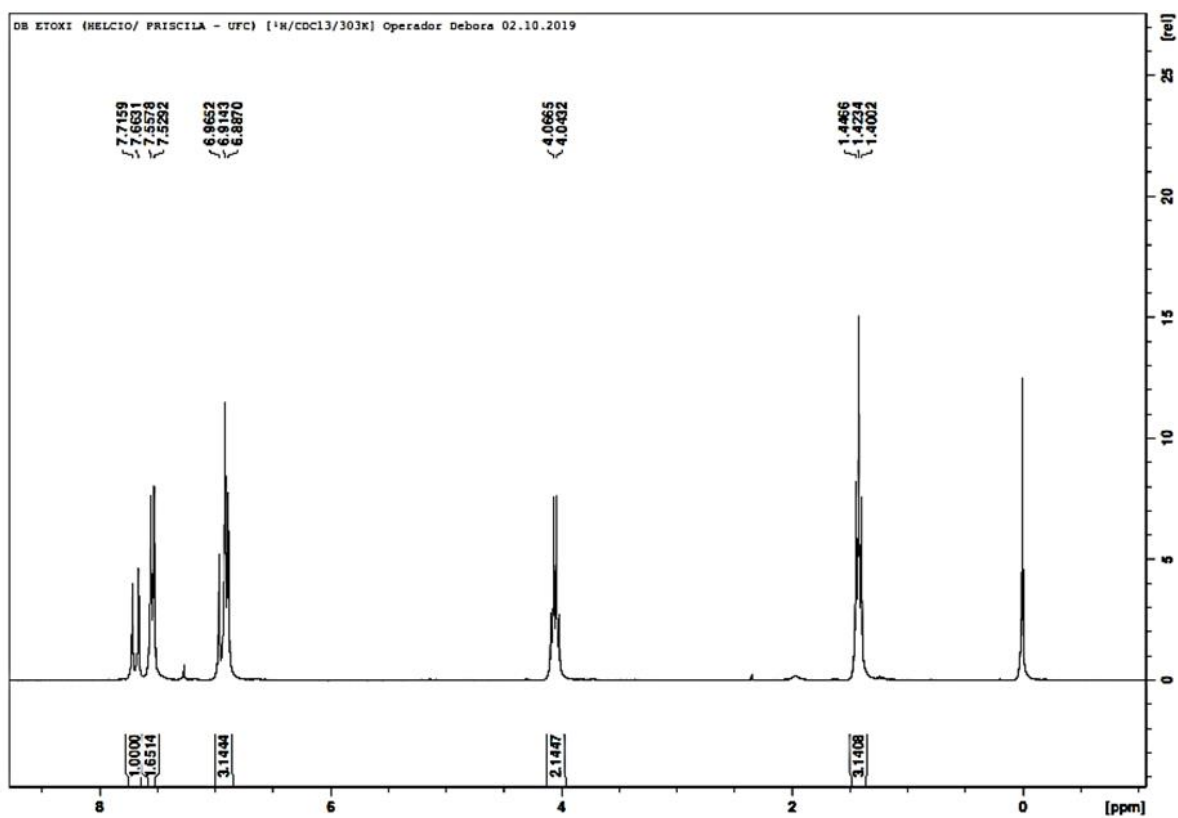
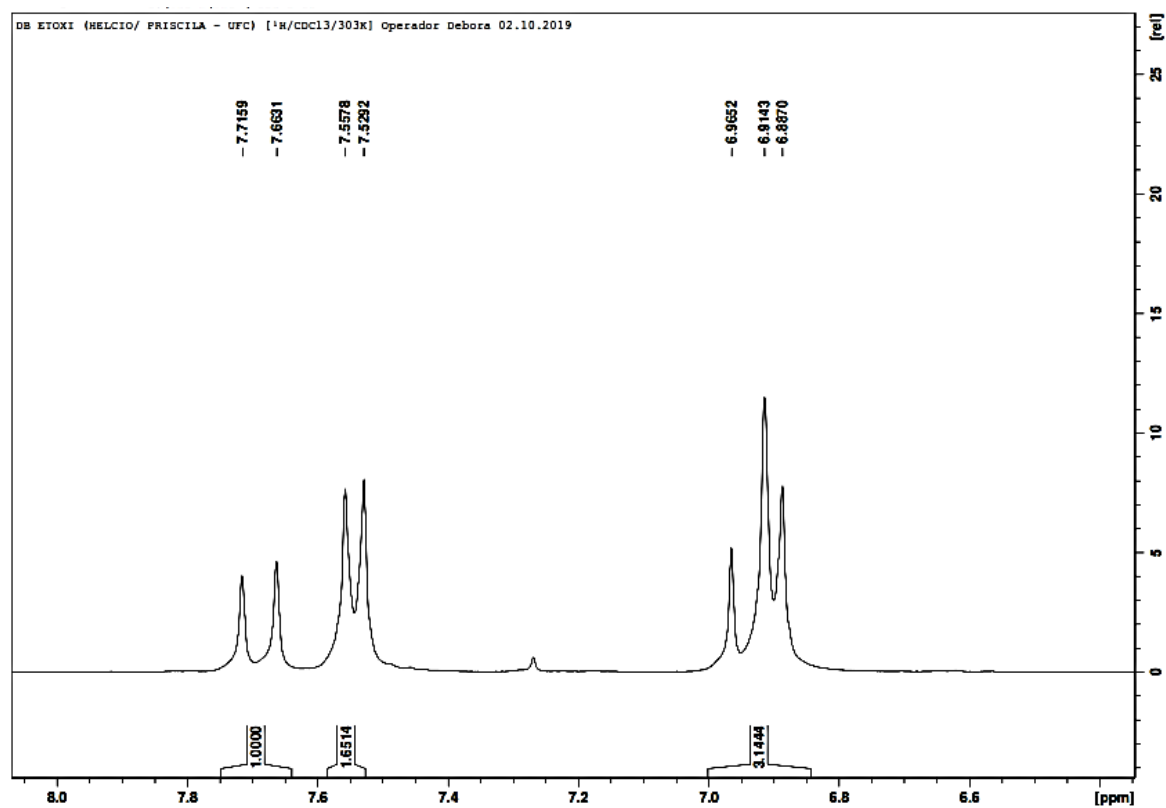
Figure S1. ^1H NMR spectrum (CDCl_3 , 300 MHz) of DB ethoxy.**Figure S2.** Expansion of ^1H NMR spectrum (CDCl_3 , 300 MHz) of DB ethoxy.

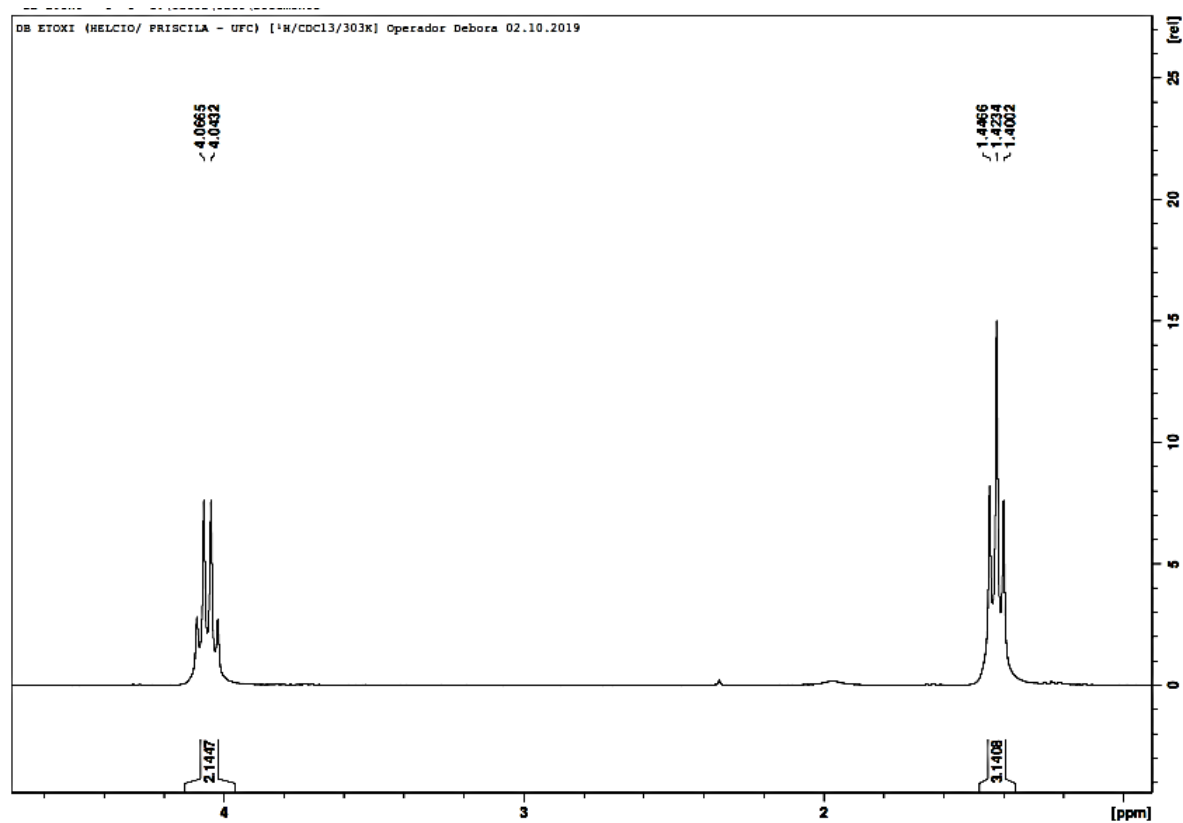
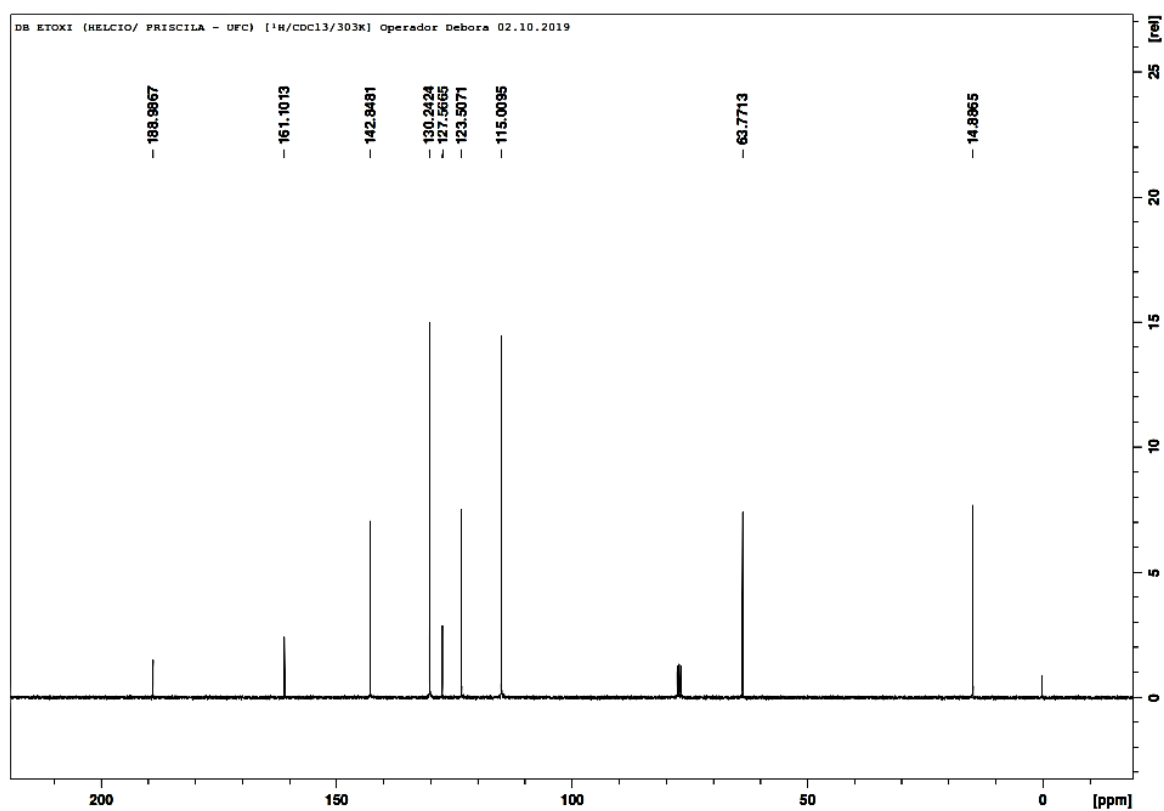
Figure S3. Expansion of ^1H NMR spectrum (CDCl_3 , 300 MHz) of DB ethoxy.**Figure S4.** ^{13}C NMR spectrum (CDCl_3 , 125 MHz) of DB ethoxy.

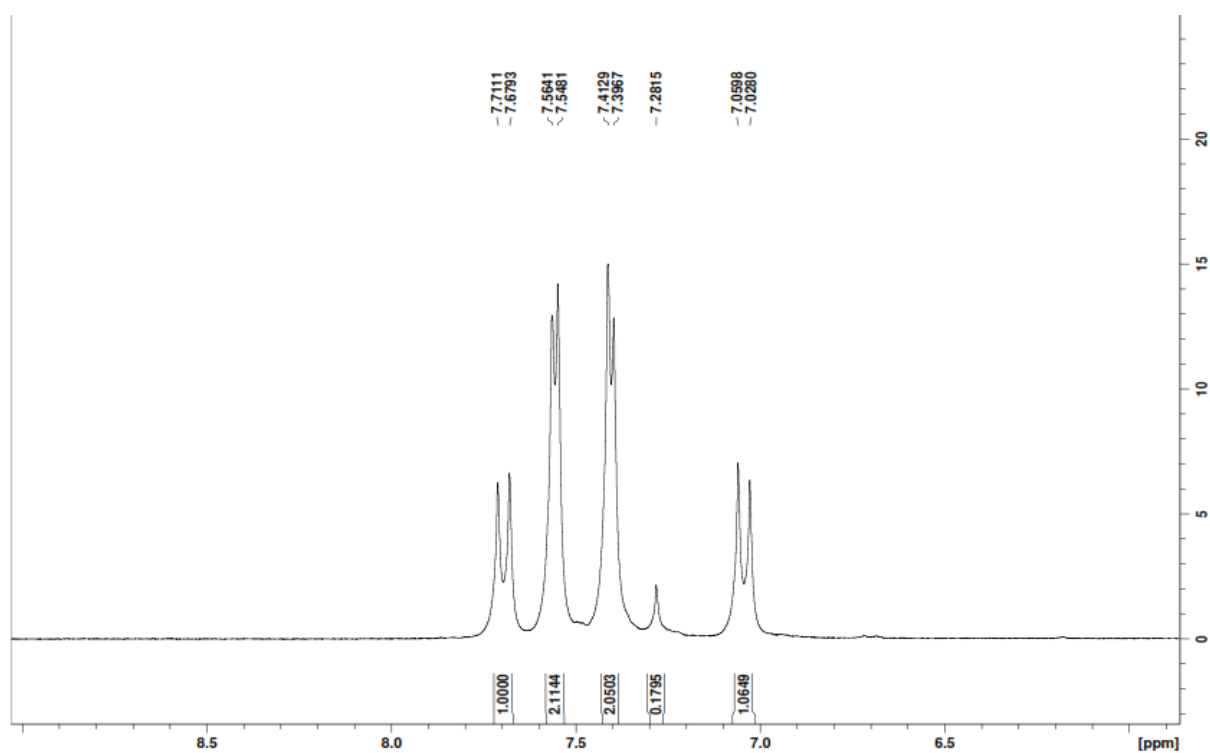
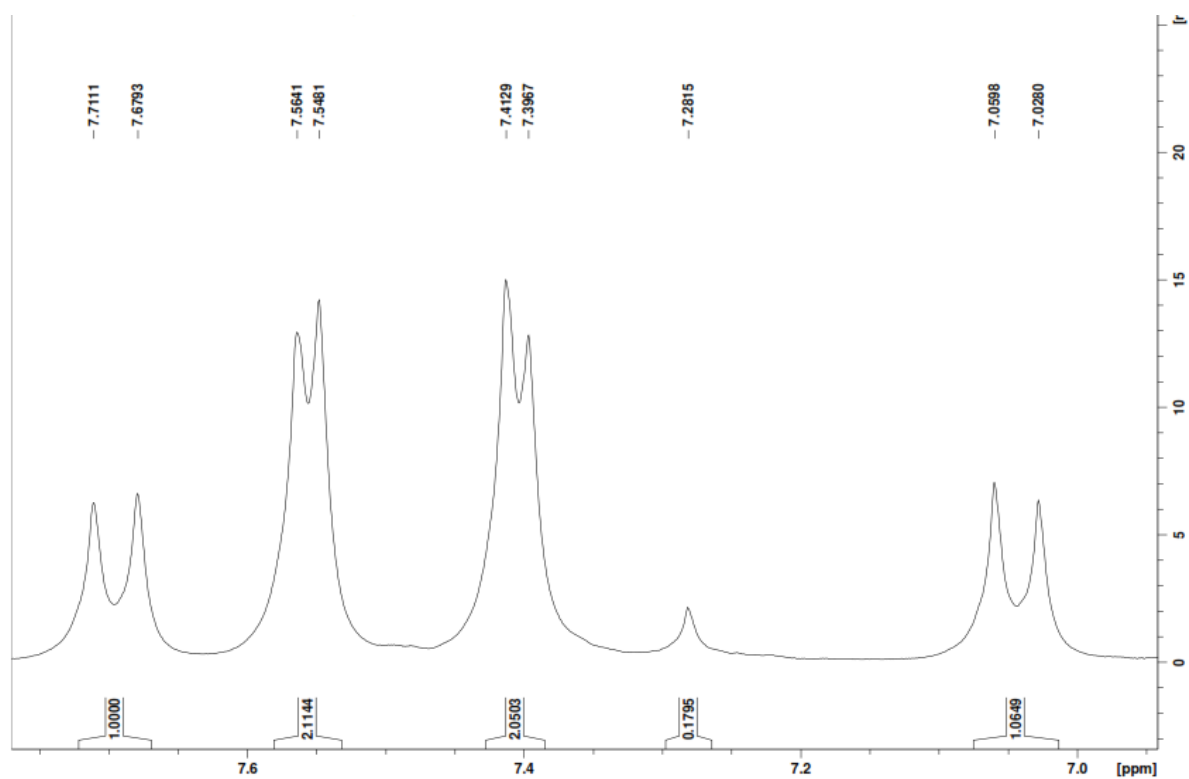
Figure S5. ^1H NMR spectrum (CDCl_3 , 300 MHz) of DB chlorine.**Figure S6.** Expansion of ^1H NMR spectrum (CDCl_3 , 300 MHz) of DB chlorine.

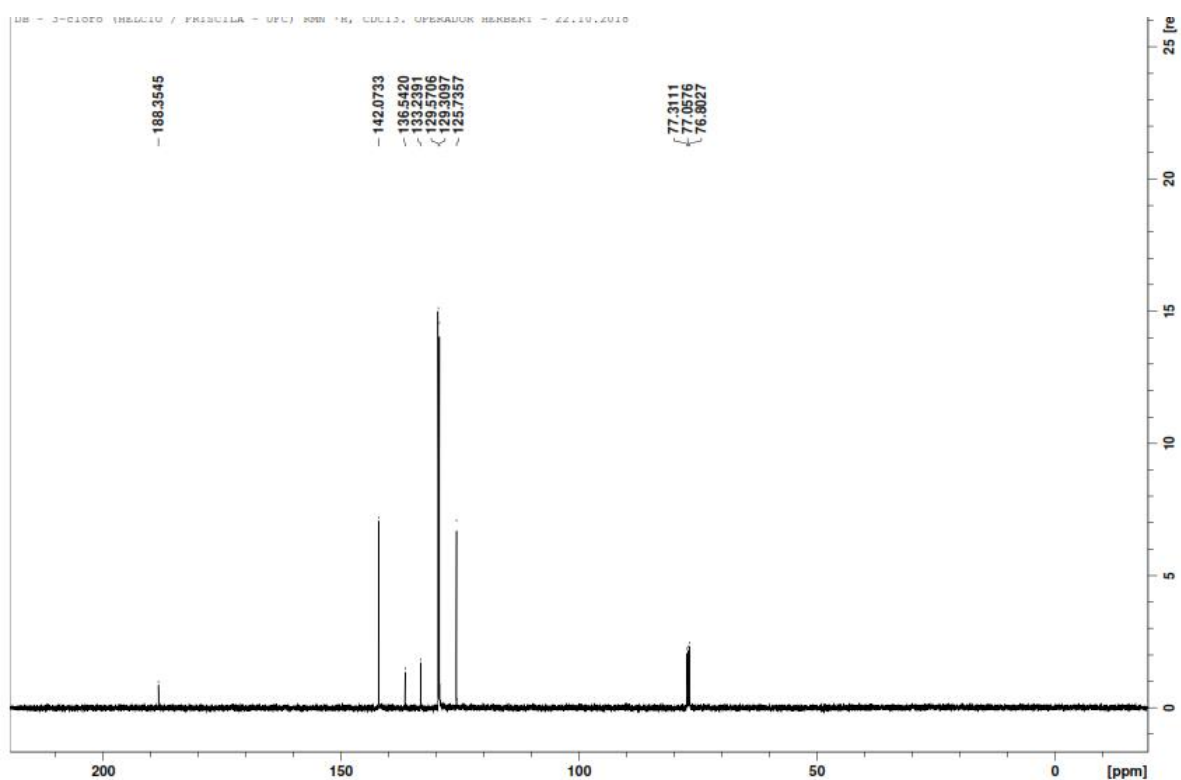
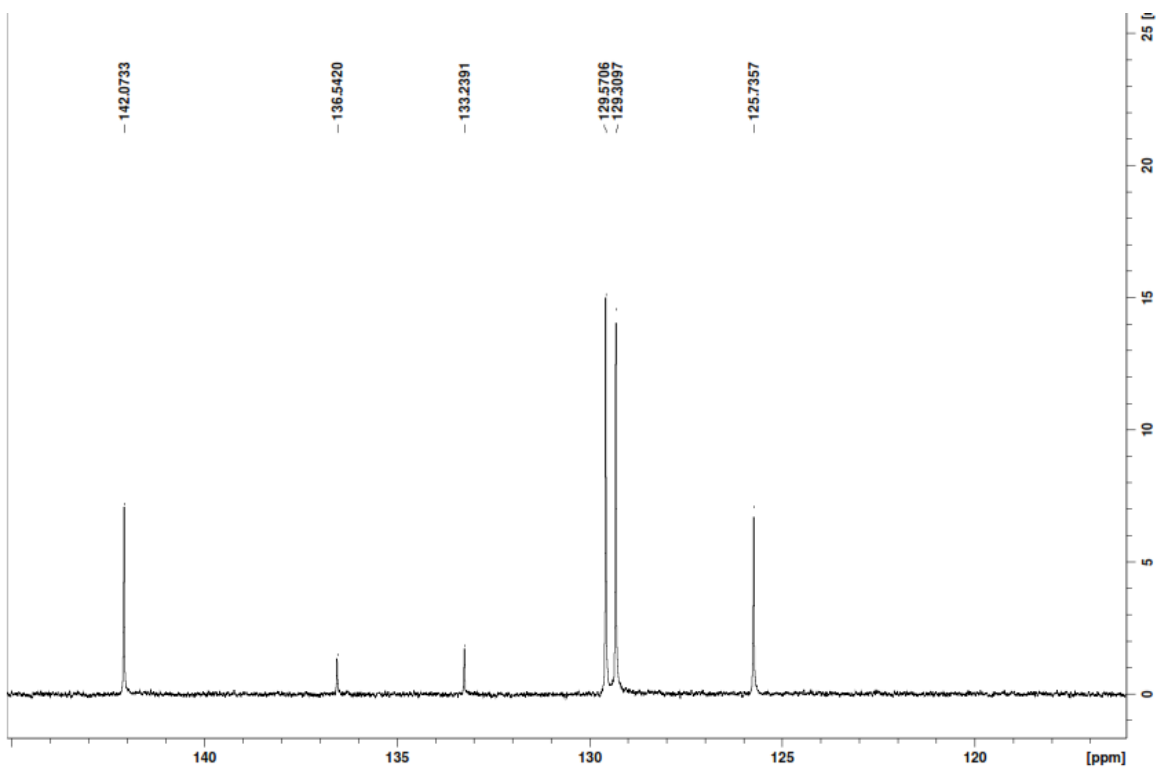
Figure S7. ^{13}C NMR spectrum (CDCl_3 , 125 MHz) of DB chlorine.**Figure S8.** Expansion of ^{13}C NMR spectrum (CDCl_3 , 125 MHz) of DB chlorine.

Figure S9. Experimental IR spectra of DB ethoxy after dehydration.

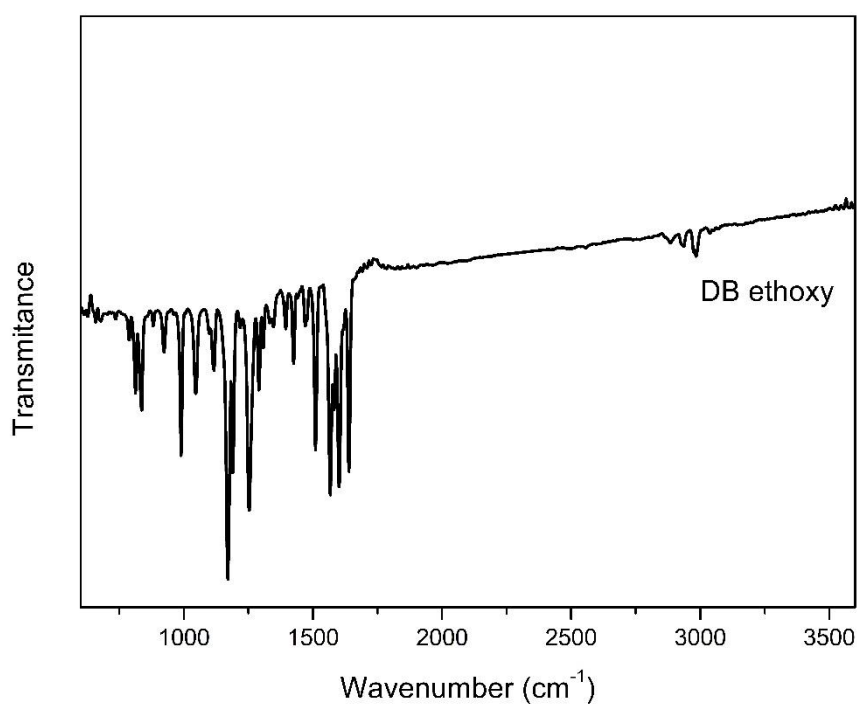


Figure S10. Comparison of the Raman calculated spectra of different DB ethoxy models along with the experimental spectrum for the trihydrated crystal in the 200 to 3450 cm⁻¹ range.

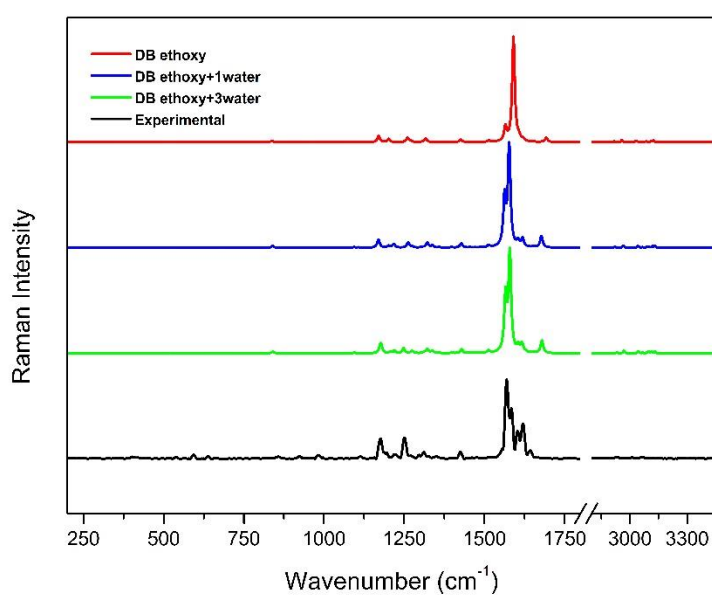


Figure S11. Comparison of the infrared calculated spectra of different DB ethoxy models along with the experimental spectrum for the trihydrated crystal in the 600 to 4000 cm^{-1} range.

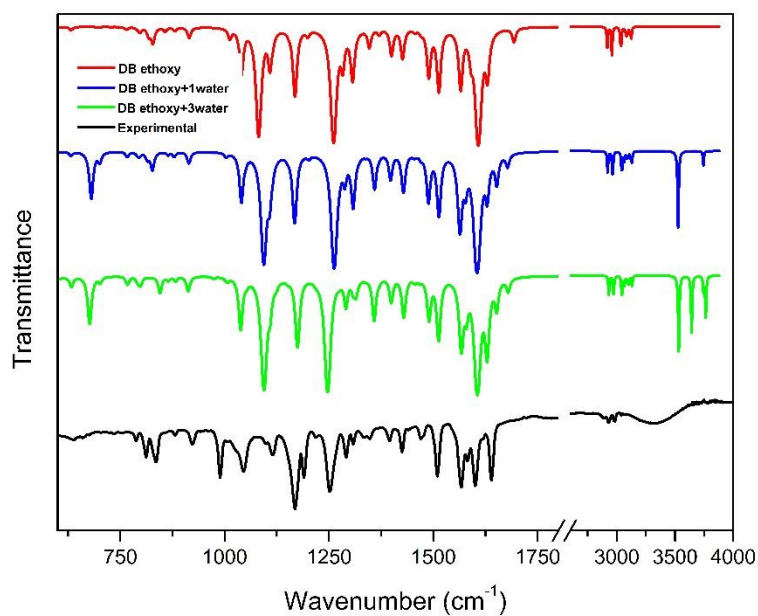


Figure S12. Comparison of the Raman calculated spectra of DB chlorine model along with the experimental spectrum for the crystal in the 200 to 3450 cm^{-1} range.

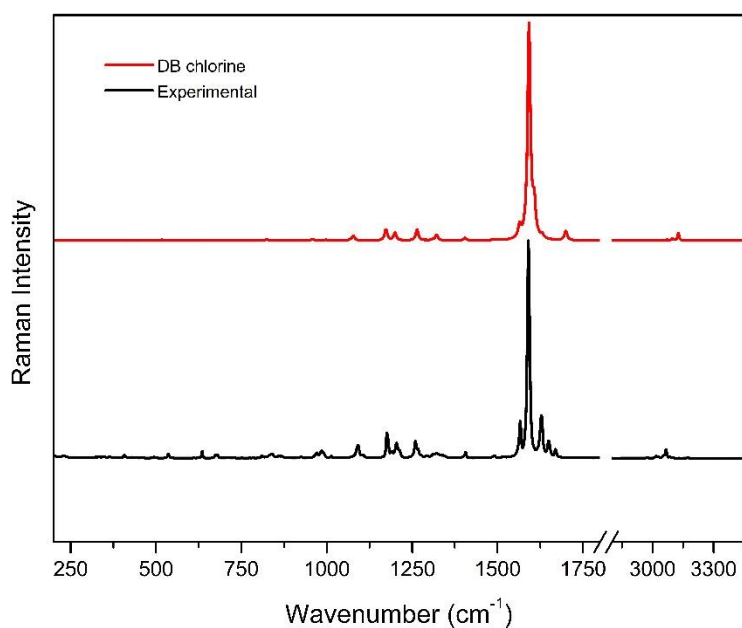


Figure S13. Comparison of the infrared calculated spectra of DB chlorine model along with the experimental spectrum for the crystal in the 600 to 4000 cm^{-1} range.

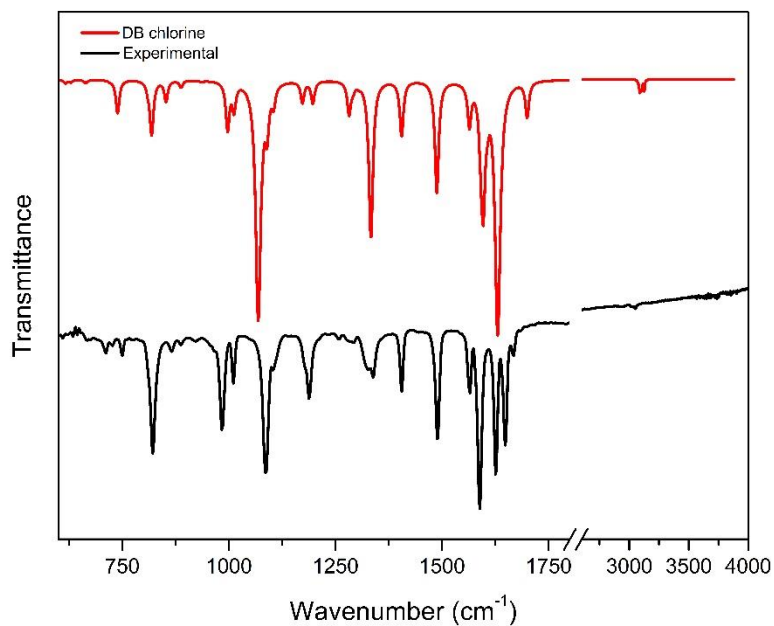


Figure S14. Mass spectra of DB chlorine.

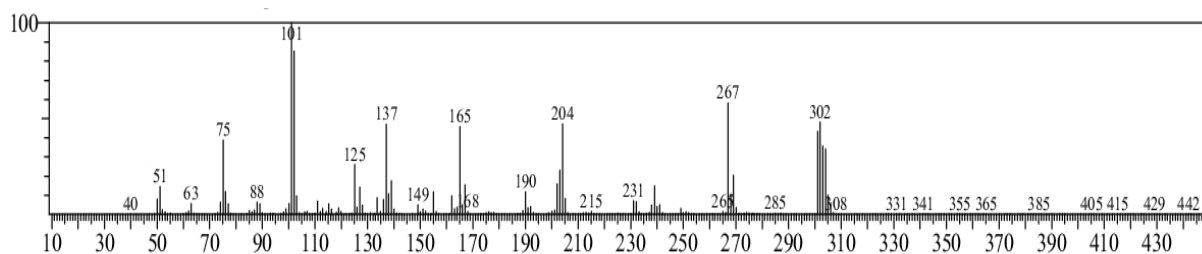


Figure S15. Mass spectra of DB ethoxy.

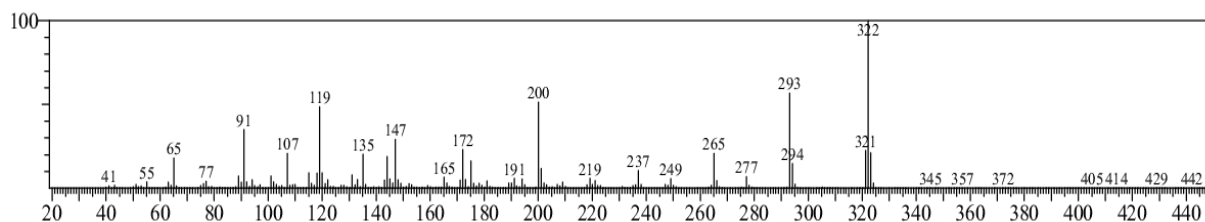


Figure S16. Dual scaling factors for DB ethoxy anhydrous, DB ethoxy monohydrated, DB ethoxy trihydrated models and DB chlorine.

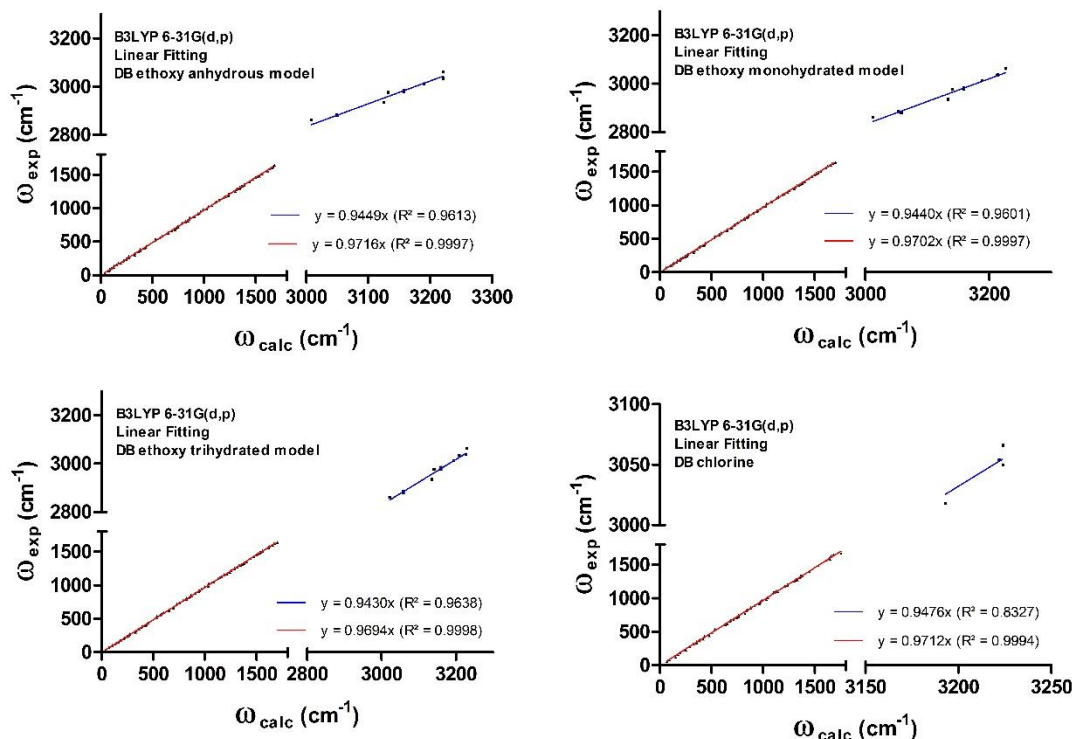


Table S1. Calculated vibrational wavenumbers (in cm^{-1}) unscaled and scaled by the scale factor 0.9716 ($\omega < 1800$) and 0.9449 ($\omega \geq 1800$), experimental Raman and infrared bands positions in units of cm^{-1} and assignment of vibrational modes for DB ethoxy in anhydrous model.

ω_{calc}	ω_{scal}	ω_{Raman}	ω_{IR}	Assignment of the molecular vibrations with PED (%)
3221	3043			$\nu(\text{C1H25})$ (49) + $\nu(\text{C14H34})$ (47)
3221	3043	3034vw/3063vw	3037vw	$\nu(\text{C1H25})$ (47) + $\nu(\text{C14H34})$ (49)
3209	3032			$\nu(\text{C3H26})$ (37) + $\nu(\text{C4H27})$ (11) + $\nu(\text{C16H35})$ (39) + $\nu(\text{C17H36})$ (12)
3209	3032			$\nu(\text{C3H26})$ (39) + $\nu(\text{C4H27})$ (12) + $\nu(\text{C16H35})$ (37) + $\nu(\text{C17H36})$ (11)
3191	3015			$\nu(\text{C3H26})$ (11) + $\nu(\text{C4H27})$ (35) + $\nu(\text{C16H35})$ (12) + $\nu(\text{C17H36})$ (36)
3190	3015	3012vw		$\nu(\text{C3H26})$ (12) + $\nu(\text{C4H27})$ (38) + $\nu(\text{C16H35})$ (11) + $\nu(\text{C17H36})$ (37)
3180	3005			$\nu(\text{C6H28})$ (50) + $\nu(\text{C13H33})$ (44)
3180	3005			$\nu(\text{C6H28})$ (44) + $\nu(\text{C13H33})$ (50)
3174	2999			$\nu(\text{C8H30})$ (45) + $\nu(\text{C10H31})$ (45)
3165	2991			$\nu(\text{C8H30})$ (45) + $\nu(\text{C10H31})$ (45)
3158	2984			$\nu(\text{C7H29})$ (47) + $\nu(\text{C11H32})$ (48)
3158	2984	2977vw	2985vw	$\nu(\text{C7H29})$ (46) + $\nu(\text{C11H32})$ (45)
3132	2959		2976vw	$\nu(\text{C22H40})$ (38) + $\nu(\text{C22H41})$ (38) + $\nu(\text{C24H45})$ (11) + $\nu(\text{C24H46})$ (11)
3132	2959			$\nu(\text{C22H40})$ (11) + $\nu(\text{C22H41})$ (11) + $\nu(\text{C24H45})$ (38) + $\nu(\text{C24H46})$ (38)
3125	2953			$\nu(\text{C22H39})$ (16) + $\nu(\text{C24H44})$ (46) + $\nu(\text{C24H45})$ (14) + $\nu(\text{C24H46})$ (14)
3125	2953	2936vw	2935vw	$\nu(\text{C22H39})$ (46) + $\nu(\text{C22H40})$ (14) + $\nu(\text{C22H41})$ (14) + $\nu(\text{C24H44})$ (16)
3050	2882			$\nu(\text{C22H39})$ (19) + $\nu(\text{C22H40})$ (16) + $\nu(\text{C22H41})$ (16) + $\nu(\text{C24H44})$ (19) + $\nu(\text{C24H45})$ (15) + $\nu(\text{C24H46})$ (15)
3050	2882	2881vw		$\nu(\text{C22H39})$ (19) + $\nu(\text{C22H40})$ (15) + $\nu(\text{C22H41})$ (15) + $\nu(\text{C24H44})$ (19) + $\nu(\text{C24H45})$ (16) + $\nu(\text{C24H46})$ (16)
3049	2881		2885vw	$\nu(\text{C21H37})$ (34) + $\nu(\text{C21H38})$ (34) + $\nu(\text{C23H42})$ (15) + $\nu(\text{C23H43})$ (15)

3049	2881			$v(C21H37)$ (15) + $v(C21H38)$ (15) + $v(C23H42)$ (34) + $v(C23H43)$ (34)
3008	2843	2862vw		$v(C21H37)$ (26) + $v(C21H38)$ (26) + $v(C23H42)$ (23) + $v(C23H43)$ (23)
3008	2842			$v(C21H37)$ (23) + $v(C21H38)$ (23) + $v(C23H42)$ (26) + $v(C23H43)$ (26)
1745	1695			$v(O18C9)$ (51) + $v(C7C8)$ (12) + $v(C10C11)$ (12)
1680	1632	1643w	1640vs	$v(C7C8)$ (19) + $v(C10C11)$ (19)
1669	1622	1620s	1620vw	$v(C4C3)$ (14) + $v(C17C16)$ (14)
1657	1610	1603m	1601vs	$v(C4C3)$ (11) + $v(C17C16)$ (11)
1639	1593	1584s	1583s	$v(O18C9)$ (30) + $v(C7C8)$ (14) + $v(C10C11)$ (14)
1613	1568			$v(C2C1)$ (14) + $v(C15C14)$ (15)
1613	1567	1556w/1569vs	1568vs	$v(C2C1)$ (15) + $v(C15C14)$ (14)
1560	1515			$\delta(H25C1C2)$ (11) + $\delta(H26C3C4)$ (11) + $\delta(H27C4C5)$ (12) + $\delta(H28C6C1)$ (12) + $\delta(H43C23H42)$ (15) + $\delta(C3C2C1)$ (10) + $\delta(H33C13C14)$ (13) + $\delta(H34C14C15)$ (11) + $\delta(H35C16C17)$ (10) + $\delta(H36C17C16)$ (12) + $\delta(H38C21H37)$ (14)
1560	1515	1515vw	1510vs	$\delta(H25C1C2)$ (11) + $\delta(H26C3C4)$ (11) + $\delta(H27C4C5)$ (12) + $\delta(H28C6C1)$ (12) + $\delta(H43C23H42)$ (15) + $\delta(C3C2C1)$ (10) + $\delta(H33C13C14)$ (13) + $\delta(H34C14C15)$ (11) + $\delta(H35C16C17)$ (10) + $\delta(H36C17C16)$ (12) + $\delta(H38C21H37)$ (14)
1535	1492			$\delta(H38C21H37)$ (32) + $\delta(H43C23H42)$ (32)
1535	1491	1497vw	1477w	$\delta(H38C21H37)$ (31) + $\delta(H43C23H42)$ (31)
1516	1473			$\delta(H41C22H40)$ (16) + $\delta(H46C24H45)$ (36)
1516	1473	1475vw	1469vw	$\delta(H41C22H40)$ (36) + $\delta(H46C24H45)$ (16)
1500	1457			$\delta(H39C22H41)$ (19) + $\delta(H40C22H39)$ (19) + $\delta(H44C24H46)$ (19) + $\delta(H45C24H44)$ (19)
1500	1457	1459vw		$\delta(H39C22H41)$ (19) + $\delta(H40C22H39)$ (19) + $\delta(H44C24H46)$ (19) + $\delta(H45C24H44)$ (19)
1470	1428	1425w	1425m	$v(C4C3)$ (13) + $v(C17C16)$ (10) + $v(C1C6)$ (12)
1470	1428			$v(C4C3)$ (12) + $v(C17C16)$ (12) + $v(C1C6)$ (10) + $v(C14C13)$ (10)
1443	1402			$\delta(H39C22H41)$ (10) + $\delta(H40C22H39)$ (10) + $\delta(H44C24H46)$ (10) + $\delta(H45C24H44)$ (10) + $\tau(H43C23O20C2)$ (10)
1442	1402	1396vw	1396w	$\delta(H39C22H41)$ (10) + $\delta(H40C22H39)$ (10) + $\delta(H44C24H46)$ (10) + $\delta(H45C24H44)$ (10) + $\tau(H38C21O19C15)$ (10)
1412	1372			$\tau(H38C21O19C15)$ (10) + $\tau(H43C23O20C2)$ (14)
1412	1372			$\tau(H38C21O19C15)$ (14) + $\tau(H43C23O20C2)$ (10)
1388	1348	1351vw	1348vw	$\delta(H30C8C9)$ (10)
1368	1330	1325vw	1333w	$v(C6C5)$ (12) + $v(C13C12)$ (11) + $\delta(H32C11C12)$ (11)
1357	1318	1311w		$\delta(H29C7C8)$ (14) + $\delta(H30C8C9)$ (10) + $\delta(H31C10C11)$ (14)
1347	1308		1308w	$\delta(H28C6C1)$ (11) + $\delta(H33C13C14)$ (11)
1345	1307	1295w		$\delta(H25C1C6)$ (10) + $\delta(H28C6C1)$ (12) + $\delta(H30C8C9)$ (11)
1341	1303			$\delta(H25C1C2)$ (10) + $\delta(H28C6C1)$ (10)
1322	1285		1292m	$\delta(H29C7C8)$ (17) + $\delta(H32C11C12)$ (11)
1311	1273	1272vw		$\delta(H30C8C9)$ (11)
1309	1272			$\delta(H37C21C22)$ (30) + $\delta(H42C23C24)$ (34)
1309	1272			$\delta(H37C21C22)$ (34) + $\delta(H42C23C24)$ (30)
1300	1263			$v(O19C15)$ (21) + $v(O20C2)$ (21)
1299	1262	1251m	1252vs	$v(C1C6)$ (11) + $v(C14C13)$ (11) + $v(O19C15)$ (14) + $v(O20C2)$ (14)
1238	1203	1220w	1219vw	$\delta(H29C7C8)$ (11) + $\delta(H32C11C12)$ (12)
1236	1201	1196w	1190s	$\delta(H32C11C12)$ (11)
1206	1172	1176m	1169vs	$\delta(H25C1C6)$ (10) + $\delta(H28C6C1)$ (14) + $\delta(H33C13C14)$ (14)
1204	1170			$\delta(H25C1C6)$ (11) + $\delta(H28C6C1)$ (14) + $\delta(H33C13C14)$ (14)
1189	1155			$\delta(H37C21C22)$ (12) + $\delta(H42C23C24)$ (12) + $\tau(H37C21O19C15)$ (13) + $\tau(H42C23O20C2)$ (13)
1189	1155	1158vw		$\delta(H37C21C22)$ (12) + $\delta(H42C23C24)$ (12) + $\tau(H37C21O19C15)$ (13) + $\tau(H42C23O20C2)$ (13)
1149	1116			$\delta(H26C3C4)$ (14) + $\tau(H45C24C23O20)$ (10) + $\tau(H46C24C23O20)$ (10)
1149	1116	1127vw		$\delta(H35C16C17)$ (15) + $\tau(H40C22C21O19)$ (12) + $\tau(H41C22C21O19)$ (11)
1142	1110			$\tau(H45C24C23O20)$ (11) + $\tau(H46C24C23O20)$ (11)
1142	1110	1113w	1115w	$\delta(H36C17C16)$ (11) + $\tau(H40C22C21O19)$ (10) + $\tau(H41C22C21O19)$ (10)
1114	1083	1098vw	1097vw	$v(C8C9)$ (25) + $v(C9C10)$ (25) + $\delta(H30C8C9)$ (15) + $\delta(H31C10C11)$ (15)
1073	1042	1049vw	1045s	$v(C22C21)$ (20) + $v(C24C23)$ (20) + $v(O19C21)$ (20) + $v(O20C23)$ (21)
1072	1042			$v(C22C21)$ (20) + $v(C24C23)$ (20) + $v(O19C21)$ (21) + $v(O20C23)$ (20)
1043	1014		1025vw	$\tau(H29C7C8C9)$ (39) + $\tau(H32C11C12C13)$ (38)
1035	1006			$\tau(H29C7C8C9)$ (38) + $\tau(H30C8C9C10)$ (10) + $\tau(H32C11C12C13)$ (38)
1027	997	996vw	989s	$\delta(C4C3C2)$ (22) + $\delta(C17C16C15)$ (22) + $\delta(C2C1C6)$ (12) + $\delta(C15C14C13)$ (12)
1026	997			$\delta(C4C3C2)$ (22) + $\delta(C17C16C15)$ (22) + $\delta(C2C1C6)$ (12) + $\delta(C15C14C13)$ (12)
990	962	981w		$v(O18C9)$ (10) + $v(C8C9)$ (23) + $v(C9C10)$ (23)
966	938			$\tau(H26C3C4C5)$ (17) + $\tau(H27C4C3C2)$ (21) + $\tau(H35C16C17C12)$ (17) + $\tau(H36C17C16C15)$ (20)
966	938	944vw		$\tau(H26C3C4C5)$ (17) + $\tau(H27C4C3C2)$ (20) + $\tau(H35C16C17C12)$ (17) + $\tau(H36C17C16C15)$ (21)
954	927			$\tau(H25C1C2C3)$ (14) + $\tau(H28C6C1C2)$ (25) + $\tau(H33C13C14C15)$ (25) + $\tau(H34C14C15C16)$ (14)
953	926	924w		$\tau(H25C1C2C3)$ (14) + $\tau(H28C6C1C2)$ (25) + $\tau(H33C13C14C15)$ (25) + $\tau(H34C14C15C16)$ (14)

943	916			$v(C22C21)$ (15) + $v(C24C23)$ (15)
943	916	909vw	922w	$v(C22C21)$ (15) + $v(C24C23)$ (14)
908	882	878vw	881w	$\tau(H30C8C9C10)$ (34) + $\tau(H31C10C11C12)$ (32) + $\gamma(O18C8C10C9)$ (15)
884	858			$\delta(C3C2C1)$ (10) + $\delta(C16C15C14)$ (10)
870	845			$\tau(H30C8C9C10)$ (15) + $\tau(H31C10C11C12)$ (25)
863	838	857w		$\delta(C3C2C1)$ (11) + $\delta(C16C15C14)$ (11)
853	829	830vw	835m	$\tau(H27C4C5C6)$ (16) + $\tau(H30C8C9C10)$ (12) + $\tau(H31C10C9C8)$ (13) + $\tau(H36C17C16C15)$ (15)
845	821			$\tau(H39C22C21O19)$ (11) + $\tau(H44C24C23O20)$ (11)
843	819		812m	$\tau(H26C3C4C5)$ (14) + $\tau(H27C4C3C2)$ (11) + $\tau(H35C16C17C12)$ (14) + $\tau(H36C17C16C15)$ (11)
839	815	813vw		$\tau(H26C3C4C5)$ (17) + $\tau(H27C4C3C2)$ (16) + $\tau(H35C16C17C12)$ (17) + $\tau(H36C17C16C15)$ (16)
821	798	806vw	789w	$\tau(H25C1C2C3)$ (20) + $\tau(H34C14C15C16)$ (20)
817	794	791vw		$\tau(H25C1C2C3)$ (19) + $\tau(H28C6C1C2)$ (12) + $\tau(H33C13C14C15)$ (12) + $\tau(H34C14C15C16)$ (19)
814	791			$\tau(H25C1C2C3)$ (36) + $\tau(H28C6C5C7)$ (18) + $\tau(H34C14C15C16)$ (36) + $\tau(H33C13C12C11)$ (18)
788	765	776vw		$\delta(C14C13C12)$ (10)
749	728	719vw/737vw		$\tau(C2C1C6C5)$ (17) + $\gamma(O18C8C10C9)$ (18)
725	704	704vw		$\tau(C2C1C6C5)$ (23) + $\tau(C15C14C13C12)$ (12)
718	697	679vw		$\delta(O18C9C10)$ (25)
689	669	664vw	661vw	$\gamma(O18C8C10C9)$ (38)
652	634	638w	638vw	$\delta(C1C6C5)$ (11) + $\delta(C2C1C6)$ (15) + $\delta(C15C14C13)$ (14)
651	632	625vw		$\delta(C1C6C5)$ (11) + $\delta(C2C1C6)$ (15) + $\delta(C15C14C13)$ (14)
595	578	578vw/593w		$\delta(C1C6C5)$ (11) + $\delta(C14C13C12)$ (10)
573	557	554vw/566vw		$\delta(O18C9C10)$ (22) + $\delta(C1C6C5)$ (11) + $\delta(C14C13C12)$ (10)
535	520			$\gamma(O20C1C3C2)$ (16) + $\gamma(O19C14C16C15)$ (16)
530	515	539w		$\delta(C10C11C12)$ (11) + $\delta(C8C9C10)$ (33)
528	513	516vw		$\gamma(O20C1C3C2)$ (16) + $\gamma(O19C14C16C15)$ (16)
444	431	438vw		$\delta(C6C5C7)$ (14) + $\delta(O19C15C16)$ (10) + $\delta(O20C2C3)$ (10)
435	423	420vw		$\delta(O19C15C16)$ (21) + $\delta(O20C2C3)$ (21)
433	421	414vw		$\delta(O19C15C16)$ (11) + $\delta(O20C2C3)$ (11) + $\delta(C21O19C15)$ (10) + $\delta(C23O20C2)$ (10)
427	415			$\tau(C4C3C2C1)$ (10) + $\tau(C15C14C13C12)$ (22) + $\tau(C3C2C1C6)$ (10)
427	414	403w		$\tau(C4C3C2C1)$ (10) + $\tau(C15C14C13C12)$ (21) + $\tau(C3C2C1C6)$ (10)
383	372	384vw		$\tau(C17C16C15C14)$ (14) + $\tau(C1C6C5C7)$ (13) + $\gamma(O20C1C3C2)$ (10) + $\gamma(O19C14C16C15)$ (10) + $\gamma(C11C13C17C12)$ (13)
382	371	368vw		$\tau(C17C16C15C14)$ (13) + $\tau(C1C6C5C7)$ (13) + $\gamma(C11C13C17C12)$ (13)
362	352	353vw		$\delta(C22C21O19)$ (20) + $\delta(C24C23O20)$ (20)
329	319	300vw/320vw		$\delta(C22C21O19)$ (17) + $\delta(C24C23O20)$ (17)
271	263	271vw/279vw		$\tau(H39C22C21O19)$ (24) + $\tau(H40C22C21O19)$ (10) + $\tau(H41C22C21O19)$ (10) + $\tau(H44C24C23O20)$ (24) + $\tau(H45C24C23O20)$ (10) + $\tau(H46C24C23O20)$ (10)
271	263	256vw		$\tau(H39C22C21O19)$ (24) + $\tau(H40C22C21O19)$ (10) + $\tau(H41C22C21O19)$ (10) + $\tau(H44C24C23O20)$ (24) + $\tau(H45C24C23O20)$ (10) + $\tau(H46C24C23O20)$ (10)
250	243	249vw		$\delta(O18C9C10)$ (14) + $\delta(C7C8C9)$ (15) + $\delta(C9C10C11)$ (15)
245	238	236vw		$\delta(C6C5C7)$ (18) + $\delta(C11C12C17)$ (16) + $\delta(C8C9C10)$ (22)
221	214	217vw		$\tau(C3C2C1C6)$ (11) + $\tau(C16C15C14C13)$ (12)
218	212	204vw		$\tau(C7C8C9C10)$ (12) + $\tau(C8C9C10C11)$ (16)
194	189	184vw		$\tau(C7C8C9C10)$ (15) + $\tau(C5C7C8C9)$ (20) + $\tau(C8C9C10C11)$ (19) + $\tau(C9C10C11C12)$ (15)
170	165	176vw		$\delta(O19C15C16)$ (10) + $\delta(O20C2C3)$ (10) + $\delta(C21O19C15)$ (20) + $\delta(C23O20C2)$ (20)
151	147	153vw		$\delta(C7C8C9)$ (10) + $\delta(C9C10C11)$ (10)
128	125	138vw		$\delta(C21O19C15)$ (16) + $\delta(C23O20C2)$ (16)
126	123	122vw		$\tau(C21O19C15C14)$ (32) + $\tau(C23O20C2C1)$ (32)
126	123			$\tau(C21O19C15C14)$ (35) + $\tau(C23O20C2C1)$ (35)
104	101	103w/106w		$\tau(C7C8C9C10)$ (11) + $\tau(C6C5C7C8)$ (10) + $\tau(C8C9C10C11)$ (12) + $\tau(C22C21O19C15)$ (12) + $\tau(C24C23O20C2)$ (12)
86	84	84w		$\tau(C22C21O19C15)$ (19) + $\tau(C24C23O20C2)$ (19)
84	82			$\tau(C10C11C12C13)$ (13) + $\tau(C6C5C7C8)$ (14) + $\tau(C5C7C8C9)$ (20) + $\tau(C9C10C11C12)$ (18)
76	73	65w		$\delta(C10C11C12)$ (15) + $\delta(C6C5C7)$ (15) + $\delta(C5C7C8)$ (16) + $\delta(C11C12C17)$ (15)
53	51			$\tau(C7C8C9C10)$ (10) + $\tau(C8C9C10C11)$ (10) + $\tau(C22C21O19C15)$ (14) + $\tau(C24C23O20C2)$ (14)
39	38	38w		$\tau(C1C6C5C7)$ (16) + $\tau(C5C7C8C9)$ (13) + $\tau(C9C10C11C12)$ (12) + $\gamma(C11C13C17C12)$ (12)
27	26			$\tau(C10C11C12C13)$ (34) + $\tau(C6C5C7C8)$ (33)
24	23			$\delta(C7C8C9)$ (18) + $\delta(C10C11C12)$ (16) + $\delta(C5C7C8)$ (16) + $\delta(C8C9C10)$ (17) + $\delta(C9C10C11)$ (18)
20	19			$\tau(C7C8C9C10)$ (20) + $\tau(C10C11C12C13)$ (17) + $\tau(C6C5C7C8)$ (16) + $\tau(C8C9C10C11)$ (19)

Abbreviation: ν , stretching; δ , bending; τ , torsion; γ , out-of-plane; vw, very weak; w, weak; m, medium; s, strong; vs, very strong.

Table S2. Calculated vibrational wavenumbers (in cm^{-1}) unscaled and scaled by the scale factor 0.9702 ($\omega < 1800$) and 0.9440 ($\omega \geq 1800$), experimental Raman and infrared bands positions in units of cm^{-1} and assignment of vibrational modes for DB ethoxy in a monohydrated model.

ω_{calc}	ω_{scal}	ω_{Raman}	ω_{IR}	Assignment of the molecular vibrations with PED (%)
3860	3644			$\nu(\text{O47H49})$ (96)
3636	3432			$\nu(\text{O47H48})$ (95)
3227	3047			$\nu(\text{C1H25})$ (96)
3227	3046	3063vw		$\nu(\text{C14H34})$ (95)
3214	3034		3037vw	$\nu(\text{C3H26})$ (76) + $\nu(\text{C4H27})$ (23)
3213	3033	3034vw		$\nu(\text{C16H35})$ (79) + $\nu(\text{C17H36})$ (20)
3197	3018			$\nu(\text{C3H26})$ (22) + $\nu(\text{C4H27})$ (69)
3194	3015			$\nu(\text{C16H35})$ (20) + $\nu(\text{C17H36})$ (74)
3189	3011	3012vw		$\nu(\text{C13H33})$ (94)
3185	3007			$\nu(\text{C6H28})$ (92)
3182	3004			$\nu(\text{C8H30})$ (69) + $\nu(\text{C10H31})$ (18)
3171	2994			$\nu(\text{C8H30})$ (19) + $\nu(\text{C10H31})$ (67) + $\nu(\text{C11H32})$ (10)
3163	2986			$\nu(\text{C7H29})$ (96)
3159	2982	2977vw	2985vw	$\nu(\text{C10H31})$ (10) + $\nu(\text{C11H32})$ (87)
3141	2965		2976vw	$\nu(\text{C24H45})$ (49) + $\nu(\text{C24H46})$ (49)
3140	2964			$\nu(\text{C22H40})$ (49) + $\nu(\text{C22H41})$ (48)
3135	2959			$\nu(\text{C24H44})$ (62) + $\nu(\text{C24H45})$ (18) + $\nu(\text{C24H46})$ (19)
3134	2959	2936vw	2935vw	$\nu(\text{C22H39})$ (62) + $\nu(\text{C22H40})$ (18) + $\nu(\text{C22H41})$ (19)
3059	2887			$\nu(\text{C24H44})$ (37) + $\nu(\text{C24H45})$ (31) + $\nu(\text{C24H46})$ (31)
3058	2887	2881vw		$\nu(\text{C22H39})$ (37) + $\nu(\text{C22H40})$ (31) + $\nu(\text{C22H41})$ (31)
3053	2882		2885vw	$\nu(\text{C21H37})$ (48) + $\nu(\text{C21H38})$ (50)
3053	2882			$\nu(\text{C23H42})$ (49) + $\nu(\text{C23H43})$ (48)
3012	2843	2862vw		$\nu(\text{C21H37})$ (51) + $\nu(\text{C21H38})$ (48)
3012	2843			$\nu(\text{C23H42})$ (49) + $\nu(\text{C23H43})$ (50)
1729	1678			$\nu(\text{O18C9})$ (38) + $\nu(\text{C7C8})$ (16) + $\nu(\text{C10C11})$ (15)
1703	1652	1643w	1640vs	$\delta(\text{H49O47H48})$ (88)
1679	1629		1620vw	$\nu(\text{C7C8})$ (17) + $\nu(\text{C10C11})$ (16)
1668	1619	1620s		$\nu(\text{C4C3})$ (14) + $\nu(\text{C17C16})$ (14)
1654	1605	1603m	1601vs	$\nu(\text{C7C8})$ (11) + $\nu(\text{C17C16})$ (10)
1625	1577	1584s	1583s	$\nu(\text{O18C9})$ (33)
1612	1564	1569vs	1568vs	$\nu(\text{C2C1})$ (20) + $\nu(\text{C6C5})$ (11)
1611	1563	1556w		$\nu(\text{C15C14})$ (17)
1560	1513			$\delta(\text{H33C13C14})$ (13) + $\delta(\text{H34C14C15})$ (11) + $\delta(\text{H35C16C17})$ (10) + $\delta(\text{H36C17C16})$ (12) + $\delta(\text{H38C21H37})$ (14)
1559	1513	1515vw	1510vs	$\delta(\text{H25C1C2})$ (11) + $\delta(\text{H26C3C4})$ (11) + $\delta(\text{H27C4C5})$ (12) + $\delta(\text{H28C6C1})$ (12) + $\delta(\text{H43C23H42})$ (15) + $\delta(\text{C3C2C1})$ (10)
1534	1489			$\delta(\text{H38C21H37})$ (43) + $\delta(\text{H43C23H42})$ (21)
1534	1488	1497vw	1477w	$\delta(\text{H38C21H37})$ (21) + $\delta(\text{H43C23H42})$ (41)
1515	1470			$\delta(\text{H41C22H40})$ (51) + $\tau(\text{H40C22C21O19})$ (10) + $\tau(\text{H41C22C21O19})$ (10)
1515	1470	1475vw	1469vw	$\delta(\text{H46C24H45})$ (51) + $\tau(\text{H45C24C23O20})$ (10) + $\tau(\text{H46C24C23O20})$ (10)
1499	1454			$\delta(\text{H39C22H41})$ (38) + $\delta(\text{H40C22H39})$ (38) + $\tau(\text{H39C22C21O19})$ (14)
1499	1454	1459vw		$\delta(\text{H44C24H46})$ (38) + $\delta(\text{H45C24H44})$ (38) + $\tau(\text{H44C24C23O20})$ (14)
1473	1429	1425w	1425m	$\nu(\text{C17C16})$ (22) + $\nu(\text{C14C13})$ (18) + $\delta(\text{H34C14C15})$ (13)
1471	1427			$\nu(\text{C4C3})$ (23) + $\nu(\text{C1C6})$ (20) + $\delta(\text{H25C1C2})$ (14)
1440	1397			$\delta(\text{H44C24H46})$ (16) + $\delta(\text{H45C24H44})$ (16) + $\tau(\text{H43C23O20C2})$ (16)
1440	1397	1396vw	1396w	$\delta(\text{H39C22H41})$ (16) + $\delta(\text{H40C22H39})$ (16) + $\tau(\text{H38C21O19C15})$ (16)
1411	1368			$\delta(\text{H39C22H41})$ (14) + $\delta(\text{H40C22H39})$ (14) + $\delta(\text{H41C22H40})$ (10) + $\tau(\text{H37C21O19C15})$ (11) + $\tau(\text{H38C21O19C15})$ (19)
1410	1368			$\delta(\text{H44C24H46})$ (14) + $\delta(\text{H45C24H44})$ (14) + $\delta(\text{H46C24H45})$ (11) + $\tau(\text{H42C23O20C2})$ (13) + $\tau(\text{H43C23O20C2})$ (22)
1401	1359	1351vw	1348vw	$\delta(\text{H32C11C10})$ (21)
1379	1337	1325vw	1333w	$\nu(\text{C6C5})$ (10) + $\delta(\text{H29C7C8})$ (13) + $\delta(\text{H32C11C10})$ (17)
1363	1322	1311w		$\delta(\text{H31C10C11})$ (20)
1349	1309		1308w	$\nu(\text{C7C8})$ (13) + $\delta(\text{H29C7C8})$ (19) + $\delta(\text{H30C8C9})$ (12)
1348	1308	1295w		$\delta(\text{H25C1C6})$ (10) + $\delta(\text{H28C6C1})$ (12) + $\delta(\text{H30C8C9})$ (11)
1341	1301			$\delta(\text{H25C1C2})$ (10) + $\delta(\text{H28C6C1})$ (10)
1327	1288		1292m	$\delta(\text{H29C7C8})$ (12) + $\delta(\text{H32C11C10})$ (11) + $\delta(\text{H36C17C16})$ (13)
1315	1276	1272vw		$\nu(\text{C5C7})$ (10) + $\delta(\text{H31C10C11})$ (12)
1307	1268			$\delta(\text{H42C23C24})$ (63) + $\tau(\text{H42C23O20C2})$ (12) + $\tau(\text{H43C23O20C2})$ (11)
1307	1268			$\delta(\text{H37C21C22})$ (63) + $\tau(\text{H37C21O19C15})$ (13) + $\tau(\text{H38C21O19C15})$ (11)
1301	1262			$\nu(\text{C1C6})$ (16) + $\nu(\text{C3C2})$ (10) + $\nu(\text{O20C2})$ (36)

1301	1262	1251m	1252vs	$v(C14C13)$ (18) + $v(O19C15)$ (35)
1255	1217	1220w	1219vw	$v(C13C12)$ (17) + $v(C11C12)$ (20) + $\delta(H31C10C11)$ (13) + $\delta(H33C13C14)$ (10)
1238	1201	1196w	1190s	$v(C6C5)$ (13) + $v(C5C7)$ (15) + $\delta(H26C3C4)$ (10) + $\delta(H29C7C8)$ (21) + $\delta(H30C8C9)$ (11)
1206	1170	1176m	1169vs	$\delta(H33C13C14)$ (19) + $\delta(H34C14C15)$ (15) + $\delta(H35C16C17)$ (11)
1203	1167			$\delta(H25C1C2)$ (15) + $\delta(H26C3C4)$ (10) + $\delta(H28C6C1)$ (22)
1187	1151			$\delta(H42C23C24)$ (24) + $\tau(H42C23O20C2)$ (26)
1187	1151	1158vw		$\delta(H37C21C22)$ (24) + $\tau(H37C21O19C15)$ (26)
1148	1114	1127vw		$\delta(H26C3C4)$ (14) + $\tau(H45C24C23O20)$ (10) + $\tau(H46C24C23O20)$ (10)
1148	1114			$\delta(H35C16C17)$ (15) + $\tau(H40C22C21O19)$ (12) + $\tau(H41C22C21O19)$ (11)
1141	1107	1113w	1115w	$\tau(H45C24C23O20)$ (11) + $\tau(H46C24C23O20)$ (11)
1141	1107			$\delta(H36C17C16)$ (11) + $\tau(H40C22C21O19)$ (10) + $\tau(H41C22C21O19)$ (10)
1127	1093	1098vw	1097vw	$v(C9C10)$ (24) + $v(C8C9)$ (25) + $\delta(H30C8C9)$ (16) + $\delta(H31C10C11)$ (13)
1083	1050	1049vw		$\tau(H32C11C12C13)$ (82)
1072	1040		1045s	$v(C22C21)$ (19) + $v(C24C23)$ (22) + $v(O19C21)$ (19) + $v(O20C23)$ (22)
1072	1040			$v(C22C21)$ (22) + $v(C24C23)$ (19) + $v(O19C21)$ (22) + $v(O20C23)$ (19)
1034	1003		1025vw	$\tau(H29C7C5C6)$ (72) + $\tau(H30C8C9C10)$ (14)
1027	996	996vw	989s	$\delta(C17C16C15)$ (40) + $\delta(H34C14C15)$ (10) + $\delta(C15C14C13)$ (21) + $\delta(C16C15C14)$ (12)
1026	995			$\delta(C4C3C2)$ (35) + $\delta(C2C1C6)$ (19) + $\delta(C3C2C1)$ (11)
1002	972	981vw		$v(O18C9)$ (12) + $v(C9C10)$ (22) + $v(C8C9)$ (22) + $\delta(C9C10C11)$ (10)
970	941	944vw		$\tau(H33C13C14C15)$ (53) + $\tau(H34C14C15C16)$ (23)
964	935			$\tau(H26C3C4C5)$ (31) + $\tau(H27C4C5C6)$ (44) + $\tau(C4C3C2C1)$ (13)
963	934			$\tau(H35C16C17C12)$ (30) + $\tau(H36C17C16C15)$ (38) + $\tau(C17C16C15C14)$ (14)
951	923	924w		$\tau(H25C1C2C3)$ (25) + $\tau(H28C6C5C7)$ (58)
942	914			$v(C24C23)$ (27) + $v(O20C23)$ (15) + $\tau(H45C24C23O20)$ (10) + $\tau(H46C24C23O20)$ (10)
942	914	909vw	922w	$v(C22C21)$ (27) + $v(O19C21)$ (15) + $\tau(H40C22C21O19)$ (10) + $\tau(H41C22C21O19)$ (10)
906	879	878vw	881w	$\tau(H30C8C9C10)$ (33) + $\tau(H31C10C11C12)$ (38) + $\gamma(O18C8C10C9)$ (13)
890	864			$\delta(C16C15C14)$ (11)
869	843			$\tau(H30C8C9C10)$ (18) + $\tau(H31C10C11C12)$ (26)
865	839	857w		$\delta(C3C2C1)$ (13)
852	827	830vw	835m	$\tau(H27C4C5C6)$ (16) + $\tau(H30C8C9C10)$ (12) + $\tau(H31C10C9C8)$ (13) + $\tau(H36C17C16C15)$ (15)
843	818		812m	$\tau(H44C24C23O20)$ (10)
839	814			$\tau(H26C3C4C5)$ (15) + $\tau(H27C4C5C6)$ (10) + $\tau(H35C16C17C12)$ (13) + $\tau(H36C17C16C15)$ (10)
837	812	813vw		$\tau(H26C3C4C5)$ (17) + $\tau(H27C4C5C6)$ (14) + $\tau(H35C16C17C12)$ (19) + $\tau(H36C17C16C15)$ (17)
820	796	806vw	789w	$\tau(H33C13C14C15)$ (14) + $\tau(H34C14C15C16)$ (34)
816	792			$\tau(H34C14C15C16)$ (36) + $\tau(H33C13C12C11)$ (18)
816	791	791vw		$\tau(H25C1C2C3)$ (36) + $\tau(H28C6C5C7)$ (18)
790	766	776vw		$\delta(C14C13C12)$ (10)
751	728	737vw		$\tau(C2C1C6C5)$ (14) + $\gamma(O18C8C10C9)$ (21)
726	704	719vw		$\tau(C2C1C6C5)$ (18) + $\tau(C15C14C13C12)$ (12)
722	700	704vw		$\delta(O18C9C10)$ (17) + $\delta(C16C15C14)$ (10)
701	680	679vw		$\delta(O47H48O18)$ (34) + $\delta(H48O18C9)$ (13) + $\delta(O18C9C10)$ (10) + $\tau(H49O47H48O18)$ (19)
692	672	664vw	661vw	$\gamma(O18C8C10C9)$ (37)
652	633	638w	638vw	$\delta(C4C3C2)$ (11) + $\delta(C1C6C5)$ (14) + $\delta(C2C1C6)$ (21)
651	631	625vw		$\delta(C2C1C6)$ (10) + $\delta(C15C14C13)$ (20)
595	577	578vw/593w		$\delta(C1C6C5)$ (11)
574	557	554vw/566vw		$\delta(O18C9C10)$ (20) + $\delta(C1C6C5)$ (11) + $\delta(C14C13C12)$ (10)
535	519			$\gamma(O19C14C16C15)$ (19)
535	519	539w		$\delta(C8C9C10)$ (26)
528	513	516vw		$\gamma(O20C1C3C2)$ (20)
444	431	438vw		$\delta(C6C5C7)$ (14) + $\delta(O19C15C16)$ (12)
436	423			$\delta(O20C2C3)$ (33) + $\delta(C23O20C2)$ (15)
434	421	420vw		$\delta(O19C15C16)$ (22) + $\delta(C21O19C15)$ (17)
428	415	414vw		$\tau(H36C17C16C15)$ (10) + $\tau(C17C16C15C14)$ (11) + $\tau(C15C14C13C12)$ (40)
426	414	403w		$\tau(C4C3C2C1)$ (20) + $\tau(C2C1C6C5)$ (28) + $\tau(C3C2C1C6)$ (18)
388	377	384vw		$\tau(C17C16C15C14)$ (23) + $\tau(C9C10C11C12)$ (13) + $\gamma(O19C14C16C15)$ (14) + $\gamma(C11C13C17C12)$ (19)
382	370	368vw		$\tau(C4C3C2C1)$ (19) + $\tau(C1C6C5C7)$ (28) + $\tau(C5C7C8C9)$ (13) + $\gamma(O20C1C3C2)$ (20)
362	351	353vw		$\delta(C22C21O19)$ (21) + $\delta(C24C23O20)$ (20)
335	325	320vw		$\delta(O47H48O18)$ (19) + $\tau(O47H48O18C9)$ (11)
328	318	300vw		$\delta(O47H48O18)$ (23) + $\tau(O47H48O18C9)$ (12)
274	266	279vw		$\tau(H49O47H48O18)$ (43) + $\tau(C8C9C10C11)$ (14)
270	262	271vw		$v(O18H48)$ (13) + $\delta(C7C8C9)$ (11)
260	252	256vw		$\tau(H44C24C23O20)$ (46) + $\tau(H45C24C23O20)$ (18) + $\tau(H46C24C23O20)$ (18)
258	251	249vw		$\tau(H39C22C21O19)$ (45) + $\tau(H40C22C21O19)$ (15) + $\tau(H41C22C21O19)$ (16)
246	239	236vw		$\delta(C6C5C7)$ (13) + $\delta(C11C12C17)$ (20) + $\delta(C8C9C10)$ (13)
218	211	217w		$\tau(C7C8C9C10)$ (15) + $\tau(C3C2C1C6)$ (19)

203	197	204vw	$\tau(\text{O47H48O18C9})$ (17) + $\tau(\text{C16C15C14C13})$ (21) + $\tau(\text{C9C10C11C12})$ (11)
194	188	184vw	$\tau(\text{C7C8C9C10})$ (15) + $\tau(\text{C5C7C8C9})$ (16) + $\tau(\text{C9C10C11C12})$ (11) + $\tau(\text{C8C9C10C11})$ (17)
171	166	176vw	$\delta(\text{O20C2C3})$ (11) + $\delta(\text{C21O19C15})$ (13) + $\delta(\text{C23O20C2})$ (22)
160	155	153vw	$\nu(\text{O18H48})$ (18) + $\delta(\text{C9C10C11})$ (12)
135	131	138vw	$\nu(\text{O18H48})$ (42) + $\delta(\text{H48O18C9})$ (11)
126	123	122vw	$\delta(\text{O19C15C16})$ (11) + $\delta(\text{C21O19C15})$ (17) + $\delta(\text{C23O20C2})$ (15)
117	113		$\tau(\text{C21O19C15C14})$ (48) + $\tau(\text{C23O20C2C1})$ (20)
116	112	106w	$\tau(\text{C21O19C15C14})$ (20) + $\tau(\text{C23O20C2C1})$ (48)
101	98	103w	$\tau(\text{C7C8C9C10})$ (10) + $\tau(\text{C8C9C10C11})$ (12) + $\tau(\text{C22C21O19C15})$ (10) + $\tau(\text{C24C23O20C2})$ (10)
99	96		$\delta(\text{H48O18C9})$ (35) + $\tau(\text{O47H48O18C9})$ (12)
92	90		$\tau(\text{H48O18C9C8})$ (23) + $\tau(\text{C1C6C5C7})$ (11) + $\tau(\text{C5C7C8C9})$ (18)
82	80	84w	$\tau(\text{C9C10C11C12})$ (18) + $\tau(\text{C22C21O19C15})$ (21) + $\tau(\text{C24C23O20C2})$ (14) + $\gamma(\text{C11C13C17C12})$ (12)
61	60	65w	$\delta(\text{C7C8C9})$ (11) + $\delta(\text{C10C11C12})$ (16) + $\delta(\text{C6C5C7})$ (10) + $\delta(\text{C5C7C8})$ (13) + $\delta(\text{C11C12C17})$ (10)
49	47		$\tau(\text{H48O18C9C8})$ (15) + $\tau(\text{C7C8C9C10})$ (11) + $\tau(\text{C22C21O19C15})$ (12) + $\tau(\text{C24C23O20C2})$ (18)
40	39	38w	$\tau(\text{H48O18C9C8})$ (25) + $\tau(\text{C10C11C12C13})$ (20) + $\tau(\text{C8C9C10C11})$ (12)
31	30		$\tau(\text{H48O18C9C8})$ (11) + $\tau(\text{C10C11C12C13})$ (10) + $\tau(\text{C1C6C5C7})$ (15) + $\tau(\text{C5C7C8C9})$ (10)
23	23		$\tau(\text{H48O18C9C8})$ (10) + $\tau(\text{C7C8C9C10})$ (11) + $\tau(\text{C10C11C12C13})$ (18) + $\tau(\text{C6C5C7C8})$ (40)
21	21		$\delta(\text{C7C8C9})$ (16) + $\delta(\text{C10C11C12})$ (19) + $\delta(\text{C5C7C8})$ (14) + $\delta(\text{C9C10C11})$ (18) + $\delta(\text{C8C9C10})$ (15)
14	13		$\tau(\text{C7C8C9C10})$ (21) + $\tau(\text{C10C11C12C13})$ (10) + $\tau(\text{C6C5C7C8})$ (16) + $\tau(\text{C8C9C10C11})$ (15)

Abbreviation: ν , stretching; δ , bending; τ , torsion; γ , out-of-plane; vw, very weak; w, weak; m, medium; s, strong; vs, very strong.

Table S3. Calculated vibrational wavenumbers (in cm^{-1}) unscaled and scaled by the scale factor 0.9694 ($\omega < 1800$) and 0.9430 ($\omega \geq 1800$), experimental Raman and infrared bands positions in units of cm^{-1} and assignment of vibrational modes for DB ethoxy in a trihydrated model.

ω_{calc}	ω_{scal}	ω_{Raman}	ω_{IR}	Assignment of the molecular vibrations with PED (%)
3880	3659			$\nu(\text{O53H55})$ (75) + $\nu(\text{O53H54})$ (19)
3880	3659			$\nu(\text{O50H52})$ (76) + $\nu(\text{O50H51})$ (18)
3860	3640			$\nu(\text{O47H49})$ (96)
3756	3541			$\nu(\text{O53H55})$ (20) + $\nu(\text{O53H54})$ (79)
3755	3541			$\nu(\text{O50H52})$ (19) + $\nu(\text{O50H51})$ (80)
3641	3434			$\nu(\text{O47H48})$ (94)
3229	3045	3063vw		$\nu(\text{C14H34})$ (96)
3227	3044		3037vw	$\nu(\text{C1H25})$ (95)
3209	3026	3034vw		$\nu(\text{C16H35})$ (47) + $\nu(\text{C17H36})$ (50)
3208	3025			$\nu(\text{C3H26})$ (58) + $\nu(\text{C4H27})$ (41)
3197	3014			$\nu(\text{C16H35})$ (49) + $\nu(\text{C17H36})$ (41)
3195	3013	3012vw		$\nu(\text{C3H26})$ (38) + $\nu(\text{C4H27})$ (53)
3191	3009			$\nu(\text{C6H28})$ (91)
3188	3006			$\nu(\text{C13H33})$ (92)
3185	3004			$\nu(\text{C8H30})$ (17) + $\nu(\text{C10H31})$ (70)
3174	2993			$\nu(\text{C8H30})$ (70) + $\nu(\text{C10H31})$ (19)
3165	2984			$\nu(\text{C11H32})$ (96)
3160	2980	2977vw	2985vw	$\nu(\text{C7H29})$ (89)
3141	2962		2976vw	$\nu(\text{C22H40})$ (29) + $\nu(\text{C22H41})$ (64)
3140	2961			$\nu(\text{C24H45})$ (64) + $\nu(\text{C24H46})$ (27)
3136	2957			$\nu(\text{C22H39})$ (60) + $\nu(\text{C22H40})$ (36)
3136	2957	2936vw	2935vw	$\nu(\text{C24H44})$ (59) + $\nu(\text{C24H46})$ (37)
3066	2891			$\nu(\text{C23H42})$ (54) + $\nu(\text{C23H43})$ (43)
3065	2890			$\nu(\text{C21H37})$ (53) + $\nu(\text{C21H38})$ (44)
3059	2885		2885vw	$\nu(\text{C22H39})$ (34) + $\nu(\text{C22H40})$ (35) + $\nu(\text{C22H41})$ (31)
3059	2885	2881vw		$\nu(\text{C24H44})$ (34) + $\nu(\text{C24H45})$ (30) + $\nu(\text{C24H46})$ (35)
3022	2850			$\nu(\text{C23H42})$ (43) + $\nu(\text{C23H43})$ (55)
3022	2850	2862vw		$\nu(\text{C21H37})$ (45) + $\nu(\text{C21H38})$ (54)
1731	1678			$\nu(\text{O18C9})$ (39) + $\nu(\text{C8C7})$ (15) + $\nu(\text{C11C10})$ (16)
1702	1650	1643w	1640vs	$\delta(\text{O47H48O18})$ (25) + $\tau(\text{O47H48O18C9})$ (36) + $\tau(\text{H48O18C9C10})$ (21)
1679	1627			$\nu(\text{C8C7})$ (16) + $\nu(\text{C11C10})$ (17)
1679	1627			$\delta(\text{H52O50H51})$ (87)
1678	1627		1620vw	$\delta(\text{H55O53H54})$ (92)

1666	1615	1620s		$v(C4C3)$ (13) + $v(C17C16)$ (16)
1655	1604	1603m	1601vs	$v(C4C3)$ (11) + $v(C17C16)$ (11)
1628	1578	1584s	1583s	$v(O18C9)$ (32)
1616	1566	1569vs	1568vs	$v(C2C1)$ (11) + $v(C15C14)$ (19)
1614	1564	1556w		$v(C2C1)$ (16)
1559	1512	1515vw	1510vs	$\delta(H25C1C6)$ (11) + $\delta(H26C3C4)$ (15) + $\delta(H27C4C5)$ (11) + $\delta(H28C6C1)$ (14) + $\delta(H43C23H42)$ (11)
1559	1511			$\delta(H33C13C14)$ (12) + $\delta(H34C14C13)$ (11) + $\delta(H35C16C17)$ (14) + $\delta(H36C17C16)$ (13) + $\delta(H38C21H37)$ (12)
1536	1489	1497vw		$\delta(H38C21H37)$ (39) + $\delta(H43C23H42)$ (31)
1535	1488		1477w	$\delta(H38C21H37)$ (31) + $\delta(H43C23H42)$ (39)
1513	1466			$\delta(H45C24H44)$ (11) + $\delta(H46C24H45)$ (54) + $\tau(H45C24C23O20)$ (12) + $\tau(H46C24C23O20)$ (10)
1513	1466	1475vw	1469vw	$\delta(H39C22H41)$ (10) + $\delta(H41C22H40)$ (55) + $\tau(H40C22C21O19)$ (10) + $\tau(H41C22C21O19)$ (11)
1500	1455	1459vw		$\delta(H44C24H46)$ (39) + $\delta(H45C24H44)$ (35) + $\tau(H44C24C23O20)$ (16)
1500	1454			$\delta(H39C22H41)$ (36) + $\delta(H40C22H39)$ (39) + $\tau(H39C22C21O19)$ (16)
1474	1428	1425w	1425m	$v(C4C3)$ (20) + $v(C1C6)$ (19) + $\delta(H25C1C6)$ (13)
1471	1426			$v(C17C16)$ (22) + $v(C14C13)$ (21) + $\delta(H34C14C13)$ (13)
1442	1398			$\delta(H44C24H46)$ (14) + $\delta(H45C24H44)$ (14) + $\tau(H43C23O20C2)$ (11)
1442	1398	1396vw	1396w	$\delta(H39C22H41)$ (14) + $\delta(H40C22H39)$ (14) + $\tau(H38C21O19C15)$ (11)
1412	1368			$\tau(H38C21O19C15)$ (10) + $\tau(H43C23O20C2)$ (13)
1411	1368			$\tau(H38C21O19C15)$ (15) + $\tau(H43C23O20C2)$ (10)
1400	1357	1351vw	1348vw	$\delta(H29C7C8)$ (24)
1379	1337	1325vw	1333w	$v(C8C7)$ (10) + $v(C13C12)$ (10) + $\delta(H29C7C8)$ (16) + $\delta(H32C11C12)$ (15)
1363	1321	1311w		$\delta(H30C8C9)$ (15)
1355	1313		1308w	$\delta(H32C11C12)$ (10) + $\delta(H33C13C14)$ (14) + $\delta(H35C16C17)$ (11) + $\delta(H36C17C16)$ (13)
1349	1308	1295w		$\delta(H25C1C6)$ (10) + $\delta(H28C6C1)$ (12) + $\delta(H30C8C9)$ (11)
1347	1306			$\delta(H31C10C11)$ (17)
1330	1289		1292m	$\delta(H29C7C8)$ (12) + $\delta(H32C11C12)$ (10)
1313	1273	1272vw		$v(C12C11)$ (13) + $\delta(H30C8C9)$ (15)
1308	1268			$\delta(H37C21C22)$ (63) + $\tau(H37C21O19C15)$ (12) + $\tau(H38C21O19C15)$ (11)
1307	1267			$\delta(H42C23C24)$ (63) + $\tau(H42C23O20C2)$ (12) + $\tau(H43C23O20C2)$ (11)
1286	1247	1251m	1252vs	$v(C14C13)$ (10) + $v(O19C15)$ (22) + $v(O20C2)$ (18)
1285	1245			$v(O19C15)$ (20) + $v(O20C2)$ (23)
1256	1218	1220w	1219vw	$v(C5C6)$ (15) + $v(C7C5)$ (17) + $\delta(H28C6C1)$ (11)
1242	1204	1196w	1190s	$v(C13C12)$ (12) + $v(C12C11)$ (11) + $\delta(H32C11C12)$ (19) + $\delta(H35C16C17)$ (13)
1213	1176	1176m	1169vs	$\delta(H25C1C6)$ (16) + $\delta(H26C3C4)$ (12) + $\delta(H27C4C5)$ (12) + $\delta(H28C6C1)$ (17)
1210	1173			$\delta(H33C13C14)$ (21) + $\delta(H34C14C13)$ (14) + $\delta(H36C17C16)$ (11)
1186	1150			$\delta(H42C23C24)$ (24) + $\tau(H42C23O20C2)$ (25)
1186	1150	1158vw		$\delta(H37C21C22)$ (24) + $\tau(H37C21O19C15)$ (25)
1157	1122	1127vw		$v(C1C6)$ (11) + $\delta(H25C1C6)$ (11) + $\delta(H26C3C4)$ (10)
1157	1122			$v(C14C13)$ (10) + $\delta(H34C14C13)$ (12) + $\delta(H35C16C17)$ (10) + $\delta(H36C17C16)$ (10)
1143	1108	1113w	1115w	$\tau(H45C24C23O20)$ (14) + $\tau(H46C24C23O20)$ (14)
1142	1107			$\tau(H40C22C21O19)$ (14) + $\tau(H41C22C21O19)$ (14)
1127	1093	1098vw	1097vw	$v(C9C8)$ (25) + $v(C10C9)$ (26) + $\delta(H30C8C9)$ (13) + $\delta(H31C10C11)$ (15)
1085	1052	1049vw		$\tau(H49O47H29C7)$ (17) + $\tau(H29C7C8C9)$ (53) + $\tau(H30C8C9C10)$ (14)
1070	1038		1045s	$v(C22C21)$ (12) + $v(C24C23)$ (28) + $v(O19C21)$ (12) + $v(O20C23)$ (28)
1070	1037			$v(C22C21)$ (28) + $v(C24C23)$ (12) + $v(O19C21)$ (29) + $v(O20C23)$ (12)
1036	1005		1025vw	$\tau(H31C10C9C8)$ (15) + $\tau(H32C11C12C13)$ (71)
1032	1001			$\delta(H27C4C5)$ (10) + $\delta(C4C3C2)$ (37) + $\delta(C2C1C6)$ (23) + $\delta(C3C2C1)$ (12)
1031	1000	996vw	989s	$\delta(C17C16C15)$ (45) + $\delta(C15C14C13)$ (26) + $\delta(C16C15C14)$ (15)
1004	973			$\tau(H35C16C17C12)$ (54) + $\tau(H36C17C16C15)$ (21) + $\tau(C17C16C15C14)$ (15)
1003	972			$\tau(H26C3C4C5)$ (50) + $\tau(H27C4C5C6)$ (26) + $\tau(C4C3C2C1)$ (13)
1001	970	981w		$v(O18C9)$ (12) + $v(C9C8)$ (22) + $v(C10C9)$ (22)
968	939	944vw		$\tau(H25C1C6C5)$ (13) + $\tau(H28C6C5C4)$ (54) + $\tau(C5C6C1C2)$ (20)
951	922	924w		$\tau(H33C13C14C15)$ (49) + $\tau(H34C14C15O19)$ (29) + $\tau(C15C14C13C12)$ (10)
941	912			$v(C22C21)$ (23) + $v(O19C21)$ (14)
940	912	909vw	922w	$v(C24C23)$ (23) + $v(O20C23)$ (14) + $\tau(H45C24C23O20)$ (10)
909	881	878vw	881w	$\tau(H30C8C9C10)$ (39) + $\tau(H31C10C9C8)$ (32) + $\gamma(O18C10C8C9)$ (12)
891	864			$\delta(C3C2C1)$ (11)
877	851			$\tau(H26C3C4C5)$ (10) + $\tau(H27C4C5C6)$ (12) + $\tau(H31C10C9C8)$ (10) + $\tau(H36C17C16C15)$ (14)
871	844		835m	$\tau(H26C3C4C5)$ (15) + $\tau(H27C4C5C6)$ (22) + $\tau(H35C16C17C12)$ (15) + $\tau(H36C17C16C15)$ (26)
866	839	857w		$\delta(C16C15C14)$ (13)
858	832	830vw		$\tau(H27C4C5C6)$ (16) + $\tau(H30C8C9C10)$ (12) + $\tau(H31C10C9C8)$ (13) + $\tau(H36C17C16C15)$ (15)
847	821			$\tau(H25C1C6C5)$ (13) + $\tau(H44C24C23O20)$ (13)
844	818	813vw	812m	$\tau(H39C22C21O19)$ (16)
823	798	806vw	789w	$\tau(H25C1C6C5)$ (33) + $\tau(H28C6C5C4)$ (11)
817	792			$\tau(H33C13C14C15)$ (18) + $\tau(H34C14C15O19)$ (37)
815	790	791vw		$\tau(H25C1C2C3)$ (36) + $\tau(H28C6C5C7)$ (18) + $\tau(H34C14C15C16)$ (36) + $\tau(H33C13C12C11)$ (18)

790	766	776vw	v(O19C15) (10)
751	728	737vw	τ (C15C14C13C12) (18) + γ (O18C10C8C9) (19)
727	705	719vw	τ (C15C14C13C12) (23)
723	701	704vw	δ (O18C9C8) (18) + δ (C3C2C1) (10)
697	676	679vw	δ (O47H48O18) (33) + δ (H48O18C9) (16) + τ (O47H48O18C9) (16)
693	672	664vw	τ (C15C14C13C12) (10) + γ (O18C10C8C9) (36)
654	634	638w	δ (C14C13C12) (14) + δ (C15C14C13) (19)
652	632	625vw	δ (C5C6C1) (13) + δ (C4C3C2) (11) + δ (C2C1C6) (19)
599	581	593w	δ (C14C13C12) (10)
581	563	578vw	δ (O50H51O20) (20) + δ (C23O20H51) (16)
575	557	566vw	δ (O50H51O20) (21) + δ (O53H54O19) (21) + δ (H51O20C2) (17)
570	553	554vw	δ (O53H54O19) (23) + δ (H54O19C15) (17)
541	525		γ (O20C1C3C2) (23) + γ (O19C14C16C15) (10) + γ (C7C4C6C5) (12)
535	518	539w	δ (C10C9C8) (24)
533	517	516vw	γ (O19C14C16C15) (17)
447	433	438vw	δ (C13C12C11) (12) + δ (C7C5C6) (12) + δ (O19C15C16) (12) + δ (O20C2C3) (17) + δ (C24C23O20) (10)
440	426		δ (O19C15C16) (27) + δ (O20C2C3) (14)
435	422	420vw	δ (O20C2C3) (11)
428	415	414vw	τ (H25C1C6C5) (11) + τ (C4C3C2C1) (16) + τ (C3C2C1C6) (16) + τ (C5C6C1C2) (32)
427	414	403w	τ (H33C13C14C15) (11) + τ (H36C17C16C15) (12) + τ (C17C16C15C14) (20) + τ (C14C13C12C11) (17) + τ (C16C15C14C13) (21)
390	378	384vw	τ (H49O47H29C7) (13) + τ (C4C3C2C1) (20) + γ (O20C1C3C2) (16) + γ (C7C4C6C5) (16)
384	372	368vw	τ (C17C16C15C14) (17) + τ (C14C13C12C11) (28) + τ (C12C11C10C9) (13) + γ (O19C14C16C15) (20)
362	351	353vw	δ (C22C21O19) (21) + δ (C24C23O20) (21)
336	326	320vw	δ (C22C21O19) (10) + τ (H48O18C9C10) (10)
328	318	300vw	δ (O47H48O18) (14) + τ (H49O47H29C7) (10) + τ (O47H48O18C9) (16) + τ (H48O18C9C10) (21)
274	266	279vw	δ (H49O47H29) (53)
270	262	271vw	v(O18H48) (13) + δ (C11C10C9) (11)
261	253	256vw	τ (H52O50H51O20) (14) + τ (O50H51O20C2) (12) + τ (H44C24C23O20) (27) + τ (H46C24C23O20) (10)
259	251	249vw	τ (H55O53H54O19) (13) + τ (O53H54O19C15) (11) + τ (H39C22C21O19) (32) + τ (H40C22C21O19) (11) + τ (H41C22C21O19) (12)
252	244		δ (C13C12C11) (11) + δ (C7C5C6) (16) + δ (C10C9C8) (11)
245	237	236vw	τ (H52O50H51O20) (46) + τ (C23O20H51O50) (18)
242	234		τ (H55O53H54O19) (50) + τ (C21O19H54O53) (18)
228	221		τ (H55O53H54O19) (13) + τ (O53H54O19C15) (25)
223	216	217vw	τ (H52O50H51O20) (21) + τ (O50H51O20C2) (34)
209	203	204vw	τ (H55O53H54O19) (11) + τ (O53H54O19C15) (23) + τ (C11C10C9C8) (10)
200	193		τ (H49O47H29C7) (14) + τ (C10C9C8C7) (16)
191	186	184vw	τ (C12C11C10C9) (13) + τ (C9C8C7C5) (15) + τ (C10C9C8C7) (10)
180	174	176vw	δ (O19C15C16) (12) + δ (C21O19H54) (11)
164	159	153vw	v(O18H48) (14) + δ (C9C8C7) (11)
139	135	138vw	v(O18H48) (45) + v(O19H54) (16) + δ (H48O18C9) (11)
133	129		v(O20H51) (27)
131	127	122vw	v(O19H54) (39) + v(O20H51) (23)
121	117		v(O19H54) (22) + v(O20H51) (19)
110	107		τ (C22C21O19C15) (11) + τ (C21O19H54O53) (13) + τ (C23O20H51O50) (14)
109	106	106w	τ (C21O19H54O53) (12) + τ (C23O20H51O50) (19)
103	99	103w	τ (C8C7C5C4) (11) + τ (C11C10C9C8) (10) + τ (C13C12C11C10) (16)
100	97		δ (H48O18C9) (27)
92	89		τ (C14C13C12C11) (13) + τ (C12C11C10C9) (13) + τ (C9C8C7C5) (11)
81	79	84w	τ (C9C8C7C5) (14) + τ (C22C21O19C15) (11) + τ (C24C23O20C2) (16) + γ (C7C4C6C5) (10)
71	69		δ (H48O18C9) (12) + δ (C21O19H54) (11) + δ (C23O20H51) (11)
68	66	65w	δ (O50H51O20) (14) + δ (O53H54O19) (13) + δ (H54O19C15) (14) + δ (H51O20C2) (17) + δ (C21O19H54) (12) + δ (C23O20H51) (13)
54	52		δ (H54O19C15) (11) + δ (C8C7C5) (12) + δ (C13C12C11) (10) + δ (C7C5C6) (11) + δ (C12C11C10) (11)
50	48		τ (C11C10C9C8) (14) + τ (C22C21O19C15) (20) + τ (C24C23O20C2) (11)
45	44		τ (H51O20C2C1) (17) + τ (C8C7C5C4) (21) + τ (C24C23O20C2) (12)
37	36	38w	τ (H54O19C15C14) (42)
32	31		γ (C7C4C6C5) (12)
25	24		τ (H48O18C9C10) (12) + τ (H54O19C15C14) (11) + τ (H51O20C2C1) (34)
18	18		δ (C8C7C5) (19) + δ (C11C10C9) (15) + δ (C12C11C10) (14) + δ (C9C8C7) (18) + δ (C10C9C8) (15)
14	13		τ (H51O20C2C1) (14) + τ (C8C7C5C4) (33) + τ (C12C11C10C9) (11) + τ (C10C9C8C7) (18)
12	11		τ (C11C10C9C8) (28) + τ (C13C12C11C10) (42)

Abbreviation: v, stretching; δ , bending; τ , torsion; γ , out-of-plane; vw, very weak; w, weak; m, medium; s, strong; vs, very strong.

Table S4. Calculated vibrational wavenumbers (in cm^{-1}) unscaled and scaled by the scale factor 0.9712 ($\omega < 1800$) and 0.9476 ($\omega \geq 1800$), experimental Raman and infrared bands positions in units of cm^{-1} and assignment of vibrational modes for DB chlorine.

ω_{calc}	ω_{scal}	ω_{Raman}	ω_{IR}	Assignment of the molecular vibrations with PED (%)
3224	3055			$\nu(\text{C1H21})$ (38) + $\nu(\text{C16H31})$ (44)
3224	3055	3066vw	3050vw	$\nu(\text{C1H21})$ (45) + $\nu(\text{C16H31})$ (39)
3222	3053			$\nu(\text{C3H22})$ (36) + $\nu(\text{C14H30})$ (39)
3222	3053	3054vw		$\nu(\text{C3H22})$ (40) + $\nu(\text{C14H30})$ (37)
3202	3034			$\nu(\text{C4H23})$ (41) + $\nu(\text{C13H29})$ (40)
3202	3034			$\nu(\text{C4H23})$ (41) + $\nu(\text{C13H29})$ (42)
3193	3026			$\nu(\text{C6H24})$ (46) + $\nu(\text{C17H32})$ (47)
3193	3026	3018vw		$\nu(\text{C6H24})$ (47) + $\nu(\text{C17H32})$ (46)
3183	3016			$\nu(\text{C8H26})$ (46) + $\nu(\text{C10H27})$ (46)
3174	3008			$\nu(\text{C8H26})$ (46) + $\nu(\text{C10H27})$ (46)
3166	3001			$\nu(\text{C7H25})$ (48) + $\nu(\text{C11H28})$ (48)
3166	3000			$\nu(\text{C7H25})$ (46) + $\nu(\text{C11H28})$ (47)
1752	1701	1670vw	1668w	$\nu(\text{O18C9})$ (53) + $\nu(\text{C7C8})$ (12) + $\nu(\text{C10C11})$ (12)
1681	1632	1649w	1649s	$\nu(\text{C7C8})$ (28) + $\nu(\text{C10C11})$ (28)
1656	1609	1629m	1626vs	$\nu(\text{O18C9})$ (20) + $\nu(\text{C7C8})$ (12) + $\nu(\text{C10C11})$ (12)
1646	1598		1589vs	$\nu(\text{C4C3})$ (14) + $\nu(\text{C13C14})$ (15)
1641	1593	1590vs		$\nu(\text{O18C9})$ (14) + $\nu(\text{C13C14})$ (10)
1612	1566			$\nu(\text{C2C1})$ (14) + $\nu(\text{C16C15})$ (14) + $\nu(\text{C6C5})$ (11) + $\nu(\text{C12C17})$ (10)
1612	1566	1566m	1566m	$\nu(\text{C2C1})$ (14) + $\nu(\text{C16C15})$ (14) + $\nu(\text{C6C5})$ (11) + $\nu(\text{C12C17})$ (10)
1533	1489			$\delta(\text{H22C3C4})$ (10) + $\delta(\text{H30C14C15})$ (10)
1533	1489	1490w	1489s	$\delta(\text{H22C3C4})$ (10) + $\delta(\text{H30C14C15})$ (10)
1448	1407	1407w	1406m	$\nu(\text{C1C6})$ (10) + $\nu(\text{C13C14})$ (11) + $\nu(\text{C17C16})$ (11)
1447	1406			$\nu(\text{C13C14})$ (11) + $\nu(\text{C17C16})$ (11)
1374	1334	1342w	1338w	$\delta(\text{H26C8C9})$ (12) + $\delta(\text{H27C10C11})$ (11)
1362	1323	1322w	1327w	$\delta(\text{H25C7C8})$ (24) + $\delta(\text{H28C11C12})$ (21)
1350	1311	1312w		$\delta(\text{H26C8C9})$ (16) + $\delta(\text{H27C10C11})$ (19)
1345	1306			$\delta(\text{H25C7C8})$ (12) + $\delta(\text{H28C11C12})$ (11)
1331	1292	1294w		$\nu(\text{C3C2})$ (12) + $\nu(\text{C15C14})$ (11)
1331	1292	1277w		$\delta(\text{H23C4C5})$ (10) + $\delta(\text{H29C13C14})$ (10) + $\delta(\text{H30C14C15})$ (10)
1321	1283	1267w	1290w	$\delta(\text{H25C7C8})$ (14)
1303	1265	1259w		$\nu(\text{C5C7})$ (11) + $\nu(\text{C11C12})$ (11) + $\delta(\text{H26C8C9})$ (14) + $\delta(\text{H27C10C11})$ (11)
1236	1201	1204w	1188m	$\delta(\text{H25C7C8})$ (10) + $\delta(\text{H28C11C12})$ (10)
1233	1198	1189w		$\nu(\text{C6C5})$ (10) + $\nu(\text{C17C12})$ (10) + $\nu(\text{C12C11})$ (10) + $\nu(\text{C7C5})$ (10) + $\delta(\text{H27C10C11})$ (10) + $\delta(\text{H28C11C12})$ (10) + $\delta(\text{H26C8C9})$ (10) + $\delta(\text{H25C7C5})$ (10)
1209	1174			$\delta(\text{H24C6C5})$ (13) + $\delta(\text{H29C13C14})$ (10) + $\delta(\text{H32C17C16})$ (14)
1208	1173	1176w	1178m	$\delta(\text{H24C6C5})$ (13) + $\delta(\text{H29C13C14})$ (10) + $\delta(\text{H32C17C16})$ (15)
1138	1106			$\nu(\text{C1C6})$ (10) + $\delta(\text{H21C1C6})$ (11) + $\delta(\text{H31C16C17})$ (11)
1138	1105	1104vw	1111m	$\nu(\text{C1C6})$ (10)
1123	1090		1103m	$\nu(\text{C8C9})$ (14) + $\nu(\text{C9C10})$ (14)
1111	1079	1092w		$\nu(\text{C2C1})$ (12) + $\nu(\text{C3C2})$ (15) + $\nu(\text{C15C14})$ (14) + $\nu(\text{C16C15})$ (12)
1101	1069		1086s	$\nu(\text{C8C9})$ (10) + $\nu(\text{C9C10})$ (10)
1043	1013	1013vw	1011m	$\tau(\text{H25C7C8C9})$ (38) + $\tau(\text{H28C11C12C13})$ (33)
1035	1005			$\tau(\text{H25C7C8C9})$ (36) + $\tau(\text{H26C8C9C10})$ (10) + $\tau(\text{H28C11C12C13})$ (33)
1028	998	985vw	984vw	$\delta(\text{C4C3C2})$ (19) + $\delta(\text{C13C14C15})$ (19) + $\delta(\text{C2C1C6})$ (12) + $\delta(\text{C17C16C15})$ (12) + $\delta(\text{C16C15C14})$ (10)
1027	998			$\delta(\text{C4C3C2})$ (19) + $\delta(\text{C13C14C15})$ (19) + $\delta(\text{C2C1C6})$ (12) + $\delta(\text{C17C16C15})$ (12) + $\delta(\text{C16C15C14})$ (10)
987	959	970vw		$\nu(\text{C8C9})$ (23) + $\nu(\text{C9C10})$ (23)
969	941			$\tau(\text{H21C1C6C5})$ (13) + $\tau(\text{H24C6C5C4})$ (21) + $\tau(\text{H31C16C17C12})$ (13) + $\tau(\text{H32C17C16C15})$ (18) + $\tau(\text{C2C1C6C5})$ (11)
969	941	950vw		$\tau(\text{H21C1C6C5})$ (13) + $\tau(\text{H24C6C5C4})$ (21) + $\tau(\text{H31C16C17C12})$ (13) + $\tau(\text{H32C17C16C15})$ (17) + $\tau(\text{C2C1C6C5})$ (11)
963	935			$\tau(\text{H22C3C4C5})$ (14) + $\tau(\text{H23C4C5C6})$ (16) + $\tau(\text{H29C13C14C15})$ (15) + $\tau(\text{H30C14C15C16})$ (16)
963	935	924vw		$\tau(\text{H22C3C4C5})$ (14) + $\tau(\text{H23C4C5C6})$ (16) + $\tau(\text{H29C13C14C15})$ (14) + $\tau(\text{H30C14C15C16})$ (16)
914	888	888vw	887vw	$\tau(\text{H26C8C9C10})$ (33) + $\tau(\text{H27C10C11C12})$ (34) + $\gamma(\text{O18C8C10C9})$ (15)
878	853	866vw	866vw	$\delta(\text{C3C2C1})$ (11) + $\delta(\text{C16C15C14})$ (12)
876	850	859vw		$\tau(\text{H26C8C9C10})$ (18) + $\tau(\text{H27C10C11C12})$ (30)
849	824	839vw		$\delta(\text{C3C2C1})$ (15) + $\delta(\text{C16C15C14})$ (16)
843	819	832vw	822s	$\tau(\text{H21C1C6C5})$ (21) + $\tau(\text{H24C6C5C4})$ (10) + $\tau(\text{H31C16C17C12})$ (21) + $\tau(\text{H32C17C16C15})$ (14)
834	810			$\tau(\text{H22C3C4C5})$ (17) + $\tau(\text{H23C4C5C6})$ (14) + $\tau(\text{H24C6C5C4})$ (10) + $\tau(\text{H29C13C14C15})$ (15) + $\tau(\text{H30C14C15C16})$ (16)

834	810	811vw		$\tau(\text{H22C3C4C5})$ (21) + $\tau(\text{H23C4C5C6})$ (17) + $\tau(\text{H29C13C14C15})$ (18) + $\tau(\text{H30C14C15C16})$ (19)
824	801	774vw		$\tau(\text{H27C10C11C12})$ (10) + $\tau(\text{H32C17C16C15})$ (10)
761	739	729vw/751vw	750vw	$\delta(\text{O18C9C10})$ (20)
742	721	713vw	711vw	$\tau(\text{C2C1C6C5})$ (10) + $\tau(\text{C12C17C16C15})$ (11) + $\gamma(\text{O18C8C10C9})$ (22)
714	693	679vw		$\tau(\text{C4C3C2C1})$ (10) + $\tau(\text{C13C14C15C16})$ (13) + $\tau(\text{C2C1C6C5})$ (16) + $\tau(\text{C12C17C16C15})$ (19)
684	664	674vw		$\nu(\text{C19I15})$ (15) + $\nu(\text{C20I2})$ (15) + $\delta(\text{C1C6C5})$ (12) + $\delta(\text{C12C17C16})$ (12)
679	659	663vw		$\tau(\text{C12C17C16C15})$ (12) + $\gamma(\text{O18C8C10C9})$ (34)
648	629	635vw		$\delta(\text{C1C6C5})$ (10) + $\delta(\text{C2C1C6})$ (15) + $\delta(\text{C17C16C15})$ (15) + $\delta(\text{C12C17C16})$ (11)
647	629	623vw		$\delta(\text{C4C3C2})$ (10) + $\delta(\text{C2C1C6})$ (16) + $\delta(\text{C17C16C15})$ (16) + $\delta(\text{C12C17C16})$ (10)
635	617	611vw		$\nu(\text{C19I15})$ (11) + $\nu(\text{C20I2})$ (11) + $\delta(\text{O18C9C10})$ (23) + $\delta(\text{C1C6C5})$ (12) + $\delta(\text{C12C17C16})$ (12)
533	517	536vw		$\delta(\text{C10C11C12})$ (13) + $\delta(\text{C5C7C8})$ (12) + $\delta(\text{C8C9C10})$ (30)
514	499			$\tau(\text{C3C2C1C6})$ (11) + $\gamma(\text{C11C13C17C12})$ (14)
505	491	494vw		$\tau(\text{C3C2C1C6})$ (11) + $\gamma(\text{C20I1C3C2})$ (10) + $\gamma(\text{C19I14C16C15})$ (10) + $\gamma(\text{C11C13C17C12})$ (13)
476	462	460vw		$\delta(\text{O18C9C10})$ (11) + $\delta(\text{C10C11C12})$ (10) + $\delta(\text{C6C5C7})$ (10) + $\delta(\text{C11C12C17})$ (10)
431	419	429vw		$\nu(\text{C19I15})$ (23) + $\nu(\text{C20I2})$ (23)
418	406	407vw		$\tau(\text{C4C3C2C1})$ (17) + $\tau(\text{C13C14C15C16})$ (16) + $\tau(\text{C17C16C15C14})$ (10) + $\tau(\text{C3C2C1C6})$ (10)
418	406	391vw		$\tau(\text{C4C3C2C1})$ (17) + $\tau(\text{C13C14C15C16})$ (16) + $\tau(\text{C17C16C15C14})$ (10) + $\tau(\text{C3C2C1C6})$ (11)
385	374	366vw		$\delta(\text{C6C5C7})$ (13) + $\delta(\text{C11C12C17})$ (13)
354	343	349vw		$\tau(\text{C1C6C5C7})$ (17) + $\tau(\text{C9C10C11C12})$ (10) + $\gamma(\text{C20I1C3C2})$ (17) + $\gamma(\text{C19I14C16C15})$ (17)
354	343	334vw		$\tau(\text{C1C6C5C7})$ (17) + $\gamma(\text{C20I1C3C2})$ (16) + $\gamma(\text{C19I14C16C15})$ (16)
326	316	323vw		$\delta(\text{C8C9C10})$ (12) + $\delta(\text{C19I15C16})$ (29) + $\delta(\text{C20I2C3})$ (30)
290	282	285vw		$\delta(\text{C19I15C16})$ (30) + $\delta(\text{C20I2C3})$ (23)
249	242	231vw		$\delta(\text{O18C9C10})$ (14) + $\delta(\text{C7C8C9})$ (20) + $\delta(\text{C9C10C11})$ (19)
197	192	190vw		$\delta(\text{C6C5C7})$ (18) + $\delta(\text{C11C12C17})$ (18) + $\delta(\text{C8C9C10})$ (22) + $\delta(\text{C19I15C16})$ (14) + $\delta(\text{C20I2C3})$ (13)
192	187			$\tau(\text{C7C8C9C10})$ (28) + $\tau(\text{C8C9C10C11})$ (35)
186	181			$\tau(\text{C9C10C11C12})$ (11) + $\gamma(\text{C20I1C3C2})$ (14) + $\gamma(\text{C19I14C16C15})$ (14)
173	168	165w		$\tau(\text{C3C2C1C6})$ (10) + $\tau(\text{C5C7C8C9})$ (22) + $\tau(\text{C9C10C11C12})$ (21) + $\gamma(\text{C20I1C3C2})$ (13) + $\gamma(\text{C19I14C16C15})$ (13)
152	148	122w		$\nu(\text{C8C9})$ (10) + $\nu(\text{C9C10})$ (10) + $\delta(\text{C7C8C9})$ (10) + $\delta(\text{C9C10C11})$ (10)
89	87	89w		$\delta(\text{C10C11C12})$ (20) + $\delta(\text{C6C5C7})$ (16) + $\delta(\text{C5C7C8})$ (19) + $\delta(\text{C11C12C17})$ (16)
79	77			$\tau(\text{C7C8C9C10})$ (11) + $\tau(\text{C10C11C12C13})$ (18) + $\tau(\text{C6C5C7C8})$ (22) + $\tau(\text{C8C9C10C11})$ (13)
69	67	64w		$\tau(\text{C1C6C5C7})$ (17) + $\tau(\text{C5C7C8C9})$ (12) + $\tau(\text{C9C10C11C12})$ (10) + $\gamma(\text{C11C13C17C12})$ (10)
48	47			$\tau(\text{C1C6C5C7})$ (22) + $\tau(\text{C17C16C15C14})$ (10) + $\tau(\text{C5C7C8C9})$ (14) + $\tau(\text{C9C10C11C12})$ (13) + $\gamma(\text{C11C13C17C12})$ (15)
26	25			$\delta(\text{C7C8C9})$ (18) + $\delta(\text{C10C11C12})$ (16) + $\delta(\text{C5C7C8})$ (16) + $\delta(\text{C8C9C10})$ (18) + $\delta(\text{C9C10C11})$ (18)
19	18			$\tau(\text{C7C8C9C10})$ (10) + $\tau(\text{C10C11C12C13})$ (33) + $\tau(\text{C6C5C7C8})$ (36) + $\tau(\text{C8C9C10C11})$ (10)
16	16			$\tau(\text{C7C8C9C10})$ (23) + $\tau(\text{C10C11C12C13})$ (13) + $\tau(\text{C6C5C7C8})$ (17) + $\tau(\text{C8C9C10C11})$ (22)

Abbreviation: ν , stretching; δ , bending; τ , torsion; γ , out-of-plane; vw, very weak; w, weak; m, medium; s, strong; vs, very strong.

CAPÍTULO 4

Cytotoxicity Activity, Efflux Pump Inhibition, Anxiolytic-Like Effect and Molecular Docking Studies of Synthetic Bis-chalcones

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Abstract

Bis-chalcones are dibenzalacetone derivatives presenting two enone moiety which can be obtained by double condensation reaction. These chalcone derivatives present several biological activities, including anti-inflammatory, antitumoral and antimicrobial activities. In this work, the cytotoxicity activity, efflux pump inhibition and anxiolytic-like effect of three bis-chalcones (*1E,4E*)-1,5-bis(4-ethoxyphenyl)penta-1,4-dien-3-one (DB ethoxy), (*1E,4E*)-1,5-bis(4-chlorophenyl)penta-1,4-dien-3-one (DB chlorine) and (*1E,4E*)-1,5-di(thiophen-2-yl)penta-1,4-dien-3-one (DB thiophene) were evaluated. DB thiophene and DB chlorine were very active in reduction of the cancer cells HCT-116 growth, as well as showed a possible anxiolytic-like effect in adult zebrafish model. Furthermore, they were able to modulate the resistance to Norfloxacin and Ciprofloxacin in *S. aureus* strains overproducers of the NorA and MepA efflux pumps, respectively, suggesting a potential action as efflux pump inhibitors. Docking studies indicated that this potential efflux inhibiting action occurs through the competition mechanism at the same binding site. Results found in the present study evidence that bis-chalcones tested, mainly DB thiophene, could be used as adjuvants of antibiotics such as Norfloxacin or Ciprofloxacin in the treatment of infections caused by *S. aureus* strains overproducing NorA or MepA efflux pumps.

Keywords: Efflux pump inhibitors, Bis-chalcones, Cytotoxicity, Anxiolytic, Docking.

1. Introduction

Several medical disorders, such as cancer[1], drug-resistant infections[2] and those related to central nervous system[3], have worried scientists worldwide, promoting a search for new bioactive compounds. The chalcone-like framework has fascinated researchers for a long time due to its versatility in obtaining different derivatives with a wide range of bioactivities[4], e.g., anti-inflammatory[5,6], antimicrobial[7,8], antioxidant[9], anticancer[10,11], anti-protozoal[12,13], anxiolytic[14], *etc.* This vast spectrum may be related to its small structure with an α,β -unsaturated carbonyl system (enone moiety) and to the large number of replaceable hydrogens[4], allowing them to bind reversibly or irreversibly[15] to several targets.

Derivatives with two enone moiety can be obtained by the double condensation of a ketone that possess two α,α' -active sites with two equivalents of aromatic aldehyde. This scaffold provides an extensive relocation of π electrons that is achieved through the dienone moiety favoring polarizability[16]. These compounds are commonly denominated as bis-chalcones[17–19], dibenzalacetone derivatives[20] or simply chalcone derivatives[21]. Divers bis-chalcones have shown remarkable anticancer activity against nasopharyngeal, oral, colon, prostate and cervical cancer[10,22]. The manipulation of its aromatic rings such as their replacement by heterocyclic rings or by ferrocene derivatives, as well as the association with other pharmacologically active structures have been reported to enhance anticancer activity[23].

In addition to anticancer activity, it is important to mention the ability of bis-chalcones to act, alone or in co-administration with an antibiotic, against pathogenic bacterial[24,25]. Bacterial resistance to antibiotics is a public health problem that causes many concern. The main mechanisms involved in the bacterial resistance to antibiotics include target site alteration, drug inactivation or modification, reduced drug accumulation, either by a decreased permeability of the cell wall or by the efflux of the antibiotic from the cell[26,27]. Multidrug efflux pumps consist of hydrophobic proteins that drives an important mechanism in the microbial resistance increasing, and the inhibition of such pumps is seen as a promising strategy for overcoming multidrug resistance (MDR) phenotype[28].

MDR efflux systems are separated into five families according to their energy needs and structures: the major facilitator superfamily (MFS); the small multidrug resistance (SMR) family; the multidrug and toxic compound extrusion (MATE) family; the resistance-nodulation-cell division (RND) superfamily; and the adenosine-triphosphate (ATP)-binding cassette (ABC) superfamily[27]. The NorA efflux pump is a MFS efflux system found in *S. aureus*, encoded by the *norA* chromosomal gene. This efflux pump confers resistance to hydrophilic fluoroquinolones, and is capable of expelling from the cell various substances such as Norfloxacin, Ciprofloxacin, Ethidium Bromide and quaternary ammonium compounds[29–31]. Overexpression of the *mepA* gene encoding the efflux pump MepA belongs to the MATE family was also observed in *S. aureus* mutant strains derived from efflux-related MDR phenotypes. MepA has a broad substrate profile, including various monovalent and divalent biocides, as well as Norfloxacin and Ciprofloxacin[32]. Inhibition of efflux pumps such as NorA and MepA could be made by different mechanisms, such as preventing overexpression of the genes encoding these proteins, disrupting pump assembly, reducing substrate binding by competitive or non-competitive means, and interrupting the required energy source of the pump. Compounds capable of performing such actions and consequently restoring antibiotic susceptibility to efflux-efficient strains, are called efflux pump inhibitors (EPI)[33,34].

Furthermore, anxiety disorders and pain affect people in all age groups today which also make them an important public health problem. Benzodiazepines (BZDs) are part of the most common group of drugs to treat these types of diseases, however they induce side effects such as sedation, muscle relaxation, amnesia, and dependence potential[3]. Dysfunctions of GABA_A receptor, responsible for phasic and tonic neuronal inhibition in the vertebrate central nervous system, are associated with epilepsy, insomnia, anxiety and chronic pain. BZDs act on positive allosteric modulation in relation to the endogenous receptor site[35]. Several studies have already reported the relevance of chalcones and their derivatives in anxiolytic-like effect including with affinity in BZD binding site[36–39]. Therefore, it is very important to develop a new chalcone-like structure that can enhance its anxiolytic action without causing side effects.

In the present study, three bis-chalcones ((1*E*,4*E*)-1,5-bis(4-ethoxyphenyl)penta-1,4-dien-3-one, (1*E*,4*E*)-1,5-bis(4-chlorophenyl)penta-1,4-dien-3-one) and (1*E*,4*E*)-1,5-Di(thiophen-2-yl)penta-1,4-dien-3-one) previously synthesized and characterized by us [40,41], were evaluated for antiproliferative activity in HCT-116, SNB and PC3 cancer

cell lines, ability to modulate the antibiotic resistance in *S. aureus* strains overproducers of NorA or MepA efflux pumps, and anxiolytic-like effect in GABAergic system. Molecular docking simulations were also carried out to understand the mechanism of interaction with NorA, MepA and GABA_A proteins. These three bis-chalcones will be referred to hereinafter as DB ethoxy, DB chlorine, and DB thiophene respectively.

2. Material and Methods

2.1. Compounds and Materials

The three bis-chalcones DB ethoxy, DB chlorine and DB thiophene were previously synthesized by us through the Claisen-Schmidt condensation reaction where their structures were also fully elucidated by mass, infrared, Raman and NMR spectroscopy[40,41]. Figure 1 shows the bis-chalcones molecular structures. The reagents dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), RPMI-1640 medium, chlorpromazine (CPZ), Ciprofloxacin, Norfloxacin, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and ethidium bromide (EtBr) were purchased from Sigma-Aldrich Inc. Fetal bovine serum (FBS) and phosphate buffered saline (PBS) were from Gibco™. Brain heart infusion (BHI) and blood agar base were purchased from KASVI company. Hydrochloric acid (HCl) and Isopropanol were from Dinâmica Química Contemporânea Ltd. and Vetec Química Fina Ltd. respectively. Drugs used for zebrafish experiments were diazepam (DZP) from Neo Química® and flumazenil (FMZ) from Sandoz.

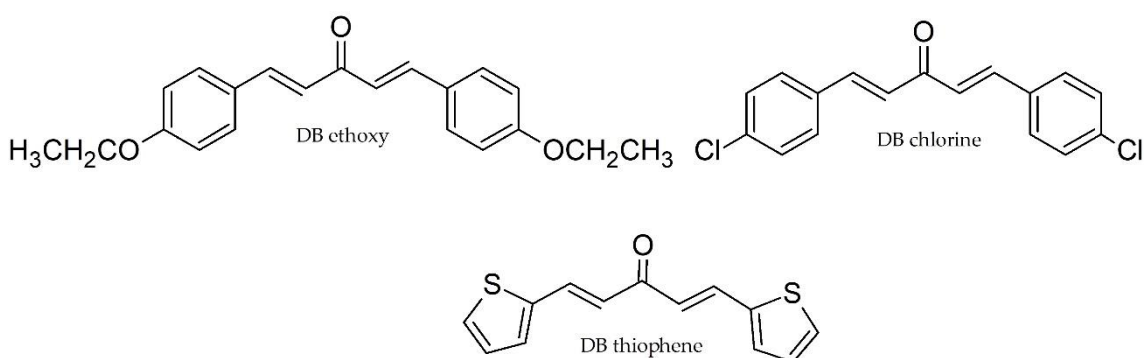


Figure 1. DB ethoxy, DB chlorine and DB thiophene molecular structures.

2.2. Cytotoxicity assay

The three human cancer cell lines HCT-116 (colon carcinoma), SNB (glioblastoma) and PC3 (prostate) were provided by National Cancer Institute (USA).

They were initially grown in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics, in 5% CO₂ atmosphere at 37 °C. The antiproliferative activity of the samples were measured by Colorimetric MTT assay[42]. This technique consists of preparing a calibration curve with cells plated in 96-well trays in double dilutions starting with 10⁵ cells/well in triplicates. First 0.01ml of MTT solution stock (MTT dissolved in PBS at 5 mg/mL) was added to each well. After 3 hours incubation at 37°C, acid isopropanol (0.1mL of HCl 0.04 M in isopropanol) was also added to each well and mixed. After few minutes at the room temperature optical density (absorbance) was read at wavelength of 595 nm. The best line that represents the relationship between the number of living cells and the absorbance of the medium is obtained by linear regression calculations using the least squares method. After this 0.1mL of neoplastic cells growth medium were plated in a 96-well tray (0.7 × 10⁵ cells/mL) and then the compounds were added after 24 hours with a final concentration of 10 μM. The plates were incubated at 37 °C in 5% CO₂ atmosphere. After 72 hours, the optical density was recorded at the same wavelength and the reduction in cell viability was obtained from calibration curve. DMSO was used as the negative control. The assay was performed in triplicates and the results were expressed as reduction in cell viability (% RCV) ± standard error of the mean (SEM). Two-Way ANOVA followed by the Bonferroni post hoc test were applied. The compounds that showed a reduction in cell viability ranging from 65 to 100% were considered very active, those ranging from 40 to 65% as moderately active, from 1 to 40% as little active, less than 1% as inactive.

2.3. Evaluation of the modulating effect on the antibiotic resistance

Initially two multiresistant strains, *S. aureus* SA1199B that overexpress the *norA* gene encoding the NorA efflux protein and the multidrug resistant (MDR) strain *S. aureus* K2068 which presents MepA efflux pump, were grown overnight at 37°C in Heart Infusion Agar slants. Before use, the bis-chalcones were dissolved in DMSO aqueous solution (5,1% v/v), CPZ and EtBr were dissolved in sterile water (1:1, v/v) and CCCP in methanol/water (1;1, v/v), all up to the final concentration of 1024μg/mL. The solutions were stored at -20°C. Then the Minimum Inhibitory Concentration (MIC), which is defined as the lowest concentration in which no microbial growth can be observed[43], of the bis-chalcones, CPZ, CCCP and EtBr were determined by broth microdilution method. Each strain was resuspended in 150μL of saline solution corresponding to approximately 10⁵ colony-forming units/mL, followed by addition of 1350μL of BHI in

ependorf's. 100 μ L of inoculum were shifted to 96-well microtiter plate with two-fold serial dilutions by adding 100 μ L of each compound solutions with final concentration ranging from 0.5 to 512 μ g/mL. The microtiter plates were incubated at 37°C for 24h and bacterial growth was revealed by staining with resazurin.

To establish the modulating effect on the resistance to Norfloxacin and Ciprofloxacin, MICs for these antibiotics were determined in the presence or absence of the bis-chalcones tested at sub-inhibitory concentration (MIC 1/8). Then a comparative study was made accessing its ability to decrease the MIC of antibiotics and EtBr, which is a substrate for MDR pumps including NorA[27,44–46] and MepA[32,47], by comparing with CPZ and CCCP, which are standard EPIs[48–51]. These results were expressed as a geometric mean \pm SEM of triplicates. They were analyzed by two-way ANOVA followed by Bonferroni post hoc test to identify statistical difference between treatments and control. Then, significant modifications of the activity were calculated as percent reduction of MIC in the presence of test compounds compared to controls (% of modulating effect).

2.4. Anxiolytic-like effect

2.4.1. Animals

The animal model used in this step was adult wild zebrafish (*Danio rerio*) (aZF) of both genders, from 60 to 90 days old, with 0.4 ± 0.1 g weight and 3.5 ± 0.5 cm long. They were purchased from Agroquímica: Comércio de Produtos Veterinários LTDA (Fortaleza, Brazil). Groups of 60 fish were acclimated for 24 hours in a glass tank (30 \times 15 \times 20cm) containing dechlorinated water (ProtecPlus®) equipped with air pumps and submerged filters at 25 °C and pH 7.0, with a cicardian cycle 14/10h of light/dark. One day before experiment, the animals were fed *ad libitum* and, after the test, they were euthanized by immersion in ice water (2 – 4°C) for 10 min until the loss of opercular movements occurred[52]. The Ethics Committee on Animal Use of the Ceará State University (CEUA-UECE) approved all experimental procedures with acceptance number 3344801/2017.

2.4.2. Acute toxicity (96 h)

The acute toxicity study was carried out in aZF according to *Ekambaram* and colleagues[53]. As an adaptation of the method, aZF (n = 6/group) were treaded

intraperitoneally (*i.p.*) with 20 μ L of bis-chalcones (4, 20 and 40 mg/kg), diluted in DMSO 3%. As a negative control, it was used only the vehicle (DMSO 3%). After 24, 48, 72 and 96 hours, the number of dead aZF were recorded and the lethal concentration capable of killing 50% of the animals (LC₅₀) was determined using the mathematical method Trimmed *Spearman-Karber* with a 95% confidence interval[54].

2.4.3. Locomotor activity (Open field test)

To assess whether the bis-chalcones could alter the motor coordination of the animals, either by sedation and/or by muscle relaxation, the open field test was carried out, according to methodologies proposed by Benneh[55] and Magalhães[56]. The aZF (n = 6/group) were treated (*i.p.*) with 20 μ L of bis-chalcone solutions in doses of 4, 20 and 40 mg/kg. The vehicle (DMSO 3%) and DZP (4 mg/kg) groups were also analyzed. After 30 min, the fishes were added in Petri dishes containing the same water from glass tank, marked with quadrants and the locomotor activity was analyzed by counting the number of crossing lines for 5 minutes. Animals that did not receive treatments (naïve) were considered as baseline (100% locomotor activity) and the percentage of locomotor activity (% LA) was calculated.

2.4.4. Light/dark test

This test was carried out in an aquarium (30 \times 15 \times 20 cm) filled with 3 cm of water, pre-treated with anti-chlorine containing a light and a dark zone. The aZF (n = 6/group) were treated (*i.p.*) with bis-chalcones (4, 20 and 40mg/kg; 20 μ L), vehicle (3% DMSO; 20 μ L) and diazepam (4 mg/kg; 20 μ L). A naïve group (no treatment) was also incorporated to the test. After 30 min, the anxiolytic-like effect was assessed by the individual introduction of the fish in the clear zone and subsequent quantification of their permanence in this zone during 5 minutes in percentage (PCZ, in%)[57].

2.4.5. Mechanism of action (GABAergic system)

According to Benneh[55], aZF (n = 6/group) were treated (*i.p.*) with FMZ (4 mg/kg; 20 μ L), after 15 min the same procedure with the three bis-chalcones (40mg/kg; 20 μ L), vehicle (3% DMSO; 20 μ L) and DZP (4 mg/kg; 20 μ L). After 30 min, the inhibition of the anxiolytic-like effect was quantified by light/dark test as described above[57]. Results were expressed as values of the mean \pm standard error of the mean

(SEM) for each group of 6 animals. After confirming the normal distribution and homogeneity of the data, the differences between the groups were analyzed using a One-way ANOVA followed by the Tukey test.

2.5. Proteins modeling

Since the crystallographic structure of NorA and MepA proteins are not available in the literature, homology models were produced in SWISS-MODEL[58]. SWISS-MODEL searches in both BLAST (Basic Local Alignment Search Tool) and HHblits (HMM-HMM-based lightning-fast iterative sequence search) to identify models. The combined use of these two approaches ensures good alignments at the high and low levels of sequence identity. The primary protein sequence of the NorA and MepA efflux pumps were recovered in Uniprot[59] with the entry codes Q5HHX4 and Q2G140. Of all the 50 templates generated in both case, and based on the Global Model Quality Estimation Score, the structure of *E. coli* YajR transporter (SMTL ID :3wdo.1) and *P. furiosus* MATE (pfMATE) transporter in complex with MaL6 (SMTL ID :3wbn.1) were selected as template of NorA and MepA molecular modeling respectively. The final models were protonated and examined using the program MolProbity[60]. In order to provide a better view of the cavities and tunnels of the transmembrane protein models, we proceed calculations with the MOLEonline 2.0 software[61], then using the USF Chimera[62], it was found which residues were in contact with main cavities. The human GABA_A receptor structure in complex with diazepam (PDB-ID: 6hup) was retrieved from the protein data bank website[63].

2.6. Molecular Docking

In order to improve the understanding of the inhibition mechanisms of the NorA and MepA efflux pumps, as well as the anxiolytic-like effect in the GABAergic system, we performed the molecular docking simulation using AutoDock Vina[64]. The theoretical models of NorA and MepA proteins prepared in SWISS MODEL and the crystallographic structure of human GABA_A were used as macromolecules, Kolman charges were added and non-polar hydrogens merged. The ligands were optimized using the PM6 forcefield in the MOPAC software[65]. Gasteiger charges were added to ligands, non-polar hydrogens were merged, and rotational bonds tree was determined. The grid box was set up at 70-70-70 Å npts around the geometrical center of the NorA and MepA models, and around the benzodiazepine binding site at the $\alpha 1-\gamma 2$ interface of the

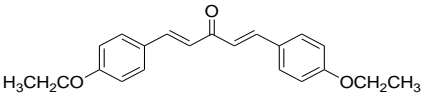
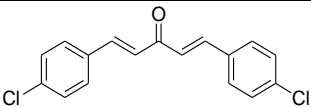
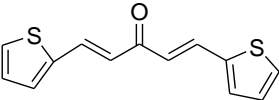
extracellular domain according to Zhu et al.[66]. The lowest energy pose of each docking calculation was then selected and analyzed.

3. Results and discussion

3.1. Cytotoxicity assay

The antiproliferative assessment of bis-chalcones DB ethoxy and DB chlorine in cancer strains HCT-116, SNB e PC3 has already been published by us recently[40]. In it, DB chlorine revealed to be very active (%RCV 85.03 ± 0.57) against colon carcinoma (HCT-116), little active against prostate cancer cells (PC3) and inactive for the glioblastoma (SNB), while DB ethoxy proved to be little active (HCT-116 and PC3) or completely inactive (SNB). In our present study, we carried out this same evaluation with DB thiophene, which demonstrated better citotoxicity effects in cancer strains tested. It was very active against HCT-116 (% RCV 85.24 ± 0.57), moderately active versus PC3 (% RCV 43.57 ± 3.09) and little active using SNB strains (% RCV 9.83 ± 4.90). Table 1 summarizes these results.

Table 1. Cytotoxicity of bis-chalcones at a single concentration (10 μ M) against HCT-116, SNB and PC3 cells after 72 h of incubation using MTT assay.

Bis-chalcones	Cancer cell lines			Ref.
	HCT-116	SNB	PC3	
Reduction in Cell Viability (% RCV)				
	8.21 \pm 4.32 ^{a1}	iav	12.59 \pm 7.32	[40]
	85.03 \pm 0.57 ^{b1,b2}	iav	30.74 \pm 3.71 ^{b3}	[40]
	85.24 \pm 0.57 ^{c1,c2}	9.83 \pm 4.90	43.57 \pm 3.09 ^{c3}	This work

Abbreviations: iav, inactive; ^{a1} $p > 0.05$ (not significant), in relation to DB ethoxy/PC3; ^{b1} $p < 0.001$, in relation to DB chlorine/PC3; ^{c1} $p < 0.001$, in relation to DB thiophene/SNB and DB thiophene/PC3; ^{b2,c2} $p < 0.001$, in relation to DB ethoxy/HCT-116; ^{b3} $p < 0.001$ in relation to DB ethoxy/PC3; ^{c3} $p < 0.001$, in relation to DB ethoxy/HCT-116 and $p < 0.01$ in relation to DB chlorine/PC3.

It is well known that various synthetic chalcones derivatives provide effective anti-cancer actions. In addition, its heterocyclic analogues are still reported to be quite powerful against resistant cancer cell lineages[67]. A serie of thiophene chalcones derivatives has demonstrated a high cytotoxicity in a variety of strains[68,69]. This is consistent with the performance presented by DB thiophene in this work. Comparing DB

ethoxy and DB chlorine structures, we realized that the ethoxy group has an electron-donating effect greater than that of chlorine at para position. Therefore, the best results of DB chlorine may be due to this difference in mesomeric effect. This is in line with what was reported by Raghavan et al.[70] in which the increasing of electron-donating effect on chalcones A ring reduced cytotoxic activity. In this same study, the -Cl substituent was found to increase cytotoxicity consistently in all the three cell lines, viz., A549, MCF7 and MIA PaCa-2. Although the bis-chalcones mechanism of action is not clear here, it is known that the main form of compound-induced cell death is apoptosis, resulting from various cellular events[68].

3.2. Modulation of the bacterial resistance to antibiotics

The compounds studied in this work did not show intrinsic antimicrobial activity (MIC > 1024µg/mL), so their modulating action on the drug resistance in multi-drug resistant SA1199B and K2068 strains was investigated aiming to evaluate their potential activity as NorA or MepA inhibitors (Table 2). It should be noted that DB chlorine and DB thiophene showed the ability to modulate the resistance to EtBr in the strain K2068 comparable to CPZ, suggesting a possible inhibition of the MepA efflux pump. The inhibition of efflux by DB thiophene had already been published by our group previously[41]. The results indicated that the three bis-chalcones are probably inhibitors of NorA-mediated EtBr efflux, whereas in MepA-mediated EtBr efflux only DB ethoxy was inactive. Holler et al.[71] investigated a series of 117 chalcones, among which five were able to inhibit the NorA-mediated EtBr efflux. However, not all were able to inhibit the NorA efflux pump when the substrate was Ciprofloxacin.

Table 2. Modulating effect of bis-chalcones, Chlorpromazine (CPZ) and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on the resistance to Ethidium Bromide in the *S. aureus* SA1199B (*norA*) and K2068 (*mepA*) strains.

Compounds ^a	Modulating effect (%)	
	SA1199B	K2068
DB ethoxy	37.0	iav
DB Chlorine	37.0	37.0
DB thiophene	(29.3)	(43.9)
CPZ	60.3	37.0
CCCP	92.1	-

Abbreviations: iav, inactive. ^aThe compounds were tested at subinhibitory concentration (MIC/8). All percent efflux inhibition were significant ($p < 0.0001$ vs control). Values in parentheses are from Oliveira and colleagues work[41].

In our previous work, DB thiophene was also able to favorably modulate the resistance to Norfloxacin and Ciprofloxacin in SA1199B and K2068, respectively. This compound reduced the MIC values for Norfloxacin by 30% and for Ciprofloxacin by half[41]. Another study also demonstrated the ability of compounds containing thiophene ring to restore the Ciprofloxacin activity against SA1199B[72]. Results found in the present study showed that DB ethoxy and DB chlorine were not able to modulate the resistance of SA1199B to Norfloxacin, and DB chlorine did not modulate the resistance of K2068 to Ciprofloxacin (data not shown). It is intriguing why these compounds have not increased the effect of antibiotics since they were active when tested in combination to EtBr. However, EtBr it is not a specific substrate for NorA or MepA[73], thus it can be extruded by other unidentified efflux pump systems present in the strains tested.

Controversially, DB ethoxy reduced the MIC of Ciprofloxacin by 50% against K2068, although it was inactive in the MepA-mediated EtBr efflux.

3.3. Acute toxicity (96 hours)

The aZF has become a complementary animal model to rodents in genetic, developmental biology, neurobiological and toxicological tests[74] since it has low cost, diverse adaptability, short reproduction cycle, high fertility and transparent embryos[75]. Its reduced dimensions in adulthood require a small amount of test compounds at doses as well as less reagents and materials used in the treatment and maintenance of the animals. The aZF also stand out for their use in assessing the toxicity of pharmaceutical compounds[76]. In this context, we used aZF as an animal model to measure the acute toxicity of DB ethoxy, DB chlorine and DB thiophene bis-chalcones. Results found showed that all tested samples proved to be safe, as they were not toxic to aZF until 96 hours of analysis (Table 3).

Table 3. Acute toxicity test of bis-chalcones in aZF.

Compounds	mortality				96 hours
	NC	D ₁	D ₂	D ₃	LC ₅₀ (mg/kg) / CI
DB ethoxy	0	0	0	0	> 40
DB chlorine	0	0	0	0	> 40
DB thiophene	0	0	0	0	> 40

Abbreviations: NC, negative control group (DMSO 3%); D₁, Dose 1 (4 mg/kg); D₂, Dose 2 (20 mg/kg); D₃, Dose 3 (40 mg/kg); LC₅₀, lethal concentration to kill 50% of adult zebrafish; CI, confidence interval.

3.4. Locomotor activity (Open field test)

Locomotor activity is one of the behavioral analysis parameters that has been used to evaluate drugs action on the central nervous system of the aZF and, eventually, induce locomotor impairment[57]. This activity can be analyzed using the open field test, in an aquarium[77], or Petri dishes[78]. The natural behavior of aZF in the open field is characterized by constant swimming activity. Manifestations of immobility are rarely observed in natural conditions, so one of the parameters that can be evaluated is its immobility, also called as freezing[79]. The analysis of locomotor activity explored through an open field can be a model used to assess hyperactivity as being indicative of anxiety[74]. Then, treatment with anxiolytic drugs can increase exploratory activity in the open field[79] or cause sedative effect and reduce locomotor activity[55].

The results of this assay showed that the bis-chalcones DB chlorine and DB thiophene (Figures 2B and 2C) provoked motor impairment in aZF. In this case, there was a significant reduction in the number of line crossing in the Petri dish by the animals compared to Naive group. In contrast, DB ethoxy (Figure 2A) did not alter the animal's locomotion during the 5 min of analysis. When analyzing the behavioral profile of aZF after treatment with DB chlorine and DB thiophene samples, the decrease in locomotor activity suggests a possible sedative action, such as benzodiazepines (anxiolytic drugs). It is known that these drugs can lower the locomotor activity (mobility) of aZF in open field tests[55]. Thus, these bis-chalcones must have their anxiolytic activity evaluated by the light/dark test and their possible mechanism of anxiolytic action enlightened. Since DB ethoxy did not alter the animals' locomotion, this may suggest a potential antinociceptive action as shown by Silva et al.[80].

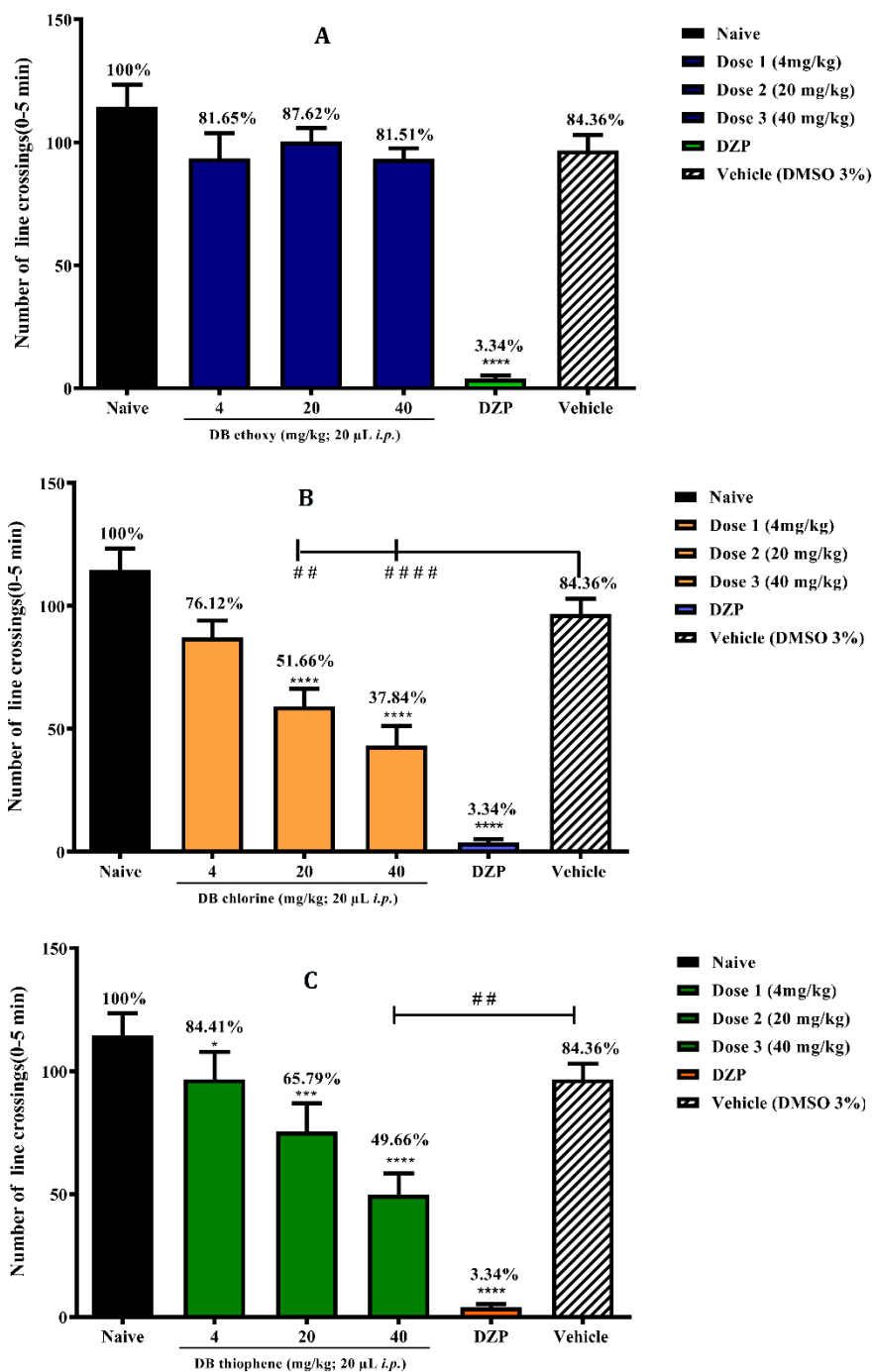


Figure 2. Effect of bis-chalcones DB ethoxy (A), DB chlorine (B) and DB thiophene (C) under the locomotor activity of the adult zebrafish (*Danio rerio*) in the Open Field Test (0 - 5min). Naive - untreated animals. DZP - diazepam (4 mg/kg; 20 μ L; i.p.). Vehicle - 3% DMSO (20 μ L; i.p.). The values represent the mean \pm standard error of the mean (SEM) for 6 animals/group. ANOVA followed by Tukey test (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$ vs. Naive); (## $p < 0.01$; #### $p < 0.0001$ vs. Vehicle).

3.5. Anxiolytic-like effect

Among the various tests to assess anxiety in adult zebrafish, the Light & Dark Test is one of the most used specific tests, as it is based on the paradigm of zebrafish's innate aversion to well-lit areas, like that of rodents. In such test, animals not treated with anxiolytic drugs present the same behavior as in mice, having aversion to clear zones[57,81,82].

In this context, we use the same animal model to investigate the anxiolytic-like effect of the synthesized compounds. Figures 3A and 3B show the anxiolytic-like effect of DB chlorine and DB thiophene, since aZF treated with both compounds (40 mg/kg; 20 μ L; i.p.) remained significantly for most of the analysis time in the clear zone (PCZ_{DB chlorine} = 54.16%; PCZ_{DB thiophene} = 78.27%), as Diazepam (4mg/kg; 20 μ L; i.p.) which is the anxiolytic control (PCZ_{DZP} = 81.05%).

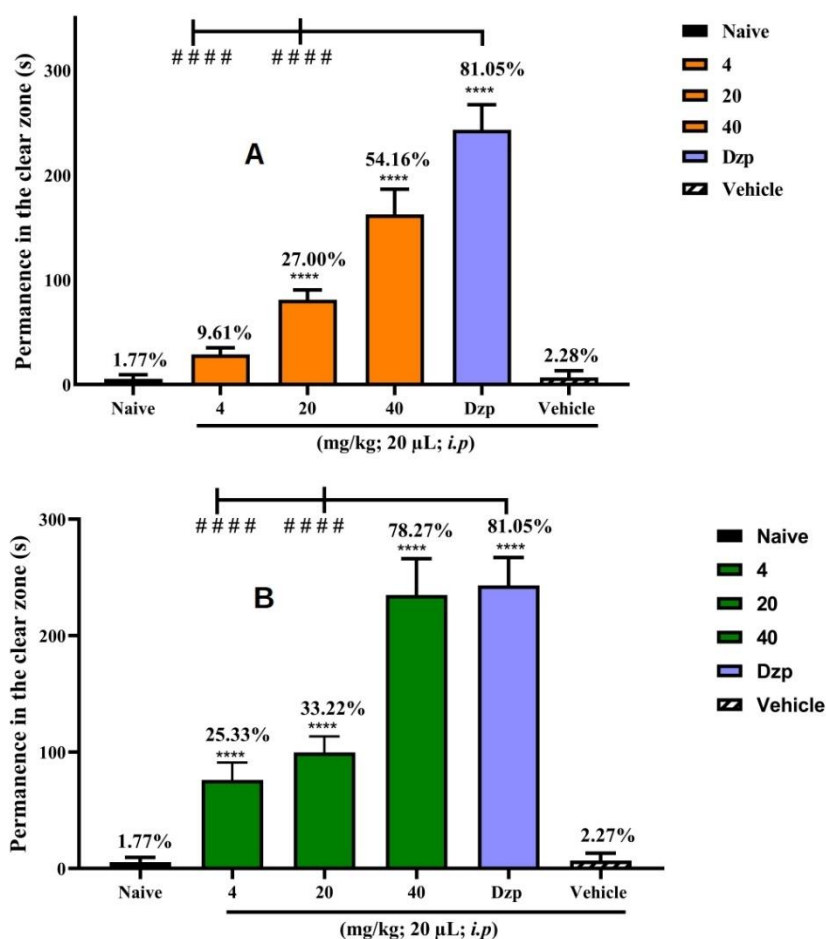


Figure 3. Anxiolytic-like effect of bis-chalcones DB chlorine (A) and DB thiophene (B) on adult zebrafish (*Danio rerio*) in the Light & Dark Test (0-5min). Naive - untreated animals. Vehicle – 3% DMSO (20 μ L; i.p.). DZP – Diazepam (4 mg/kg; 20 μ L; i.p.). The values represent the mean \pm standard error of the mean (SEM) for 6 animals/group.

ANOVA followed by Tukey test (**** $p < 0.0001$ vs. Naïve or Vehicle); (### $p < 0.0001$ vs. DZP). The numbers above each column indicate percentage of the permanence in the clear zone (PCZ).

3.6. Mechanism of action (GABAergic system)

When verifying a possible anxiolytic-like effect, we investigated the mechanism of action of this activity using the GABA_A antagonist, Flumazenil. It is a specific γ -aminobutyric acid (GABA_A) antagonist receptor complex. In this way it antagonizes the effects of benzodiazepines such as Diazepam, including anxiolytic, sedative, and hypnotic effects. Then, we used Flumazenil to investigate the mechanism of anxiolytic action at the highest dose (40 mg/kg; 20 μ L; i.p.) using the light/dark test.

Flumazenil (4 mg/kg 20 μ L; ip) significantly reversed the anxiolytic-like effect of DB chlorine and DB thiophene (40 mg/kg; 20 μ L; i.p.), in addition to exhibiting the same behavior when used with Diazepam (4 mg/kg; 20 μ L; i.p.). In this case, there was a significant decrease in the permanence in the clear zone of animals pre-treated with Flumazenil and then with bis-chalcones, very similar to the results of animals treated with Flumazenil and Diazepam (Figures 4A and 4B). These results showed that both bis-chalcones evidence an anxiolytic-like effect via the GABAergic pathway.

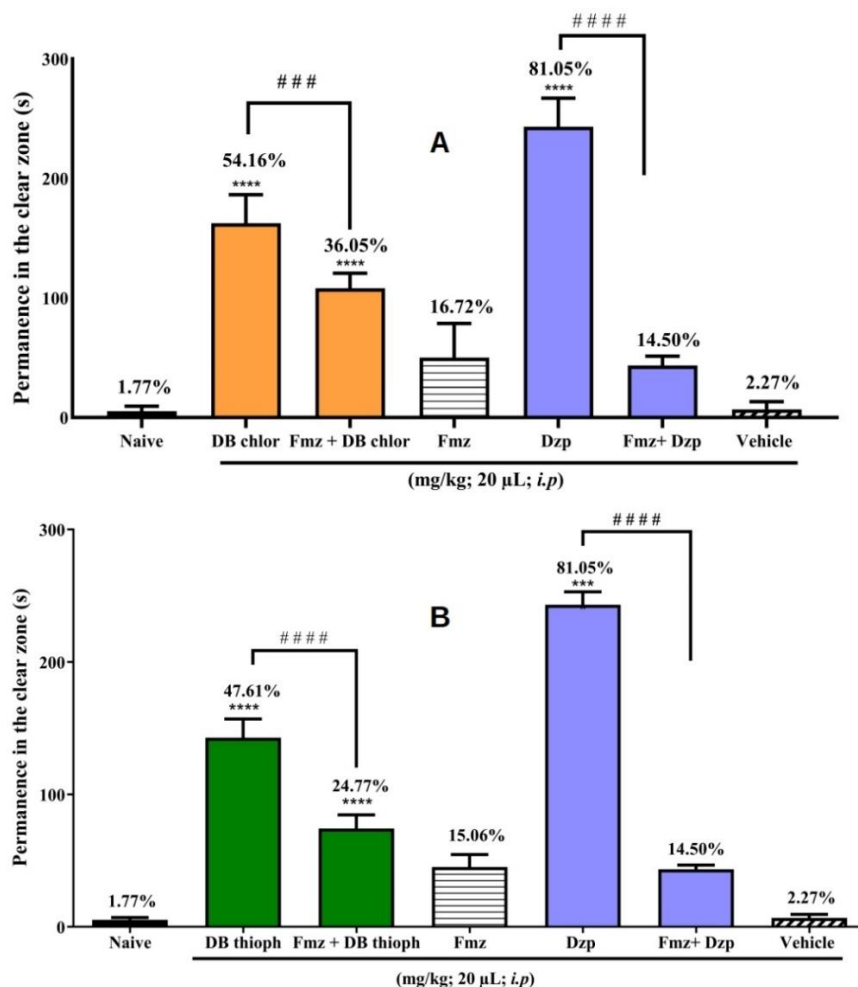


Figure 4. Mechanism of anxiolytic action of bis-chalcones DB chlorine (A) and DB thiophene (B) on adult zebrafish (*Danio rerio*) in the Light & Dark Test (0-5min). Naive - untreated animals. Vehicle – 3% DMSO (20 μ L; i.p.). DZP – Diazepam (4 mg/kg; 20 μ L; i.p.). FMZ – Flumazenil (4 mg/kg; 20 μ L; i.p.). The values represent the mean \pm standard error of the mean (SEM) for 6 animals/group. ANOVA followed by Tukey test (**** p < 0.0001 vs. Naïve or Vehicle); (### p <0.001 vs. DB chlorine; #### p <0.0001 vs. DZP or DB thiophene). The numbers above each column indicate percentage of the permanence in the clear zone (PCZ).

3.7. Protein modeling

Our NorA model (Figure 5A) contained 12-transmembrane (TM1 - TM12) helices as is common in the major facilitator superfamily (MFS). Between TM6 and TM7, a small piece of the chain can be seen, including residues 193-199, where a small helix was formed as well. According the template used[83], we presume that our homology model is in a outward-facing conformation. These structural characteristics are the basis for the H^+ -driven system responsible for alternating between the outward and inward states and the consequent expulsion of the substrate. A cleft entrance formed by TMs 1,2,5,7,6 and

11 gives access to a central cavity that allows the proton to enter and the substrate to leave the binding site. Deeper inside the cavity, a region surrounded by residues PHE16, ILE19, GLN51, MET109, ILE136, LEU218, PHE306, ARG310, THR 336, SER 337 and ASN 340 seems to be the main binding site (red spheres), however another two pockets could also anchor the substrate, one formed by residues ALA45, ALA 48, LEU49, MET52, PHE341, PRO344 and LEU 345 positioned laterally in relation to the center of the cavity (blue spheres), and other surrounded by ASN137, PHE140, LEU219, GLU222, THR225, ALA 243, SER299 and PHE303 residues (green spheres). As the protein is in the outward conformation, we argue that these two other pockets may be part of the substrate's exit path. In almost all crystalline structures of proton-driven MFS transporters, there are proton titratable amino acid residues in the central cavity. These titratable residues are important to switch outward conformation onto inward and vice versa[83]. In our NorA homology model, such residues are likely to be GLU222 and ARG310 because they are the only two residues in theory titratable inside the cavity.

The MepA model (Figure 5B) was also obtained in the outward-facing position as is presumed due to the template used. Its 3D structure also featured 12-transmembrane (TM1 - TM12) helices of which 1, 2, 5, 8 and 10 form the cleft entrance that gives access to the main pocket. The open cleft is made up of the residues ILE40, TYR41, ASP48, ILE52, ILE55, SER56, LEU57, LEU59, PRO60, LEU259, LEU 263, ASN 270, PHE271, ILE273, ALA274, ILE278, PHE280, ARG 281, PHE345 and ASP348 (gray spheres). This opening is the outlet path of the central binding site surrounded by residues MET25, THR29, SER32, VAL33, TYR35, PHE62, MET66, ASN70, VAL74, VAL149, PHE152, PHE153, GLU156, ARG160, MET172, SER175, VAL176, THR201, SER251, MET254, GLN284, MET291, ALA292, CYS294, GLU295, PHE373, THR376, GLN380, ALA392 and GLN395 (orange spheres). It is possible to notice that the binding site is formed mainly by hydrophobic residues, to a lesser extent by hydrophilic ones and very few titratables such as GLU156, GLU295 and ARG160. The Ramachandran plot for both models is shown in the supplementary material.

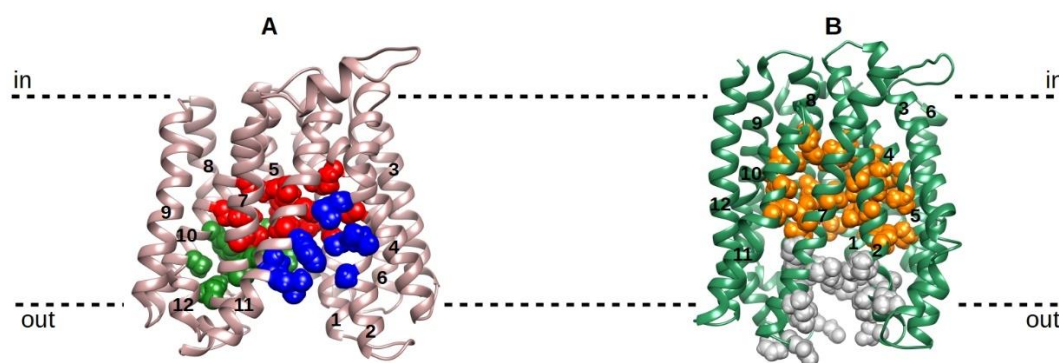


Figure 5. NorA (A) and MepA (B) homology models in outward face conformation. The colored spheres represent the side chain of the residues.

3.8. Molecular docking

3.8.1. NorA and MepA

From the best conformation of the molecular docking, it was possible to study the protein-ligand interactions in the two efflux pumps. Binding affinities were estimated with DB ethoxy and DB chlorine showing lower binding scores than EtBr in NorA, while in MepA none performed better than EtBr. Table 4 summarizes the types of interactions that the compounds and EtBr formed with the residues of the NorA and MepA proteins as well as their binding affinity.

Table 4. Interaction sites and binding energy between the compounds in the best pose and the proteins of the efflux pumps.

Protein	Ligands	Interactions with residues								Binding Energy (kcal/mol)
		Van der Walls	H-Bond	π -Sigma	Alkyl	π -alkyl	π - π stacked	π - π T-shaped	π -Sulfur	
NorA	EtBr	PHE16 ASN137 GLU222 PHE306 THR336	ASN340	ILE136	LEU218	LEU218	PHE140			-7.3
	DB thiophene	ILE12 GLU222 PHE306 THR336	ASN340			MET109 ILE136 LEU218	PHE16 PHE140			-5.8
	DB ethoxy		GLN51 ARG310	LEU218	ILE15 PHE16 ILE19 PHE140 PHE303	MET109 ILE136				-7.7
	DB Chlorine	ILE12 ASN137 SER215 GLU222 PHE306 THR336	ASN340	LEU218	ILE15 PHE16 PHE140 PHE303	MET109 ILE136				-7.7
MepA	EtBr	SER175 THR201	ASN179			MET172 VAL176	PHE153			-7.6
	DB thiophene	PHE152 PHE153 SER175 VAL176 THR201	MET172			VAL149				-6.3
	DB Chlorine	VAL176 PHE152	SER175			PHE62 PHE153	TYR35 PHE153	MET66		-7.1

It is noticed that in both cases, the compounds occupied essentially the same site of EtBr after simulation under the same conditions, although they did not interact in the same way with the residues. As can be seen in Figure 6, EtBr (A), DB thiophene (B), DB chlorine (C) and BD ethoxy (D) interact with several residues in common, *e.g.*, PHE16, ILE136, PHE140 and LEU218, indicating that the three bis-chalcones probably modulate the efflux of EtBr by competing in the same binding site. This site is contained in the region identified in Figure 5A by red spheres and has in common some residues with which Ciprofloxacin established more contacts in the molecular dynamics conducted by *Palazzotti* and colleagues[84]. This result is interesting because *Palazzotti* and colleagues used a NorA protein model in the inward-facing conformation in which our findings would correspond to a region equivalent to sites (c) and (d) showed by these authors. It is worth mentioning that residues GLN51, PHE140, LEU218, PHE303 and PHE306, belonging to the site where our compounds bind, are preserved from *E. coli* YajR transporter.

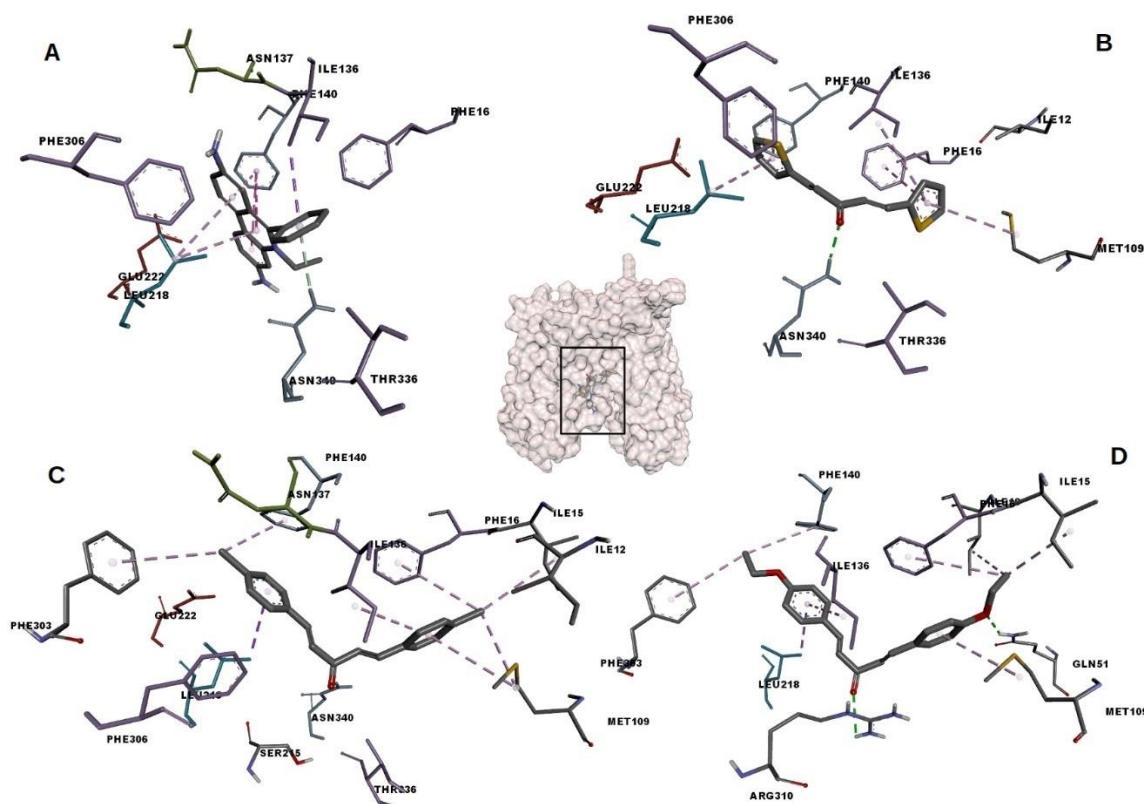


Figure 6. Interactions between EtBr (A), DB thiophene (B), DB chlorine (C) and DB ethoxy (D) in their best poses with NorA protein model.

DB thiophene and DB chlorine were found to be docked at the same cavity of EtBr in MepA, they interacted with several residues in common, *e.g.*, SER175, VAL176

and PHE 153, suggesting that the inhibition of EtBr efflux occurs by competition at the same binding site. Figure 7 shows the interactions between binding site residues and the compounds.

It is relevant to note that PRO26 has been shown to be very important in EtBr export pfMATE-mediated[85] and this residue is conserved in MepA model being occupied by PRO24, while the acidic residues ASP41 and ASP184, through protonated and non-protonated states, are involved in the antiport mechanism, essential for conformational change in pfMATE[85]. In this case only ASP184 was conserved in our model.

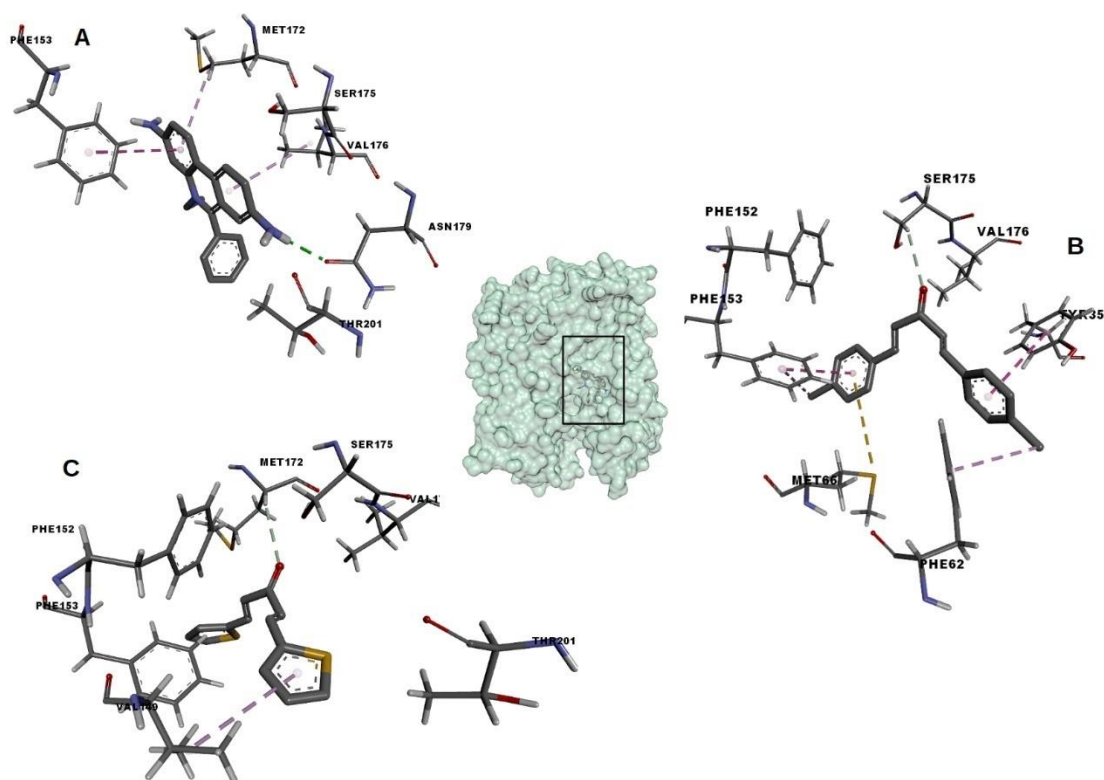


Figure 7. Interactions between EtBr (A), DB chlorine (B) and DB thiophene (C) in their best poses with MepA protein model.

Since DB thiophene showed potent EPI effect for antibiotics in the NorA and MepA efflux pumps, an analysis of the docking involving DB thiophene, Norfloxacin and Ciprofloxacin was performed, and the results are displayed in Figures 8A and 8B. Additionally, 2D ligand-protein interaction diagrams are provided in Figures 8C and 8D. DB thiophene formed on NorA model an H-bond in its carbonyl group with the residue

ASN340, their aromatic rings interacted with PHE16 and PHE 140 through π - π T-shape and with MET109, ILE136 and LEU218 through π -Alkyl interaction. The residues ILE12, GLU222, PHE306 and THR336 contributed to the stabilization of the structure with *Van Der Walls* interactions. Its binding score was -5.8kcal/mol. This corroborates with an earlier report of DB thiophene in which the compound bound to essentially the same binding site and probably functions as a competitive inhibitor[41]. Norfloxacin was strongly stabilized by the H-bonds formed with GLN51 and ARG310 as well as interactions involving residues GLU222 and PHE140 due to the protonation state of the nitrogen of the piperacinic group. The binding affinity of Norfloxacin was said - 6.7kcal/mol. Our results confirm the importance of residues GLN51, ARG310, GLU222 and PHE16 in the recognition of substrates by NorA because Dantas et al.[86] also described their interactions with Norfloxacin. These same residues played an important role in the binding site of complex NorA-reserpine, a standard inhibitor[87]. Moreover, they also proved to be a crucial part in the recognition pathway of the NorA-mediated efflux of Ciprofloxacin[84,88].

In MepA, DB thiophene and Ciprofloxacin binds roughly to the same region of the model. The bis-chalcone established *Van Der Waals* interactions with residues PHE152, PHE153, SER175, VAL176 and THR201, a π -Alkyl with VAL149 and an unconventional H-bonds with MET172, which gave it a binding score of -6.3kcal/mol. In addition to close contacts with residues PHE153, TYR35 and VAL176, Ciprofloxacin made H-bonds with ASN179 and ASN70 (unconventional), and a halogen interaction with residue GLU156. This allowed Ciprofloxacin to achieve better binding score than DB thiophene, -7.5kcal/mol. Thus, this result showed a good positioning to prevent the antibiotics binding to the MepA protein, reinforcing the results of modulating assays found by us before for K2068 overexpressing *mepA* gene[41]. Similar results corroborate the belief that the region we found is probably the MepA binding site. Rezende-Júnior et al.[89] obtained a docking of a chalcone in practically the same place, even using another template to build their MepA homology model, Xavier et al.[90] also reported similar results in the docking of chalcones, which occupied the same binding site as Ciprofloxacin.

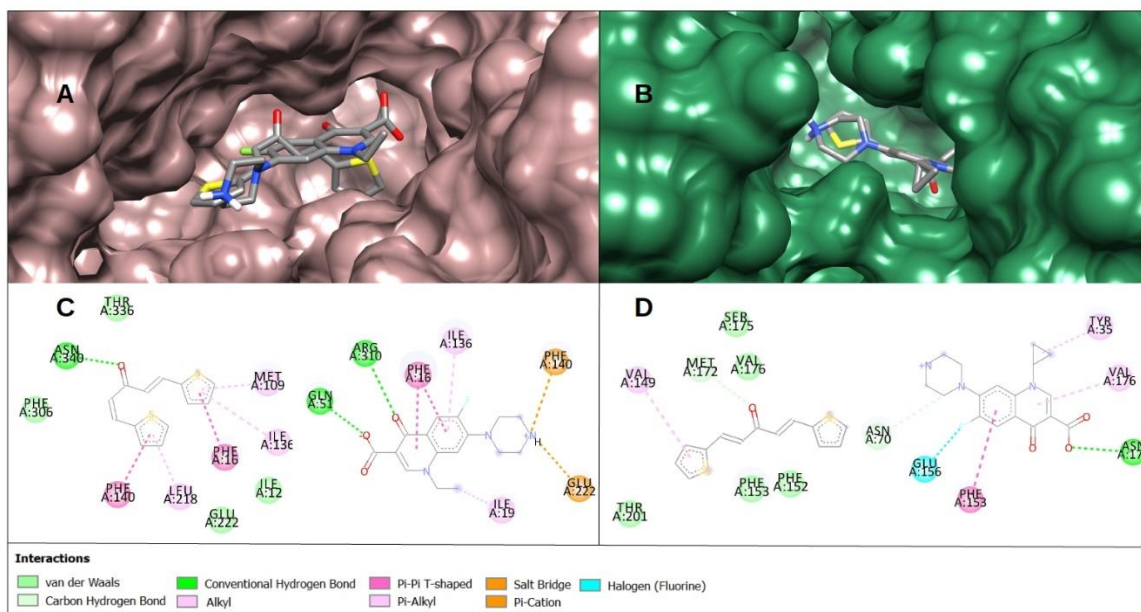


Figure 8. (A) Norfloxacin binding site in NorA with DB thiophene competition. (B) Ciprofloxacin binding site in MepA with DB thiophene competition. (C) 2D ligand-protein interaction diagram of DB thiophene (left) and Norfloxacin (right) in NorA model. (D) 2D ligand-protein interaction diagram of DB thiophene (left) and Ciprofloxacin (right) in MepA model. All the poses shown have the lowest binding energy.

3.8.2. GABA_A

In Figure 9 is shown the compatibility of DB thiophene and DB chlorine with the GABA_A receptor at benzodiazepines binding site. DB thiophene achieved a binding score of -7.1 kcal/mol through *Van Der Waals*, π - π stacked and amino- π stacked interactions with α 1 subunit residues TYR58, ALA79, and MET57, and π -sulfur interactions with the γ 2 subunit residue TYR210. Likewise, the best docking pose of DB chlorine bound at the α 1- γ 2 interface and established close contacts with γ 2 subunit residues HIS102 (conventional hydrogen bond), LYS156 (π -sulfur) and SER205 (π -donor hydrogen bond) as well as interacted through π -donor hydrogen bond and π - π stacked with the α 1 subunit residues ASN60 and PHE77, respectively. In this case the binding score was -9.1 kcal/mol. Although the binding score should not be mistaken for affinity, both compounds appear to have stronger interactions with the benzodiazepines binding site. It is noteworthy that Diazepam, at the same binding site, through a redocking process, revealed in its best pose a binding score of -9.8 kcal/mol.

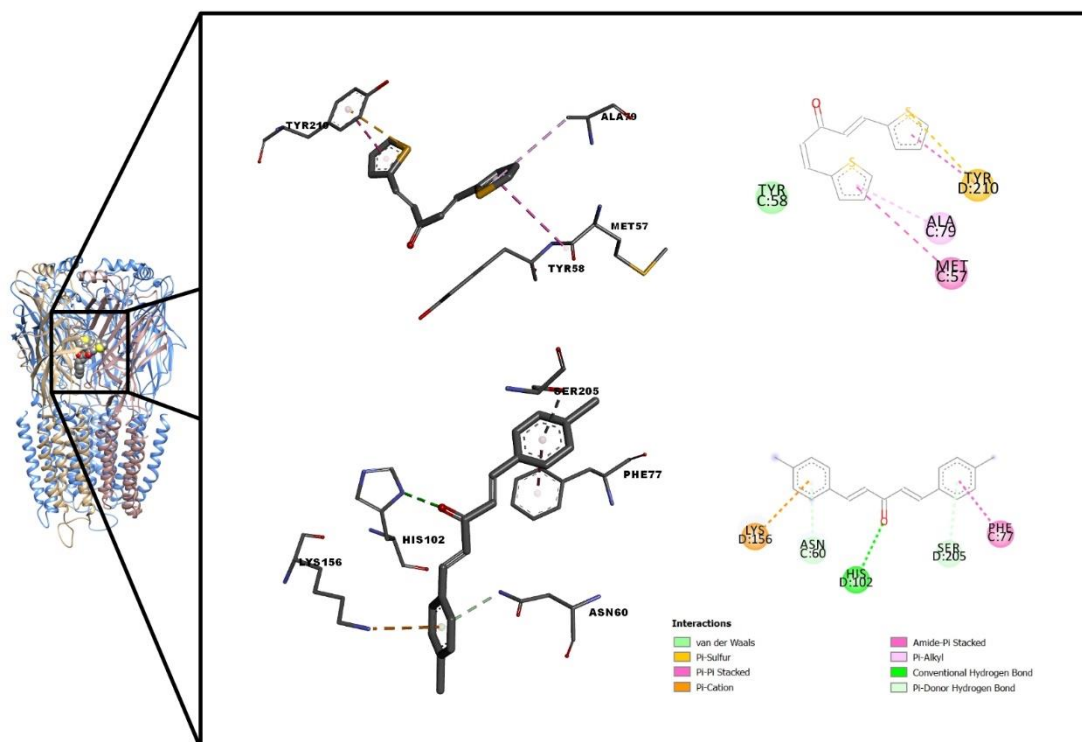


Figure 9. The best pose of DB thiophene (above) and DB chlorine (below) on the benzodiazepine site of the GABA_A receptor ($\alpha 1$ – $\gamma 2$ interface of the extracellular domain - ECD). 2D ligand-protein interaction diagram is on the right.

4. Conclusions

Bis-chalcones are a class of compounds with great biological potential. Results found in the present study showed that DB chlorine and DB thiophene were very active against colon carcinoma strain (HCT-116), suggesting a potential application as antitumoral drugs. The three bis-chalcones appear to be inhibitors of NorA-mediated EtBr efflux, while only DB chlorine and DB thiophene appear inhibit the MepA-mediated EtBr efflux. Docking studies showed that this inhibitory effect is likely due to competition for the same binding site. DB thiophene shows great potential as a modulator of the resistance to Norfloxacin and Ciprofloxacin, probably reducing their efflux by NorA and MepA efflux pumps, respectively. We proposed, through docking studies, that DB thiophene competes for the same binding site of Norfloxacin and Ciprofloxacin, acting as a competitive inhibitor of efflux pumps. DB chlorine and DB thiophene were nontoxic, also caused motor impairment in aZF suggesting a possible sedative action. Further investigation exhibited significant anxiolytic-like effect in adult zebrafish of both compounds, modulated via the GABAergic system.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary Material

Cytotoxicity Activity, Efflux Pump Inhibition, Anxiolytic-Like Effect and Molecular Docking Studies of Synthetic Bis-chalcones

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Figure S1. Ramachandran plot for NorA homology model.

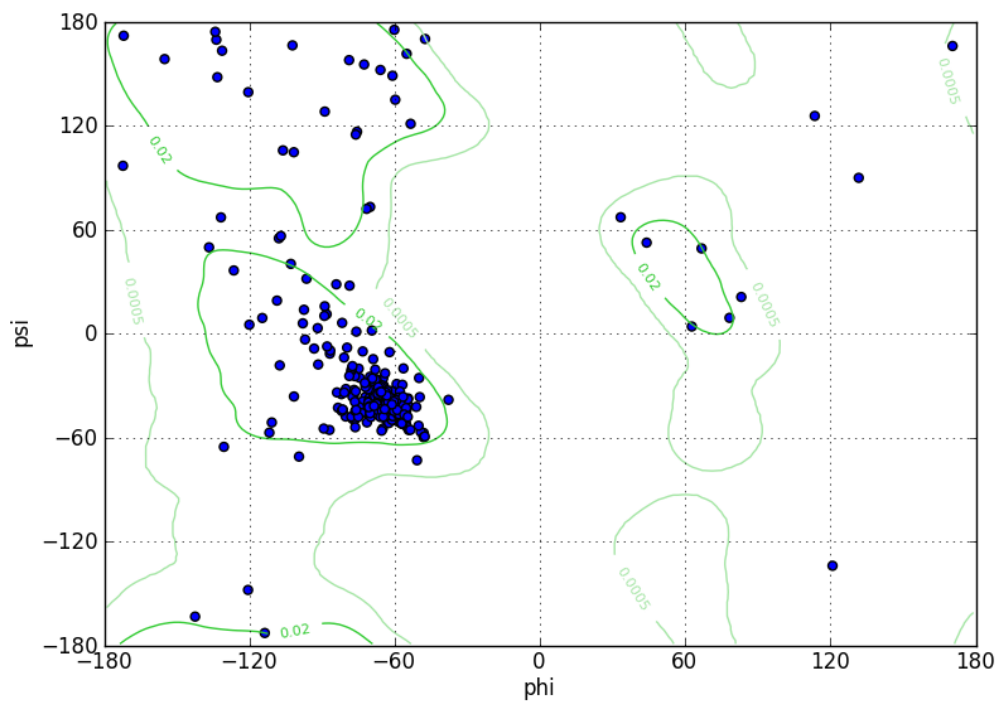
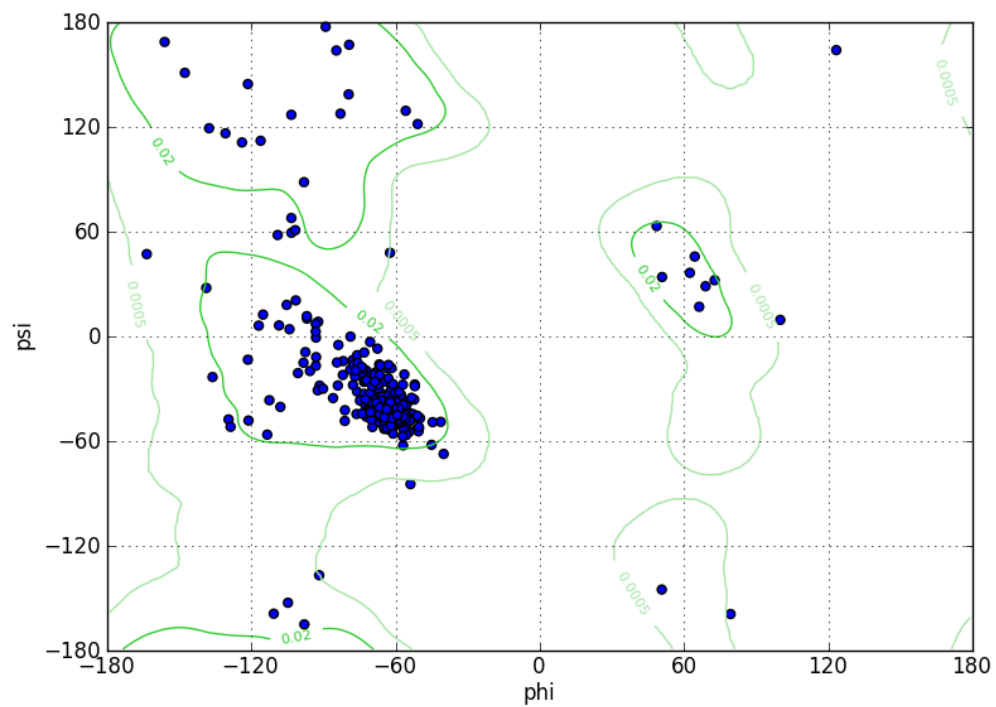


Figure S2. Ramachandran plot for MepA homology model



CAPÍTULO 5

5. CONSIDERAÇÕES FINAIS

5.1. DISCUSSÃO GERAL

Três derivados da dibenzalacetona (1*E*,4*E*)-1,5-di(tiofen-2-il)penta-1,4-dien-3-ona (DB tiofeno), (1*E*,4*E*)-1,5-bis(4-etoxifenil)penta-1,4-dien-3-ona (DB etoxi) e (1*E*,4*E*)-1,5-bis(4-clorofenil)penta-1,4-dien-3-ona (DB cloro) foram obtidos por dupla condensação de Claisen-Schmidt. Suas estruturas químicas foram determinadas por RMN e EM obtendo-se excelente correspondência com o esperado. Esses resultados confirmaram a estereoquímica tipo E das duplas ligações para os três compostos. A partir dos estudos de modelagem molecular utilizando cálculos quânticos, identificou-se 72, 132 e 90 modos normais vibração para DB tiofeno, DB etoxi e DB cloro, respectivamente. A partir da descrição de cada modo normal e posterior comparação com os modos vibracionais experimentais foi possível verificar que o perfil vibracional do DB tiofeno combina melhor com o confórmero do tipo *cis-trans* embora as diferenças com os outros tenham sido diminutas. Isto é reforçado quando comparamos o momento dipolo experimental com o momento dipolo teórico dessa conformação. No caso do DB etoxi e do DB cloro, não há outros confórmeros devido à rotação das ligações simples existentes entre os anéis aromáticos e os grupos enonas, todavia em função da descrição detalhada dos modos vibracionais desses compostos foi possível notar que o composto DB etoxi apresentava uma estrutura cristalina hidratada, porém não se sabe exatamente a proporção de moléculas de água por célula unitária do cristal. De todo modo, a completa atribuição dos modos de vibração desse três compostos é bastante relevante e até onde se sabe foi inédita.

O composto DB tiofeno mostrou ser uma molécula bastante relevante e com amplo espectro de atividade biológica. Ela exibiu alta redução da viabilidade celular de células tumorais de colon (HCT-116) em 85,24% e redução moderada da viabilidade em células tumorais de próstata (PC3) com 43,57% de redução. Ainda, apresentou pouca redução da viabilidade celular frente à linhagem de glioblastoma (SNB), em que alcançou o valor de 9,83%. Isto é expressivo uma vez que foi o único composto desse estudo que obteve alguma atividade nesse tipo agressivo de tumor cerebral. Esse composto também é um excelente candidato à inibidor de bomba de efluxo, inibiu o efluxo de EtBr em 29,3% contra a cepa SA1199B que superexpressa a bomba de efluxo NorA, e em 43,9% contra a cepa K2068 que superexpressa a bomba de efluxo MepA. Além disso, reduziu a CIM do norfloxacino contra SA1199B em quase 30%, e em 50% do ciprofloxacino contra K2068. Estudos de docking molecular revelaram que provavelmente essa inibição se dá por competição no mesmo sítio de ligação desses substratos nas bombas de efluxo. Continuando as potencialidades desse

composto, identificamos uma ação redutora da locomoção em modelos animais *zebrafish*. Atingiu-se uma redução da locomoção de 84,81%, 65,79% e 49,66% para doses de 4 mg/kg, 20 mg/kg e 40 mg/kg, respectivamente. Essa redução foi produzida devido ao efeito ansiolítico do DB tiofeno atuando em receptores GABA_A como mostrou o teste claro/escuro, sendo que na dose de 40 mg/kg obteve-se 78,27% de permanência na zona clara, semelhante estatisticamente à droga diazepam.

A atividade antiproliferativa ou citotóxica do composto DB cloro também foi bastante animadora, reduziu a viabilidade celular de HCT-116 em 85,03%, valor este considerado como um bom resultado. Mostrou-se menos ativa para a linhagem PC3, reduzindo a viabilidade celular em 30,74% e foi completamente inativa contra as células tumorais do tipo SNB. Inibiu o efluxo de EtBr em 37% tanto para SA1199B quanto para K2068. Estudos mostraram que o DB cloro ocupou essencialmente o mesmo sítio de ligação do EtBr nessas duas bombas de efluxo, configurando também uma provável inibição por competição. Contudo não foi capaz de reduzir a CIM dos antibióticos norfloxacino e ciprofloxacino, respectivamente, contra as duas cepas utilizadas. O DB cloro apresentou resultado ainda melhor que o DB tiofeno quanto a redução a atividade locomotora de *zebrafish*, 72,12%, 51,66% e 37,84% de redução para doses de 4 mg/kg, 20 mg/kg e 40 mg/kg, respectivamente. O teste claro/escuro revelou que o DB cloro também produziu aumento da permanência dos animais na zona clara indicando um potencial efeito ansiolítico. O resultado obtido na maior dose também foi estatisticamente semelhantes ao do diazepam. Tal como o DB tiofeno, o efeito ansiolítico do DB cloro ocorre por mecanismo de ação GABAérgica como revelou o teste de mecanismo de ação. Isso põe esses dois derivados da dibenzalacetona como potenciais substâncias com efeitos ansiolíticos.

Dentre os três compostos objetos desse estudo, o DB etoxi foi aquele que obteve resultados menos expressivos. Evidenciou baixa inibição do crescimento celular contra a linhagem HCT-116, exibindo 8,21% de redução de viabilidade, e 12,59% contra a linhagem PC3. Assim como o DB cloro, apresentou completa inatividade contra o glioblastoma (SNB). Inibiu o efluxo de EtBr mediado por NorA em 37%, porém mostrou-se inativa para o efluxo de EtBr mediado pela MepA. Controversamente reduziu a CIM do ciprofloxacino em K2068, porém acredita-se que essa redução não se deu pela inibição da bomba de efluxo MepA, provavelmente pode ser resultado de alguma alteração da permeabilidade da membrana celular. Esse composto não modificou significativamente a locomoção dos *zebrafish* em nenhuma das doses estudadas. A não alteração da locomoção dos animais pode sugerir uma ação antinociceptiva desse composto. Vale ressaltar que os três derivados da dibenzalacetona desse

estudo obtiveram $LC_{50} > 40$ mg/kg até 96 horas, portanto foram considerados seguros, e não demonstraram atividade intrínseca contra as cepas SA1199B e K2068.

Claramente observa-se uma ordem de desempenho entre os três derivados da dibenzalacetona estudados, em que o DB tiofeno e o DB cloro se apresentam como moléculas potencialmente candidatas a se tornarem fármacos. Isso provavelmente se dá devido aos anéis tiofeno no DB tiofeno, e do átomo de cloro substituinte na posição *para* dos anéis benzeno no DB cloro. Já é sabido que derivados de chalconas com anél heterocíclico e átomos de cloro substituintes demonstram atividade contra diversas linhagens de câncer. Já o grupo etoxi substituinte no DB etoxi, exerce um efeito doador de elétrons maior que o átomo de cloro, que possui grande eletronegatividade, portanto a diferença de desempenho do DB etoxi pode ser devido à disparidade no efeito mesomérico entre os dois grupos. Também podemos sugerir que o maior “comprimento” na molécula do DB etoxi, fruto dos seus substituintes, pode dificultar seu acesso aos sítios ativos. Testes *in silico* das três moléculas mostram que todas possuem solubilidade moderada, alta absorção gastrointestinal e que passaram no teste R05 de Lipinski, tendo apenas o DB cloro violado um dos quesitos e os outros dois compostos, nenhum. Todos eles apresentam uma alta biodisponibilidade.

5.2. CONCLUSÕES GERAIS

Os três derivados da dibenzalacetona DB tiofeno, DB etoxi e DB cloro são moléculas sintéticas que apresentam dois grupos enonas. Tiveram suas estruturas químicas caracterizadas e seus modos de vibração descritos e atribuídos pela primeira vez. O DB tiofeno e o DB cloro revelaram grande potencial em se tornarem fármacos pois possuem efeito citotóxico em células tumorais (HCT-116 e PC3) e demonstram efeito ansiolítico em sistema GABAérgico. São prováveis inibidores das bombas de efluxo NorA e MepA. Além disso, o DB tiofeno apresenta-se como provável candidato a adjuvante dos antibióticos norfloxacino e ciprofloxacino.

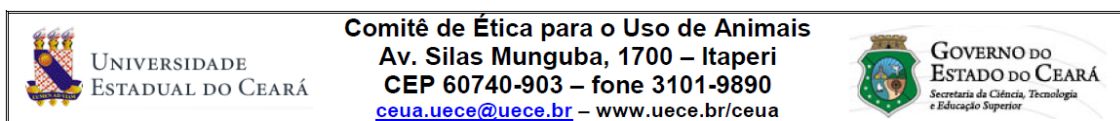
5.3. PERSPECTIVAS DE INVESTIGAÇÕES FUTURAS

Esse trabalho além de apresentar dois compostos com potencial ação citotóxica, ansiolítica e moduladora do efluxo, também traz algumas possibilidades de investigações futuras, tais como:

- Investigação do mecanismo de ação dos compostos DB tiofeno e DB cloro contra as células tumorais HCT-116 e PC3, bem como determinação da IC_{50} .
- Investigação de uma possível ação antinociceptiva do composto DB etoxi.

- Confirmação da inibição do efluxo de EtBr por ensaios fluorimétricos para corroborar com ensaios de microdiluição.
- Prosseguimento da investigação do efeito citotóxico e reversor da resistência bacteriana pelo DB tiofeno e DB cloro em modelos *in vivo*.
- Desenvolvimento de um modelo da relação quantitativa estrutura/atividade (QSAR) para prever atividades biológicas das chalconas e derivados.

ANEXO A – Certificado do Comitê de Ética para Uso de Animais da Universidade Estadual do Ceará (CEUA – UECE).



CERTIFICADO

Certificamos que o **Projeto de Pesquisa** intitulado “**Uso do zebrafish (*Danio rerio*) como biosensor em testes toxicológicos**” registrado sob o número **3344801/2017**, tendo como pesquisador principal **Francisco Ernani Alves Magalhães**, está de acordo com os Princípios Éticos de Experimentação Animal, adotados pelo **Comitê de Ética para o Uso de Animais da Universidade Estadual do Ceará** (CEUA – UECE), tendo sido aprovado em 09 de junho de 2017. Este certificado expira-se em 16 de dezembro de 2020.

CERTIFICATE

We hereby certify that the Project entitled “**Uso do zebrafish (*Danio rerio*) como biosensor em testes toxicológicos**” registered with the protocol **3344801/2017**, under the supervision of **Francisco Ernani Alves Magalhães** is in agreement with Ethical Principles in Animal Experimentation, adopted by the **Ethics Committee in Animal Experimentation of Ceará State University** (CEUA – UECE). This certificate will expire in december 16, 2020.

RESUMO

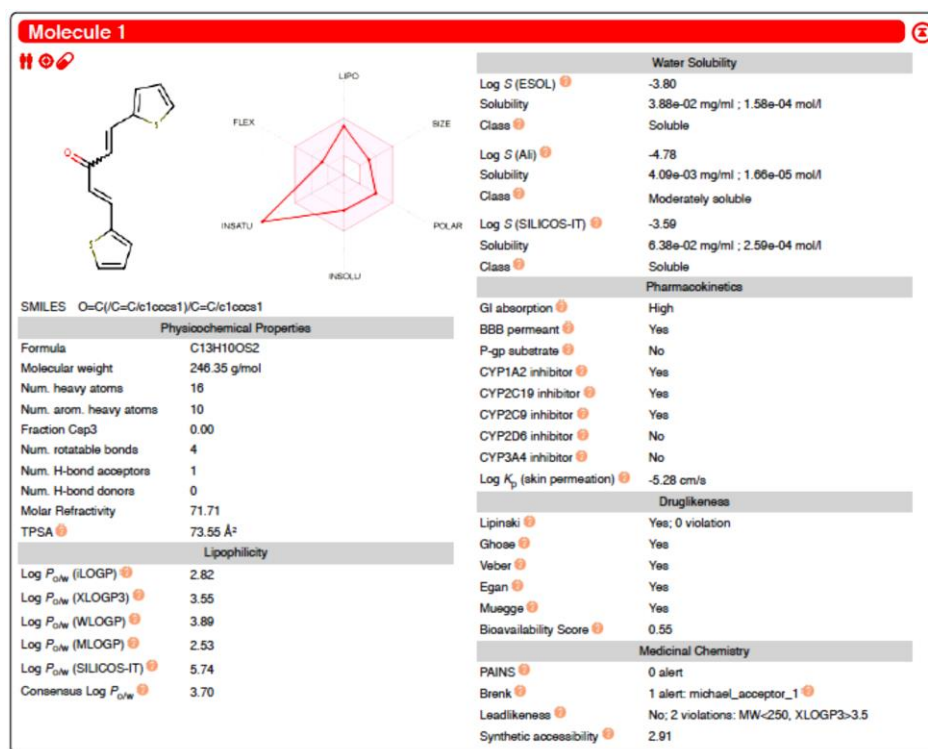
Vigência do projeto	01/08/2017 a 31/07/2020
Espécie/linhagem	Zebrafish (<i>Danio rerio</i>)
Número de animais	1000
Peso/idade	0,3 a 0,6 gr/ três meses
Sexo	500 Machos e 500 Femeas
Origem	Obtidos de um fornecedor comercial de Fortaleza

Fortaleza, 25 de julho de 2017.

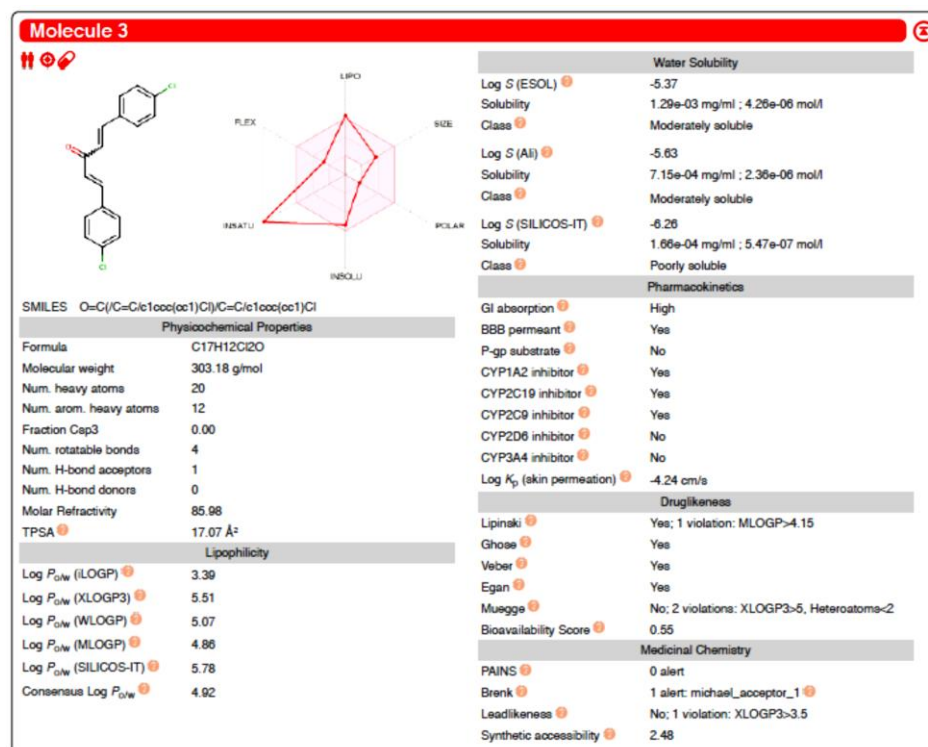
Erivalda Farias de Aragão

Maria Erivalda Farias de Aragão
Presidente CEUA-UECE

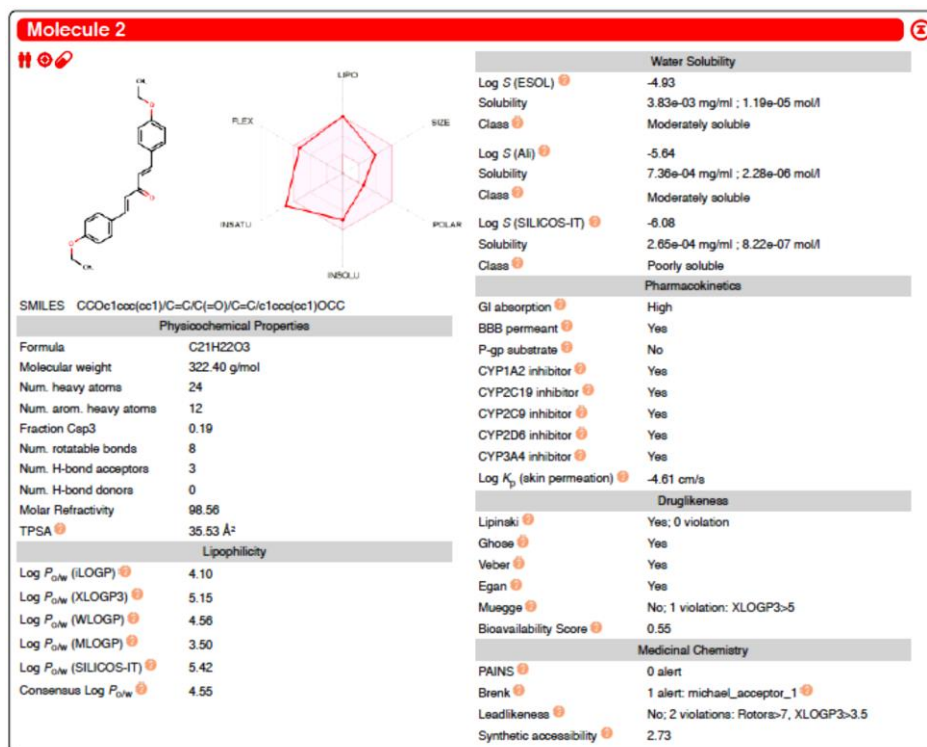
ANEXO B – Perfil físico-químico, lipofílico, farmacocinético e de semelhança com drogas do DB tiofeno realizado *in silico* no SwissADME.



ANEXO C – Perfil físico-químico, lipofílico, farmacocinético e de semelhança com drogas do DB cloro realizado *in silico* no SwissADME.



ANEXO D – Perfil físico-químico, lipofílico, farmacocinético e de semelhança com drogas do DB etoxi realizado *in silico* no SwissADME.




APÊNDICE I – PRODUÇÃO ACADÊMICA (2018 - 2021)

Artigos publicados:

- 2021


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Full Spectroscopic Characterization and Cytotoxicity Activity of Synthetic Dibenzalacetone Derivatives.



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Keywords:
 Dibenzalacetone derivatives
 Cytotoxicity
 DFT
 Spectroscopy, Cancer cells

ABSTRACT

Dibenzalacetone derivatives are organic compounds formed by two α, α' active sites that provide a large relocation of π -electrons. They have great polarizability that make them excellent chromophore and draw attention for their biological properties, mainly for their activity against nasopharyngeal, oral, colon, prostate and cervical cancer. Two dibenzalacetone derivatives, (1E,4E)-1,5-bis(4-ethoxyphenyl)pent-1,4-dien-3-one and (1E,4E)-1,5-bis(4-chlorophenyl)pent-1,4-dien-3-one, were synthesized. Spectroscopic characterizations as well as vibrational assignments were predicted using density Functional Theory (DFT) calculations with B3LYP exchange-correlation functional. Cytotoxicity assays of the synthesized compounds were performed against HCT-116, SNB and PC3 cells, showing promising results against cancer cells HCT-116.

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Synthesis, spectroscopic characterization and antibacterial evaluation by chalcones derived of acetophenone isolated from *Croton anisodontus* Müll.Arg.



P.T. da Silva^a, J. da Cunha Xavier^a, T.S. Freitas^a, M.M. Oliveira^a, H.D.M. Coutinho^a, A.L.A.B. Leal^a, H.M. Barreto^a, P.N. Bandeira^b, C.E.S. Nogueira^{a,c}, D.M. Sena Jr.^a, F.W.Q. Almeida-Neto^e, E.S. Marinho^f, H.S. Santos^{a,b}, A.M.R. Teixeira^{a,c,g}

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 NMR
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ABSTRACT

Chalcones and their derivatives have been widely studied at research institutions and industries, in order to verify their biological properties against various diseases. In this work, it was carry out the synthesis of chalcones (E)-3-(turán-2-yl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl) prop-2-en-1-one (HYTFURFURAL) and (E)-1-(2-hydroxy-3,4-dimethoxyphenyl)-3-(thiophen-2-yl) prop-2-en-1-one (HYTHIOPHENE) by Claisen-Schmidt aldolic condensation reaction. The molecular structures of these new heteroaryl chalcones have been elucidated and characterized by ¹H and ¹³C Nuclear Magnetic Resonance, Fourier transform Raman (FT-Raman), Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR), and Ultraviolet-Visible (UV-Vis) spectroscopy. Calculations using the Functional Density Theory (DFT) were performed to predict the vibrational wavenumbers and to obtain the HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) molecular orbitals, and starting from them, the quantum descriptors: vertical ionization energy (I), vertical electron affinity (A), chemical potential (μ), electronegativity (χ), global hardness (η), and electrophilicity index (ω). Antimicrobial and modulatory antibiotic activities of these chalcones were also investigated. The mechanism of the NorA and MepA efflux pump inhibition was also studied by means of molecular docking. The HYTFURFURAL and HYTHIOPHENE chalcones did not present intrinsic activity against the tested bacterial strains; however, they were able to potentiate the activity of norfloxacin against the SA1199B (NorA) strain, as well as the activity of ciprofloxacin against the K2068 (MepA) strain. These results indicate that the HYTFURFURAL and HYTHIOPHENE chalcones are promising compounds as adjuvants to the norfloxacin and ciprofloxacin antibiotics in the treatment of infections caused by *S. aureus*.

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Synthesis of a new quinine dimer biocatalysed by the coconut water

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ABSTRACT

The obtaining of bis-quinine, a novel alkaloid dimer, has been successfully achieved starting from quinine and the raw coconut juice (*Cocos nucifera*) as biocatalyst dimerization-like reaction, in mild conditions, with a mass yield of 64.7% in 72 h. The structural elucidation was made based on the spectral data, mainly by a high-field NMR and a mass spectrometry. In a second step, theoretical calculations were performed, an optimised energy structure of the new compound was obtained, the energy gap of the boundary orbitals (HOMO and LUMO) as well as the chemical reactivity descriptors were estimated.

GRAPHICAL ABSTRACT

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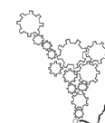
KEYWORDS

Quinine; biotransformations;
 crude enzyme;
 coconut water



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 ENGENHARIA DE PRODUÇÃO



TRIZ AND TPM METHODOLOGIES APPLIED TO MAINTENANCE PROBLEMS METODOLOGIAS TRIZ E TPM NA RESOLUÇÃO DE PROBLEMAS DE MANUTENÇÃO

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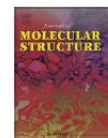
• 2020

Journal of Molecular Structure 1215 (2020) 128291



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Spectroscopic characterization and efflux pump modulation of a thiophene curcumin derivative



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ABSTRACT

Curcumin, along with its derivatives, form a large class of natural and synthetic compounds with notable biological activity. However, their highly reactive β -diketone moiety renders this type of compounds unstable at pH above 6.5. The substitution of this group for a mono-carbonyl solves this problem, while improving antibacterial and anti-inflammatory activities. A thiophene curcuminoid, (1E,4E)-1,5-Di(thiophen-2-yl)penta-1,4-dien-3-one (DB Thiophene), has been synthesised and its molecular and spectroscopic characterization is reported, as well as a complete vibrational assignment. An efflux pump inhibition assay determined that DB Thiophene exhibits a remarkable NorA and MepA efflux pump inhibition activity. Molecular docking studies were carried out in order to understand this inhibition mechanism.

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Industrial Crops and Products

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The use of new hydrogel microcapsules in coconut juice as biocatalyst system for the reaction of quinine

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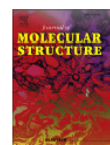
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Crystal structure, FT-Raman and FTIR spectra and DFT calculations of chalcone (2E)-1-(4-aminophenyl)-3-(furan-2-yl)prop-2-en-1-one monohydrate



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Chalcone monohydrate

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ABSTRACT

In this work we present a study of the structural and vibrational properties of the chalcone (2E)-1-(4-aminophenyl)-3-(furan-2-yl)prop-2-en-1-one monohydrate, C₁₃H₁₁O₂N·H₂O, a strong candidate for fluorescent probes, luminescent materials and optoelectronic devices. The crystalline structure of this chalcone monohydrate was determined at 273 K using single-crystal X-ray diffraction. The Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) and Fourier Transform-Raman (FT-Raman) spectra of the crystal were recorded at room temperature in the regions 600–4000 cm⁻¹ and 40–4000 cm⁻¹, respectively. Vibrational wavenumbers were predicted using Density Functional Theory (DFT) calculations with B3LYP exchange-correlation functional with the B3LYP functional and 6-31G(d) basis set. A comparison with experimental spectra using a hydrated model allowed us to assign all of the normal modes of this crystal. Additionally, we report the UV-Vis spectra and the Hirshfeld surface analysis of the AFPO crystal.

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Structural characterization, antibacterial activity and NorA efflux pump inhibition of flavonoid fisetinidol



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Staphylococcus aureus

ABSTRACT

Flavonoids belong to the extensive group of polyphenols and have several biological activities such as anti-inflammatory, anti-ulcer, anticancer, antiviral, antibacterial, and antispasmodic. These compounds can thus be explored as a tool against multidrug resistant bacteria, as nosocomial infections now constitute a major public health problem worldwide. We have carried out a structural characterization by nuclear magnetic resonance and FT-IR of fisetinidol, a flavonoid isolated from *B. pentandra* stems. In addition, this flavonoid was tested for antibacterial and modulatory activities, as well as inhibition of the NorA efflux pump. Fisetinidol has no intrinsic antibacterial activity. However, there is synergism in association with norfloxacin, with a two-fold decrease in MIC. When compared to chlorpromazine, a standard efflux pump inhibitor, a two-fold decrease in MIC was also verified when used against *S. aureus* SA-1199B, which over expresses the NorA gene encoding the NorA efflux protein. As such, it could become an alternative source to reverse the bacterial resistance if administered in association with antibiotics.

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MODELAGEM DA PREVISÃO DE VENDAS DE UMA UNIDADE AGRÍCOLA PRODUTORA DE BANANAS EM MISSÃO VELHA (CE)

BANANA SALES FORECASTING MODEL OF AN AGRICULTURAL UNIT, IN CEARÁ, BRAZIL

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Resumo: A importância de planejar as necessidades futuras surge das exigências dos processos decisórios, uma vez que todos os negócios estão suscetíveis a imprevistos e necessitam ter flexibilidade. No processo de realização do planejamento estratégico é de grande importância incluir a previsão de demanda para dimensionar a capacidade produtiva. Um estudo de previsão demanda foi desenvolvido numa empresa de 330 hectares que cultiva bananas prata e nanica, localizada na cidade de Missão Velha, estado do Ceará. Este trabalho consiste numa pesquisa explicativa com abordagem quantitativa, onde inicialmente foram levantados os dados históricos de vendas e em seguida verificados seus comportamentos a partir de testes estatísticos. Foi escolhida a banana prata pela sua representatividade para a empresa. Identificou-se comportamentos de tendência e sazonalidade. Após essa constatação aplicou-se os métodos ARIMA e Holt-Winters a fim de identificar o método que melhor se adequava, com o intuito de evitar desequilíbrios entre a equipe de produção e comercial. O método que apresentou os melhores resultados foi o ARIMA (1,1,1), tendo como critério para a validação o MAD, MAPE e MSD.

Palavras-chave: Previsão de demanda. Planejamento e controle da produção. Bananicultura.

- 2019



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Tecnologia ambiental

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Artigo Original

Enzymatic Biocatalyst using enzymes from Pineapple (*Ananas comosus*) Peel Immobilized in Hydrogel Beads.

Biocatalisador enzimático usando enzimas da casca do abacaxi (*Ananas comosus*) imobilizado em esferas de hidrogel.

Aluísio Marques da Fonseca¹, Regilany Paulo Colares², Mauro Macedo de Oliveira³,
Maria Cristiane Martins de Souza⁴, Rodolpho Ramiton de Castro Monteiro⁵, Rinaldo dos Santos
Araújo⁶, Aiala Vieira Amorim⁷, José Cleiton Sousa dos Santos⁸, Carlos Alvarado Alcócer⁹,
Olienaide Ribeiro de Oliveira Pinto⁹

Abstract

The enzymatic extract from pineapple peels, considering the time factor, low cost and ease of preparation of the reaction system, makes the use of this plant material scrap an economical alternative for some reactions of synthetic interest. Therefore, this work aimed to prepare pineapple peel enzymatic extract containing bromelain, with and without mechanical grinding for a period of up to 9-days of extraction, and then immobilizing those extracts on hydrogel beads for their application as a biocatalyst to produce energy interest esters. The best protein content obtained was 1.95 mg mL⁻¹ for 6-days with mechanical grinding, as for the hydrolysis of p-NPP (p-nitrophenyl palmitate), 0.0125 U.mL⁻¹ for 1-day extraction with trituration. The best index esterification activity achieved for the lauric acid as a substrate was 1.8 U mL⁻¹ at 1-day extraction without grinding. For the hydrogel beads immobilization, a maximum protein yield of 28.8% was obtained with the 1-day extract and mechanical grinding, and in terms of p-NPP activity, a yield of 40.6% for the immobilized with 9-day extract without mechanical grinding. The immobilized and dehydrated beads with 1-day extract without grinding took the best esterification activity, 7.2 U g⁻¹ of biocatalyst. The best conversion performance in the biocatalysis of fatty esters was by esterification of the dodecanoate n-propyl, with 95.1% for a period of 48 hours of reaction. For the transesterification reaction, the methyl oleate yield reached 47.3% after 120 hours of reaction.

Keywords: Enzymatic extract, Bromelain, Pineapple, Fatty acids, Transesterification