SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIP STUDIES OF 1,4-NAPHTHOQUINONE DERIVATIVES AS POTENTIAL ANTI-TRYPANOSOMAL AGENTS

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ABSTRACT

Human African Trypanosomiasis (HAT) is an infectious, vector-borne protozoal disease which is amongst the so-called neglected diseases. In 2000, at a summit of the United Nations, eight Millennium Development Goals (MDGs) were set, to be achieved by 2015. MDG 6 states "to combat HIV/AIDS, malaria & other diseases". With just under 2 years to go before the end of 2015, HAT is still thriving in developing countries. The drugs currently used for the treatment of HAT are in short supply, have severe side effects and those used to treat late stages of the disease are very difficult to administer. The aforementioned challenges call for research into this neglected disease in order to develop new, safe and easy-to-use medicines.

Naphthoquinones are a class of compounds shown to possess anti-parasitic activity, amongst a variety of other biological activities, and therefore this pharmacophore was selected for this study. The purpose of this study was to synthesise derivatives of 2,3-dichloro-1,4-naphthoquinone to be tested for anti-trypanosomal activity and thereafter conduct structure-activity relationship studies. A series of reactions were carried out using thiophenol, phenol and aniline nucleophiles to synthesise thioether (-S-), ether (-O-) and amino (-NH-) derivatives of 2,3-dichloro-1,4-naphthoquinone with various halogen or methyl substituents. Purification of the products was carried out by recrystallisation. Nuclear magnetic resonance (NMR), infra-red (IR) and high pressure liquid chromatography coupled to an electro-spray ionisation mass spectrometer (HPLC-ESI-MS) were the analytical methods used for structural confirmation of the products.

There were eighteen 1,4-naphthoquinone derivatives that were successfully synthesised using ethanolic solutions. Unfortunately, attempts to synthesise 1,4-naphthoquinones in reactions involving 2-(trifluoro-methyl)aniline and 2-isopropyl-5-methylphenol were unsuccessful, presumably due to steric hindrance by the bulky ortho-substituents.

Although the aims of the synthetic procedures were to obtain both mono- and disubstituted products by nucleophilic displacement of the chlorine atom(s) of 2,3-dichloro-1,4-naphthoquinone, only monosubstituted products were obtained from substitution with aniline and phenol nucleophiles. Thiol nucleophiles, however, selectively yielded disubstituted products only.

Synthesised naphthoquinone derivatives were tested against *Trypanosoma brucei* and calculation of the EC_{50} values from the obtained dose-response curves was carried out using the four parametric equation. All the 1,4-naphthoquinones showed a degree of potency, except compounds **1b**, **3c** and **3e**, which had little or lack of potency.

Structure-activity relationship studies (SARs and QSARs) were carried out to determine which structural features or functional group substituents of the naphthoquinone derivatives contribute or take away from the desired anti-trypanosomal activity.

It was found that compounds with the best *in vitro* anti-trypanosomal potencies in the series of analogous 1,4-naphthoquinone derivatives had EC_{50} values in the range 2.137 to 2.884 µM. The most potent compound in the series was 2-chloro-3-(4-(trifluoromethyl)phenylamino)-1,4-naphthoquinone **1e**; but it was 142-fold less potent than the reference standard of melarsoprol.

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To my mother Clara, I dedicate this thesis to you.

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^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{o}C	Degrees Celsius
δ	Chemical Shift
AIDS	Acquired Immunodeficiency Syndrome
CATT	Card Agglutination Test for Trypanosomiasis
$CDCl_3$	Deuterated chloroform
COSY	^{1}H-^{1}H Homonuclear Correlation Spectroscopy
d	Doublet
dd	Doublet of doublets
DEPT	Distortionless Enhancement by Polarisation Transfer
DMSO	Dimethyl sulphoxide
dt	Doublet of triplets
EC_{50}	50% of the maximal effective concentration
ESI-MS	Electro-spray Ionisation Mass Spectroscopy
EtOH	Ethanol
Et_3N	Triethylamime
g	Gram
HAT	Human African Trypanosomiasis
HPLC	High-Pressure Liquid Chromatography
HIV	Human Immunodeficiency Virus
HSQC	Heteronuclear Single Quantum Correlation
Hz	Hertz
IR	Infra-red spectroscopy
J	Spin-Spin Coupling Constant
m	Multiplet
mg	Milligram
MHz	Megahertz
ml	Millilitre

mmol	Millimolar
µM	Micromolar
MDG	Millennium Development Goals
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NECT	Nifurtimox Eflornithine Combination Therapy
NMR	Nuclear Magnetic Resonance
ppm	Parts per million
pEC_{50}	Negative logarithm of EC_{50}
QSAR	Quantitative Structure-Activity Relationship
R_f	Retardation factor
SAR	Structure-Activity Relationship
s	Singlet
t	Triplet
T. b gambiense	*Trypanosoma brucei gambiense*
T. b rhodesiense	*Trypanosoma brucei rhodesiense*
td	Triplet of doublets
TLC	Thin Layer Chromatography
UV	Ultra Violet
WHO	World Health Organization

CHAPTER I: LITERATURE REVIEW

1.1 INTRODUCTION

Human African Trypanosomiasis (HAT) - also known by its common name "sleeping sickness"[*], is a vector-borne parasitic disease that is reported in 36 sub-Saharan African countries.[1] It is amongst the so-called neglected tropical diseases. The causative organism of HAT is a protozoan parasite of the taxonomic order *Kinetoplastida* of the *Trypanosoma brucei* species. The two sub-species that are common in sub-Saharan Africa are *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, with the former being accountable for 90% of the reported cases of the disease.[2,3] In west and central Africa, *Trypanosoma brucei gambiense* is the sub-species that causes the most prevalent form of the disease, whilst the *Trypanosoma brucei rhodesiense* sub-species is prevalent in the eastern and southern parts of Africa.[1] Trypanosomes, parasites that cause HAT, are transmitted through a vector, the tsetse fly, that belongs to the *Glossina* genus.[4]

Sleeping sickness, which may lead to fatalities if untreated, occurs in two clinical stages. Stage 1, which is also known as the hemolymphatic stage, occurs early on and it involves the presence of trypanosomes in the haemolymphatic system. Non-specific symptoms such as malaise, fever, headaches and peripheral oedema are typical of stage 1. Stage 2, occurs later on and is also called the neurological or meningoencaphalic stage. It is depicted by neurological symptoms such as behavioural changes, convulsions and sleeping disorders. It is from the sleeping disorders that HAT derived its common name of sleeping sickness. It is only after the trypanosomes cross the blood-brain barrier into the brain that stage 2 occurs. At this stage, if the patient does not receive any treatment, the disease progresses and death may result. The treatment of HAT is prescribed based on the causative sub-species of the parasite, *T.b gambiense* or *T.b rhodesiense*, as well as the stage of the disease - either stage 1 or 2.[1] In order to prescribe correctly, diagnostic tests to distinguish between the two types of parasites and the two forms of the disease are crucial.

Availability of HAT medicines declined in the 1990s as the pharmaceutical industry had stopped production due to non-profitability, amongst other reasons.[5] The World Health Organization came to the rescue by establishing a public-private partnership with Sanofi-

[*]HAT and sleeping sickness will be used interchangeably

Aventis (now Aventis) and Bayer in the year 2000 which was renewed in the years to follow. This partnership saw the donation and distribution of medicines in countries where HAT is endemic. Efforts made by the World Health Organization (WHO) to curb waves of HAT epidemics might be paying off, as the organisation stated in its 2009 report that numbers of new cases in that year had dropped to below 10 000 for the first time in 50 years. Non-governmental organisations such as Médecins Sans Frontières (Doctors Without Borders) have also been playing a pivotal role in the distribution of HAT medicines. [5,6]

1.2 EPIDEMIOLOGY OF SLEEPING SICKNESS

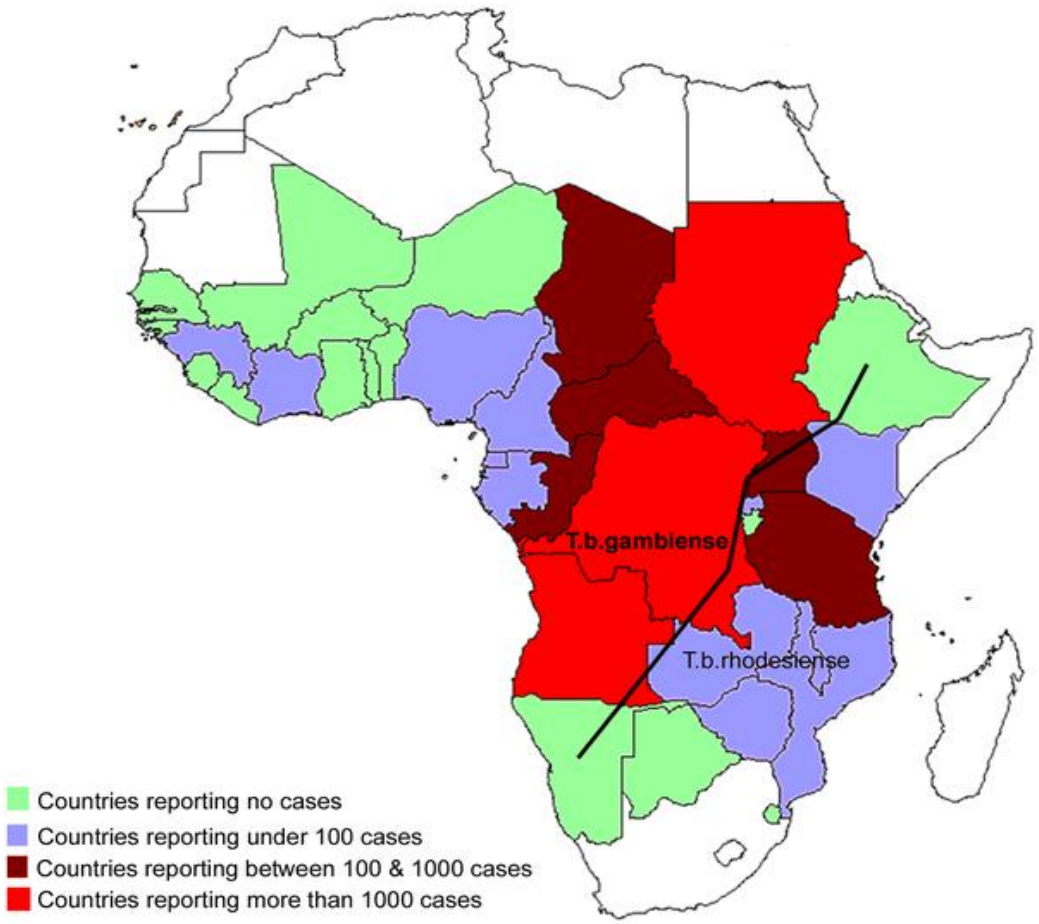


Figure 1.1: A map of Africa highlighting the regions affected by sleeping sickness. The black line demarcates the regions where the *T.b gambiense* sub-specie is prevalent (west and central Africa) from the regions of *T.b rhodesiense* (eastern and southern Africa). Countries with no colour allocation (white) are not considered endemic for the disease.

Image Credit: Adapted from Simarro *et al.*[7]
http://www.plosmedicine.org/article/info:doi/10.1371/journal.pmed.0050055

Sleeping sickness is considered to be endemic in 36 sub-Saharan countries but reports in 2011 stated that six of these (Botswana, Burundi, Ethiopia, Gambia, Namibia and Niger) had not reported any cases for two decades. Between 2000 and 2009, a majority of the countries considered endemic for the disease received support from either WHO alone or from both WHO and non-governmental organisations.[8]

In addition to ensuring the availability of these medicines in the endemic countries, WHO also provides support in terms of assessing the epidemiological status of HAT or by establishing control and surveillance programmes in line with the 1997 World Health Assembly elimination resolution.[6-9]

In its 2009 report, WHO stated that the number of new cases that year had dropped to below 10 000 for the first time in 50 years, showing that efforts made by the organisation in curbing the waves of HAT epidemics might be paying off.[6,8]

Land-mapping and tsetse fly (vector) distribution is similar to the disease mapping shown in the African map in **Figure 1.1** above.[10] Tsetse flies are inhabitants of mainly the mid-continent of Africa (south of the Sahara and north of the Kalahari) and are needed for the transmission of the disease. HAT is thus a health burden in regions where tsetse flies are found.[4,9]

1.3 LIFECYCLE OF TRYPANOSOMES

There are typically three parties that harbour the trypanosomes which cause both types of sleeping sickness namely tsetse flies, humans and wild/domestic animals. Tsetse flies ingest trypanosomes by taking a blood meal from an infected host and become infective about three weeks later. During these three weeks, the ingested parasites go through a series of morphological and biochemical changes in the midgut of the tsetse fly. Modified parasites then move from the midgut to the salivary glands where they become infective metacyclic trypanosomes transmitted through another blood meal. The trypanosomes then enter the host's bloodstream, lymph nodes and if untreated they can end up in the central nervous system. The parasites also undergo transformation in the human body just like in the tsetse fly (**Figure 1.2**). One of the most crucial components of the disease is contact between a human and a tsetse fly. Once a fly becomes infected, it remains a vector for the rest of its life posing as one of the biggest challenges in disease control.[11]

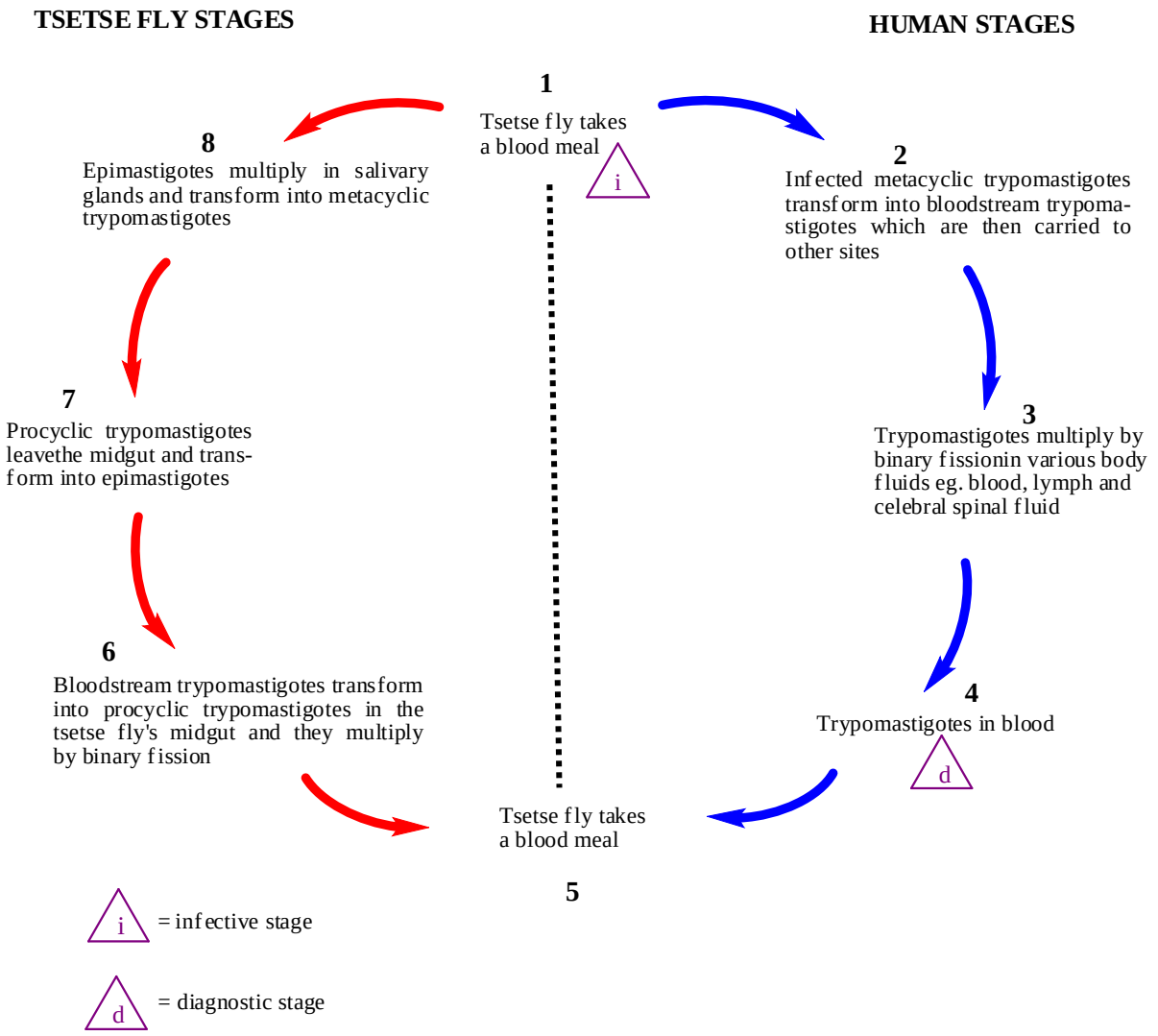
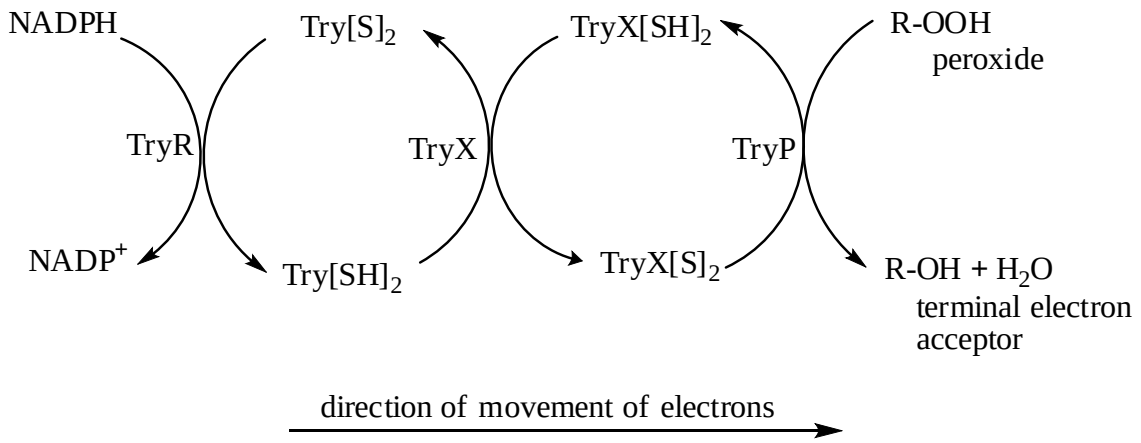


Figure 1.2: The lifecycle of *Trypanosoma brucei* in the human and tsetse fly. Adapted and re-drawn from Centers for Disease Control and Prevention.[12]

1.4 BIOCHEMISTRY OF TRYPANOSOMES

Trypanosomes and other protozoan parasites have been shown to possess a unique form of glutathione called trypanothione. Trypanothione consists of two glutathione molecules joined by spermidine, a polyamine linker. Trypanothione is the parasite equivalent of gluthathione and is essential for the survival of these parasites. There are enzymes that rely on trypanothione, known as trypanothione-dependent enzymes, and these include reductase, peroxidase and transferase enzymes (**Scheme 1.1**). Trypanothione reductase is an NADPH-dependent flavoenzyme which assists in defence against oxidative stress by upholding sufficient levels of trypanothione, which is responsible for the reduction of hydroperoxides. Gluthathione reductase is the human equivalent and it plays a similar role against oxidative stress. Variation between these two systems and differences in their enzyme active sites has been explored as a possible drug target which could offer selective toxicity. The end result of the inhibition of trypanothione reductase would be a reduction in trypanothione levels and thus oxidative stress. New agents that selectively inhibit trypanothione reductase enzyme with less toxicity would be advantageous.[13,14]



Scheme 1.1: NADPH-dependent redox cycle. TryR = trypanothione reductase enzyme, $Try[SH]_2$ = trypanothione, TryX = tryparedoxin enzyme, TryP = tryparedoxin peroxide enzyme, R-OOH = alkyl hydroperoxide. Adapted and re-drawn from Flohé *et al.*[14]

1.5 PREVENTION AND CONTROL OF SLEEPING SICKNESS

Efforts to develop a vaccine for sleeping sickness have been unsuccessful mainly due to the complexity of the biology of the trypanosomes where an antigen variation mechanism has hindered the development of vaccines. There are no available drugs for prophylaxis against sleeping sickness.[2,15]

Vector control programmes have long been thought to offer great hope in the curbing of sleeping sickness. Tsetse flies are ubiquitous, making their eradication a challenge. However, measures have been put in place to control this vector and efforts have been made to try to break the transmission cycle.[2] Various approaches explored in trying to control the tsetse flies include insecticide spraying, which has been successful but gives rise to ecological considerations that hamper their use. The use of tsetse traps impregnated with attractants has also been explored.[2] Another means of vector control is releasing sterile male tsetse flies in a bid to promote unproductive mating with females. This method was effective in eradicating *Glossina austeni* from a small island off the coast of Tanzania.[4]

1.6 DIAGNOSIS AND STAGING OF SLEEPING SICKNESS

The simplest means of parasite detection in blood is the use of a finger-prick test but this is the least sensitive method, with a detection limit of 10 000 parasites per millilitre. Diagnosis of sleeping sickness depends on identifying *T.b gambiense* or *T.b rhodesiense* trypanosomes and this clinical evidence of an infection still relies on visualisation of the parasites in blood, lymph and cerebrospinal fluid (CSF). Such evidence is provided by parasitological tests as well as light microscopy examinations. Concentration of parasites can be done to increase the sensitivity of the tests. Techniques that are used to do this include haematocrit centrifugation (HCT) with fluorescent detection of the parasites and the mini anion exchange centrifugation technique (mAECT). The mAECT allows for separation of the parasites from red blood cells before centrifugation is carried out. A major disadvantage of microscopy is that it does not provide information of whether the infection is caused by either the *T.b gambiense* or *T.b rhodesiense* trypanosomes. Discrimination between the two types of trypanosomes is critical as the effective selection of treatment depends on the causative sub-species. Therefore, once an infection with trypanosomes has been confirmed by microscopy, the next step is to carry out serological tests to discriminate between the two sub-species. The most practical

technique used to detect humoral response in blood, lymph and CSF is the card agglutination test for trypanosomiasis (CATT). CATT is capable of detecting an infection with *T.b gambiense* trypanosomes on the basis of a variable surface antigen type LiTat 1.3. There are short-comings with CATT; these include false positives and negatives, inability to indicate cure as the antibodies persist for long periods after treatment, it requires patient follow-ups and it only detects antibodies against *T.b gambiense* and not the *T.b rhodesiense* sub-species.[7,16]

Both *T.b gambiense* and *T.b rhodesiense* infections can progress from stage 1 (haemolymphatic) to stage 2 (neurological) and again the choice of treatment is dependent on the causative sub-species as well as the stage of the disease.[11] It is therefore critical that disease staging is carried out soon after parasite detection and identification. Patients are staged upon examination of their CSF, requiring a lumbar puncture on the spine to collect a sample of the CSF. A white cell count of the CSF of less than 5 per microlitre and the absence of trypanosomes in the CSF is indicative of stage 1 HAT, whilst presence of trypanosomes in CSF and a white cell count of greater than 20 per microlitre are indicative of stage 2 HAT. [11,16]

Although the diagnosis and staging of HAT is a complex process that requires skilled personnel, it is very important for treatment success as the medicines are specific for a causative sub-species and disease staging. There is great need for new drugs that are effective against both *T.b gambiense* and *T.b rhodesiense* infections as well as drugs that are capable of treating both stage 1 and 2 HAT. The late stage of the disease requires drugs that can cross through the blood-brain barrier to attain effective concentrations in the brain. Such new drugs will enable us to do away with the painful, risky lumbar punctures for collecting CSF samples and also to cut down on the costs incurred during diagnosis.[1,4,17]

1.7 CURRENT TREATMENT OR THERAPIES

To date, there are only four drugs registered for the treatment of HAT, namely suramin, pentamidine, melarsoprol and eflornithine.[18] The latter has also been registered as a combination therapy with a drug called nifurtimox which is used to treat another trypanosomal disease called Chagas disease.[19]

1.7.1 Suramin

Suramin (shown in **Figure 1.3**) is a poly-anionic sulphonated napthylamine drug synthesised in 1916 and introduced in 1922.[3,20] Suramin is still the drug of choice, nearly a century later, for the treatment of stage 1 of the *T. b. rhodesiense* form of the disease.[20] The drug's highly ionic nature does not enable it to cross the blood-brain barrier into the central nervous system (CNS) and it is therefore only useful for stage 1 of the disease which does not involve the CNS.[3,20]

Suramin is a highly water soluble drug that is administered intravenously. Upon administration, the immediate life-threatening side effects a patient may experience include nausea, vomiting and shock. Delayed severe side effects of suramin include renal malfunction, agranulocytosis, jaundice and exfoliative dermatitis. Suramin is highly protein bound in serum and this is responsible for its long half-life of 90 days; the drug also has a slow onset of trypanocidal action.[20] The mode of action of suramin is unknown.[3,20] Suramin is manufactured by Bayer and it is marketed under the trade name Germanin.[20]

1.7.2 Pentamidine

Pentamidine (shown in **Figure 1.3**) is an aromatic diamine compound synthesised in 1937 and introduced in the 1940s. It is indicated for the treatment of stage 1 of the *T.b. gambiense* form of sleeping sickness. Its other indications include antimony-resistant leishmaniasis and *Pneumocystis carinii* infections in patients that are non-tolerant to trimethoprim-sulfamethoxazole (co-trimoxazole). Pentamidine is administered parenterally owing to its protonation at physiological pH, which results in poor bioavailability when administered orally. The intramuscular route of administration is preferred to the intravenous as the latter route has been reported to cause severe hypotensive reactions. Serious side effects of pentamidine include liver, pancreas and kidney damage. The drug's mode of action is unclear, although several hypotheses have been put forward; including the inhibition of a plasma-membrane Ca^{2+} ATPase in *Trypanosoma brucei*. Pentamidine is manufactured by Aventis and it is marketed under the trade name Lomidine.[20]

1.7.3 Melarsoprol

Melarsoprol (shown in **Figure 1.3**) is a melaminophenyl-based organic arsenical compound[3] that was the drug of choice for the treatment of stage 2 of either the *T.b. gambiense* form or the *T. b. rhodesiense* form of the disease. Melarsoprol, introduced in 1949, is manufactured by Aventis and sold under the trade names Mel B and Arsobal.[20]

Melarsoprol is administered intravenously over a period of 10 days, thus requiring hospitalisation and medical supervision for the entire treatment duration.[3] The drug is completely insoluble in water therefore; it is dissolved in an alcohol (propylene glycol) to facilitate intravenous administration. However, propylene glycol is extremely irritating to the tissue and causes thrombophlebitis at the site of injection.[20] A fatal side effect of melarsoprol is reactive encephalopathy and it is reported that 5-10% of patients treated with the drug suffer from this serious complication.[3,20] At least 1-5% of the patients die during the course of treatment as a result of the toxic nature of the drug.[21] In order to minimise the chances of patients experiencing episodes of reactive encephalopathy, melarsoprol is co-administered with corticosteroids such as prednisolone.[3] Discontinuation of melarsoprol is advised if a patient experiences convulsions or deterioration in mental state.[21]

Melarsoprol is a pro-drug that is rapidly converted to its active metabolite, melarsen oxide, which has an elimination half life of 3.5 hours.[3] The mode of action of melarsoprol on trypanosomes is unknown, although several hypotheses have been put forward. These include competitive inhibition of an enzyme crucial to the parasites called trypanothione reductase as well as the rapid and selective uptake through the P2-purine transporter which contributes to selective toxicity.[3,20]

1.7.4 Eflornithine Monotherapy

Eflornithine (shown in **Figure 1.3**) is an ornithine analogue whose mode of action is the irreversible inhibition of ornithine decarboxylase, a polyamine biosynthetic enzyme.[3] Registered in 1990[7], eflornithine is manufactured by Aventis; it is the only new candidate registered for the treatment of HAT in the last 50 years[15]and is sold under the trade name Ornidyl.[20] Eflornithine is the drug of choice for the treatment of stage 2 of HAT caused by *T.b. gambiense*. This drug is less effective against *T. b. rhodesiense* HAT and is therefore not recommended for this form of the disease.[3,20] Eflornithine has its own shortcomings that

include difficult intravenous administration of 4 times daily infusions for 14 days and high costs of the drug. It is critical that the infusion be administered slowly over 2 hours and this requires the patient to be hospitalised for the entire duration of treatment.[20,21] The frequent administration of the drug is due to its short plasma half-life of 3 hours.[3] Side effects of eflornithine include fever, headaches, hearing loss, facial oedema, convulsions, dizziness, anaemia as well as gastrointestinal problems such as vomiting and diarrhoea.[3,21]

1.7.5 Nifurtimox Eflornithine Combination Therapy (NECT)

Combination therapy of nifurtimox and eflornithine (shown in **Figure 1.3**) was introduced in 2009 for the treatment of stage 2 HAT caused by *T.b. gambiense* and was added to the World Health Organization's Essential Medicines List in the same year.[19,22] Nifurtimox is an oral drug used for the treatment of Chagas disease, another trypanosomal disease, also known as American trypanosomiasis.[19] NECT is the only major advancement that has occurred in the course of 25 years in the treatment of sleeping sickness.[1,18] The combination improved and simplified administration of the intravenous course of eflornithine from four times daily infusions for 14 days to twice daily infusions for 7 days. Thus the combination treatment has reduced the number of eflornithine doses from four daily doses with the entire course comprising of 56 doses, to two daily doses, which adds up to 14 doses for the entire treatment course.[19] The treatment course with NECT and the hospital stay are therefore shorter which is useful in the resource-deprived settings affected by HAT.[23] The shorter treatment course has a huge impact on the cost of treatment, as intravenous drugs are expensive and combining with an already cheap oral drug lowers costs.[23] Yun *et al*. (2010)[19] reported the cost of NECT kits to be €39 per patient, which was almost three times cheaper than eflornithine monotherapy kits, which cost €107 per patient.[19] Reduction in toxicity and a possible delay in the emergence of drug-resistance are some of the advantages of this combination therapy.[23] Although it is an improvement to current HAT therapies, NECT has its short-comings. There is still need for intravenous infusions of eflornithine despite the reduction in the number of doses and the need for trained care-givers is still inevitable.[1,19]

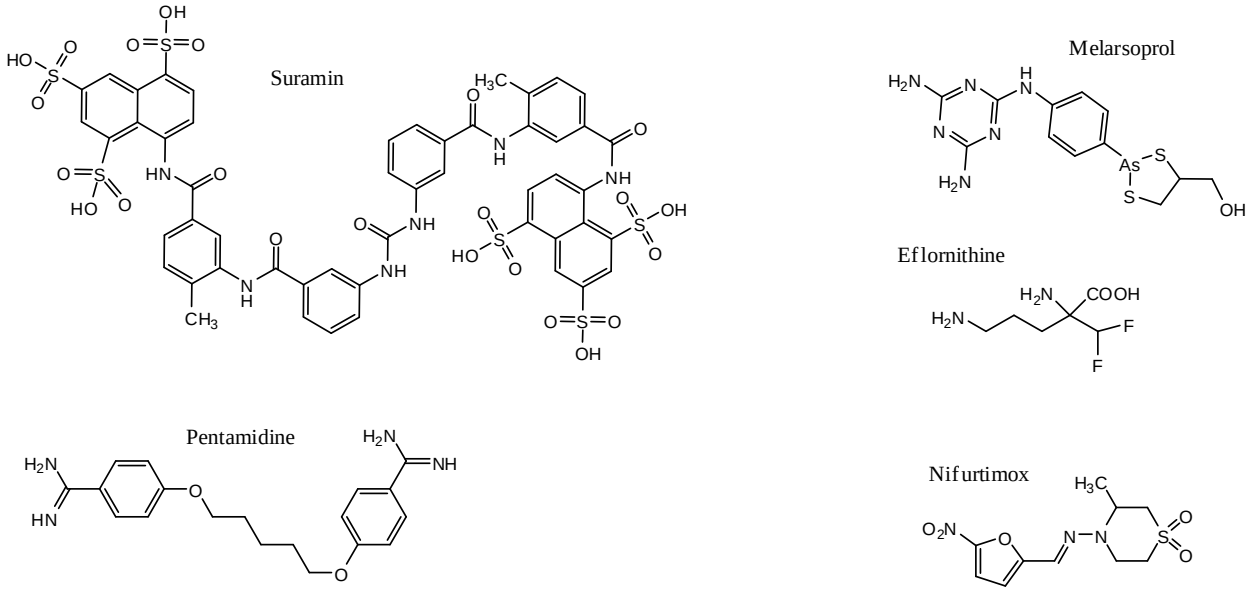


Figure 1.3: Structures of drugs used in the treatment of Human African Trypanosomiasis (sleeping sickness).

1.8 LIMITATIONS OF CURRENT TREATMENT

Melarsoprol, eflornithine and suramin all require intravenous administration whilst pentamidine requires intramuscular administration. This means that patients need to be hospitalised for the treatment of HAT - a challenge, considering that HAT mainly affects rural populations which are, in most cases, in remote areas. The disease also thrives in the war torn regions of central Africa. In addition, nifurtimox is the only orally administered drug used for HAT at present.

Adverse events resulting from treatment with HAT therapies are fatal and life-threatening. For example, 5-10% of the patients treated with melarsoprol suffer from serious complications with the most frequent complication being reactive encephalopathy. Treatment failures are also being reported and this calls for research into safe and effective therapies.[3]

There is a need for therapies that cross the blood-brain barrier with ease and concentrate in the brain to treat the neurological stages (stage 2) of the disease. Suramin, the drug of choice for the treatment of stage 1 of the *T. b. rhodesiense* form of the disease, has minimal permeation across the blood-brain barrier and this limits its use to the treatment of stage 1

only.[3] With melarsoprol, its active metabolite permeates through the blood-brain barrier but up to 1-2% of maximum plasma concentration is attained. [3]

Cost effective therapies are also crucial and high costs may be reduced through the development of orally administered therapies as opposed to expensive intravenous therapies. Costs may be further reduced if patients are treated by oral drugs whilst staying at home.

1.9 THERAPIES IN THE PIPELINE

1.9.1 Fexinidazole

The year 2006 saw the rediscovery of a potential drug candidate which was never pursued in the 1980s.[1,3] Fexinidazole is a promising orally administered treatment for sleeping sickness. This potential candidate entered phase I of clinical trials in 2009 and is currently in phase II/III trials. It is the first new drug candidate for stage 2 of sleeping sickness to enter these trials in the last 30 years. An organisation called the Drugs for Neglected Disease initiative (DNDi) carried out an extensive compound mining and profiling of more than 700 nitroheterocyclic compounds, the majority of which were nitroimidazoles. Compounds were assessed for their anti-parasitic and mutagenic potential. After a systematic review of these, fexinidazole, a 2-substituted 5-nitroimidazole, showed potential and was a promising candidate for the treatment of sleeping sickness. Fexinidazole had reached pre-clinical development as a broad spectrum antimicrobial agent in the late 1970s and early 1980s, and the work was conducted by Hoechst AG which became Sanofi-Aventis. At that time, fexinidazole had been favoured amongst a series of compounds because of the relative ease of its chemical synthesis, the broad spectrum of its action as well as its low toxicity profile.[1]

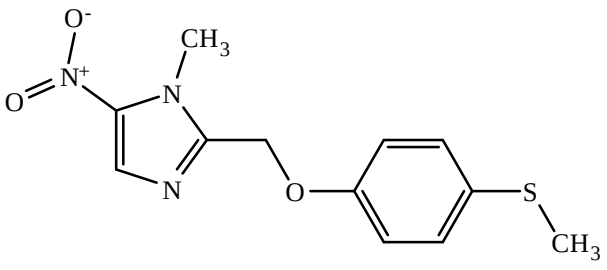


Figure 1.4: The structure of fexinidazole, a potential drug candidate for the treatment of sleeping sickness.

The *in vivo* activity of fexinidazole against sleeping sickness trypanosomes was substantiated in 1983 but the development of this promising compound was never pursued at the time.[1]

Fexinidazole is administered orally and is quickly metabolised to give two active metabolites with equivalent activities to that of the parent compound. If it passes the clinical trials, fexinidazole has the potential to become a novel, oral and short-course treatment for both stage 1 and stage 2 of sleeping sickness and supersede the old and problematic therapies currently in use.[1,17,18] Fexinidazole, therefore, has the potential to revolutionise the treatment of sleeping sickness. It will also reduce the cost of treatment in the resource-constrained settings affected by the disease, as doing away with intravenously administered drugs will lower the costs. The need for skilled professionals and hospitalisation for up to two weeks will be reduced with the introduction of an orally administered drug; which are necessary for the current therapies as they are injectables, difficult to administer and cause severe adverse effects that need to be monitored.[1]

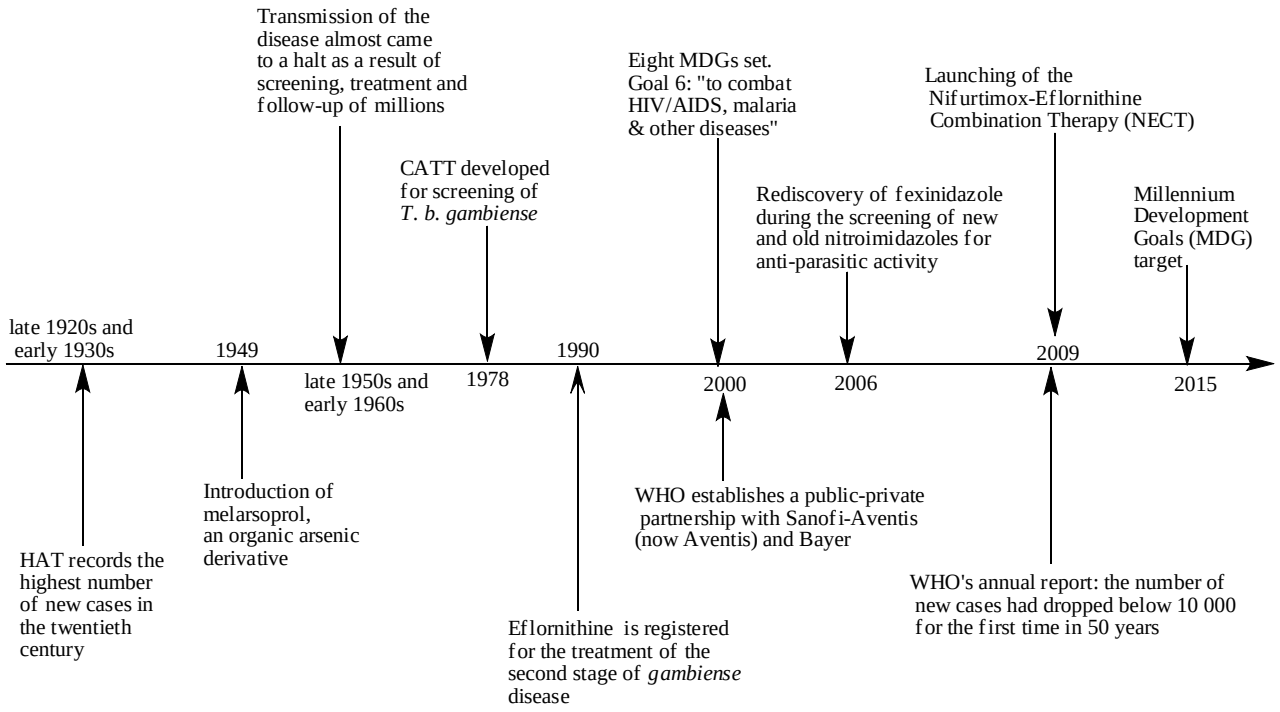


Figure 1.5: Timeline of some of the major highlights in the history of sleeping sickness. Information gathered from Simarro *et al.*[7], Fairlamb[20], Yun *et al.*[19], Torreele *et al.*[1] and World Health Organization[6].

1.10 NAPHTHOQUINONES

1.10.1 Naphthoquinones as potential drug candidates

In the year 2000 at a summit of the United Nations, eight millennium development goals (MDGs) were set to be achieved by 2015. MDG 6 states "to combat HIV/AIDS, malaria & other diseases". With just under 2 years to go before the end of 2015, malaria and HAT are still thriving in developing countries.[24] The drugs currently used for the treatment of HAT have fatal side effects, are limited in range and are in short supply. The drugs are also old and those used to treat late stages of the disease are very difficult to administer.[1] The aforementioned challenges call for research and development into this neglected disease in order to come up with new, safe and easy-to-use medicines.

This study focuses on a class of compounds called naphthoquinones which have been reported to possess a variety of biological activities, including anti-parasitic, anti-fungal, anti-cancer, anti-bacterial, anti-inflammatory, anti-molluscicidal and anti-viral activities.[25-27] A wide spectrum of activities exhibited by naphthoquinones are believed to be due to their ability to generate free radicals. Natural and synthetic naphthoquinones have the potential to accept one and/or two electron(s) to form radical anion or dianion species.[26] Some studies have gone to greater heights to demonstrate the production of free radicals or reactive oxygen species (ROS) by naphthoquinones derivatives at mitochondrial level. [28] A study conducted by Pieretti and colleagues demonstrated the capability of 2-phenoxy-1,4-naphthoquinone to generate ROS. This was proven by measuring radical formation during respiration in a mitochondrial fraction of *T.brucei* both in the presence and absence of 2-phenoxy-1,4-naphthoquinone. Further experiments carried out during this study using trypanosomal isolated mitochondria also demonstrated that 2-phenoxy-1,4-naphthoquinone was able to interfere with the respiratory chain through production of ROS.[28] Other researchers have used electrochemical studies whereby cyclic voltammetry measurements, in aprotic media, were done to demonstrate the potential of naphthoquinones to accept electrons and form free radicals.[29,30] A cyclic voltammogram of 2,3-dichloro-1,4-naphthoquinone (starting material of this study) obtained by Sayil and colleagues[30] reviewed two reversible monoelectric waves. The first reversible reduction wave was attributed to the formation of a semiquinone anion radical ($Q^{\cdot -}$):

$$Q + e^- \rightleftharpoons Q^{\cdot -} \text{ (reversible)} \qquad (1)$$

The second wave was attributed to the reduction of the semiquinone anion radical to a dianion (Q^{2-}):

$$Q^{\cdot -} + e^{-} \rightleftharpoons Q^{2-} \text{ (quasi-reversible)} \qquad (2)$$

Numerous studies have reported this typical behavior of the reduction of naphthoquinones in aprotic conditions.[29-31] Formation of ROS and the redox potential of synthetic as well as natural naphthoquinones are believed to cause oxidative stress, giving this class of compounds its wide spectrum of biological activities.[28,32]

1.10.2 Naphthoquinone derivatives on the market

Atovaquone is a 1,4-naphthoquinone derivative currently on the market and is registered for the prevention of malaria, treatment of *Pneumocystis pneumonia* and toxoplasmosis. Atovaquone is a derivative of lapachol (**Figure 1.6**), a naturally occurring 1,4-naphthoquinone.[33] Other marketed 1,4-naphthoquinones are K vitamins, phylloquinone and menaquinone (**Figure 1.7**). Phylloquinone, also known as vitamin K_1, is used as an antidote for treating an overdose of warfarin, a vitamin K antagonist.[21]

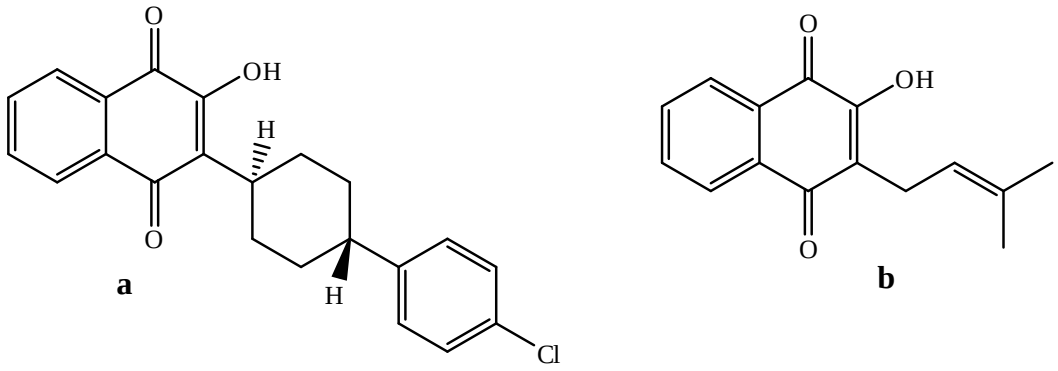


Figure 1.6: a) Structure of atovaquone, a 1,4-naphthoquinone compound and derivative of lapachol, currently on the market for prevention of malaria. **b)** Structure of lapachol, a naturally occurring 1,4-naphthoquinone derivative explored for various activities including anti-tumuor activity.[33] Structures adapted and re-drawn from Epifano *et al.*[33]

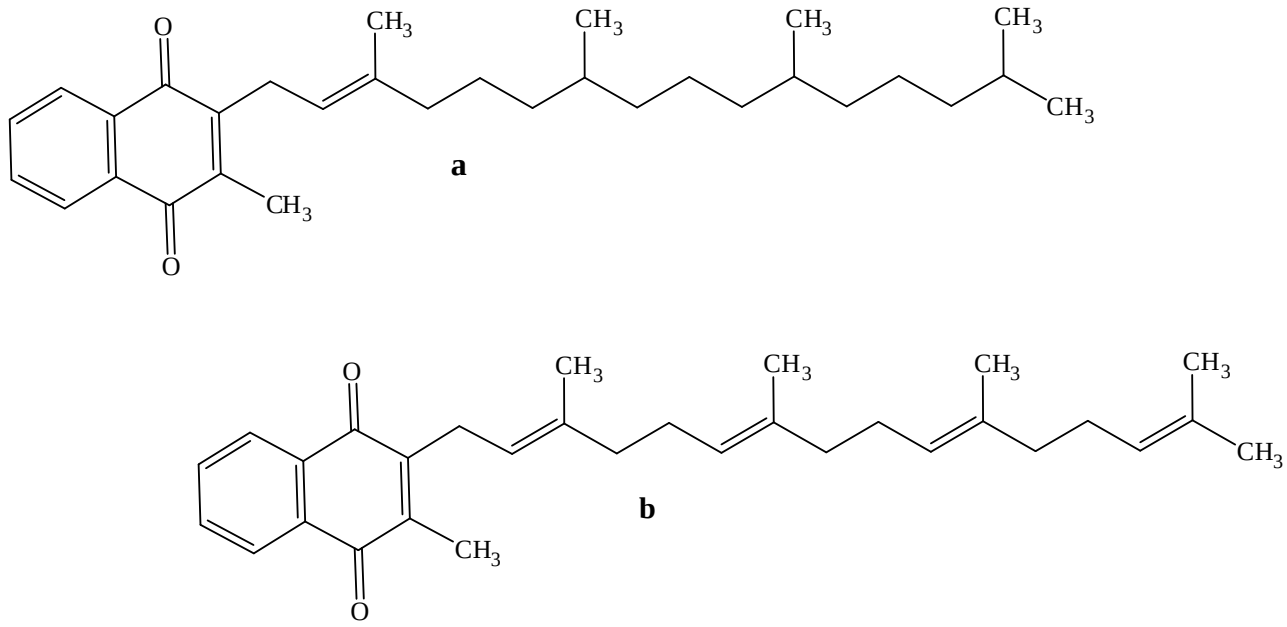


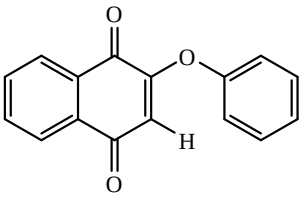
Figure 1.7: **a)** Structure of vitamin K_1, composed of a 1,4-naphthoquinone ring and an aliphatic side chain **b)** Structure of vitamin K_2, composed of 1,4-naphthoquionone backbone and a side chain with isoprenoid units which can vary with four units being common.

1.10.3 Previous studies of anti-trypanosomal assays of 1,4-naphthoquinone derivatives

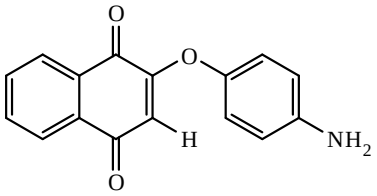
Extensive studies have been conducted on the synthesis and analysis of biological activities of naphthoquinone derivatives but a few of them have focused on their potential anti-trypanosomal activity.

Pieretti *et al.*[28] carried out a structure-activity relationship study of 1,4-naphthoquinones shown in **Figure 1.8** below. In this study, different positions of hydroxyl groups on the naphthoquinone backbone and the presence of an amino substituent on the phenoxy moiety were investigated with respect to trypanosomal activity. The study revealed that shifting of the hydroxyl group from the eighth to the fifth position on the naphthoquinone backbone reduced anti-trypanosomal activity by 5-fold and the addition of an amino group on the phenoxy moiety reduced the activity by 7-fold (**Figure 1.8**).[28]

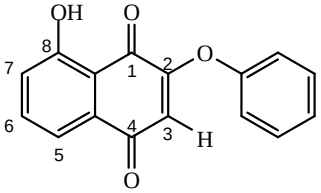
Furthermore, the study pursued 2-(4-aminophenoxy)-1,4-naphthoquine (**Figure 1.8**) to determine its mechanism of action against *T.b. rhodesiense*. The study concluded that naphthoquinones (**Figure 1.8**) exert their anti-trypanosomal activity, through a multi-target mechanism, including interference of parasite mitochondrial respiratory chain as a result of the generation of reactive oxygen species by the naphthoquinones.[28]



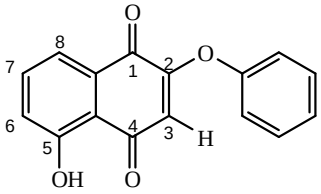
2-phenoxy-1,4-naphthoquinone
IC_{50} = 0.08µM



2-(4-aminophenoxy)-1,4-naphthoquinone
IC_{50} = 0.57µM



8-hydroxy-2-phenoxy-1,4-naphthoquinone
IC_{50} = 0.85µM



5-hydroxy-2-phenoxy-1,4-naphthoquinone
IC_{50} = 4.4µM

Figure 1.8: 1,4-naphthoquinone derivatives with their respective trypanosomal activities against *T.b. rhodesiense* parasites as reported by Pieretti *et al* (2012).[28]

1.11 AIMS AND OBJECTIVES

There has been an increasing interest in the development of new naphthoquinone derivatives in which the 1,4-naphthoquinone pharmacophore is retained. Bio-assaying these naphtho-quinone derivatives for various activities in search of new hit candidates, which can further progress to the hit-to-lead and lead optimisation stages of drug discovery, is on the rise.

The research questions for this study were whether the electronegativity of substituents, the position of substituents (*para*, *ortho* and *meta*), linkages and either monosubstitution or disubstitution of 1,4-naphthoquinone derivatives have an effect on anti-trypanosomal activity. In light of the above, the aims and objectives of this study were:

- To synthesise derivatives of 2,3-dichloro-1,4-naphthoquinone:
    - With thioether (-S-), ether (-O-) and amino (-NH-) linkages by coupling the naphthoquinone with three types of nucleophiles namely thiols, phenols and anilines.
    - From various nucleophiles with electronegative halogen substituents and electron-donating methyl substituents on *para*, *meta* and *ortho* positions.
    - Which are monosubstituted and disubstituted.

- To screen the synthesised naphthoquinone derivatives against *T.brucei*.
- To conduct structure-activity relationship (SAR) and quantitative structure-activity relationship (QSAR) studies to determine which structural features or functional group substituents of the naphthoquinone derivatives contribute or take away from the desired anti-trypanosomal activity.

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CHAPTER II: METHODOLOGY

2.1 INTRODUCTION

Synthesis of 1,4-naphthoquinone derivatives from aniline, phenol and thiophenol nucleophiles will be discussed in this chapter. The synthetic procedures were adopted and modified from available literature.[1,2] While various methods have been proposed for the synthesis of naphthoquinone derivatives, a single-step reaction using ethanolic solutions was used for this project. Other methods put forward in literature for the synthesis of naphthoquinone derivatives include:

- 'On-water'/'in-water' reactions which make use of aqueous micellar solutions of sodium lauryl sulphate with micelles acting as micro-reactors or catalysts[3,4]
- Buchwald-Hartwig cross-coupling reactions which make use of transition metals such as palladium for catalysis[5,6]
- One-pot laccase enzyme catalyzed method[7]

2.2 EXPERIMENTAL

2.2.1 Equipment and materials

Reactants and reagents were obtained from Sigma-Aldrich® (South Africa) and Saarchem (Pty) Ltd (South Africa). Organic solvents (methanol, n-hexane and ethyl acetate) used for chemical synthesis and thin layer chromatographic analysis were LiChrosolv® liquid chromatography grades from Merck KGaA (Germany). The chloroform used for recrystallisation was a chemically pure grade from Minema Chemicals (Pty) Ltd (South Africa). Deuterated chloroform for the NMR analysis and the absolute ethanol for chemical synthesis were obtained from Merck KGaA (Germany). No further purification of any of the organic solvents was carried out. Silicone oil for an oil bath was obtained for Sigma-Aldrich® (USA).

Chemical reactions were carried out in round bottom flasks that were submerged in a silicone oil bath placed on top of a Fried Electric (Israel) hot plate/magnetic stirrer. A 76 mm immersion

thermometer was used to alter the temperature of the silicone oil bath. Thin layer chromatography (TLC) was carried out on TLC silica gel $60F_{254}$ aluminium plates from Merck KGaA (Germany) and, the TLC plates were visualised using Syngene 254, 365 nm UV lamb. Melting point determinations were carried out using a Stuart® SMP30 digital and advanced melting point apparatus (United Kingdom). Synthesised products were stored in size 6 glass vials from Lasec (South Africa).

Nuclear magnetic resonance (NMR) spectra were recorded on a 600MHz Bruker 600 UltraShield™ spectrometer and 400MHz Bruker spectrometer in a chloroform-d1 ($CDCl_3$) solution. Chemical shifts (δ) of the NMR spectra are reported in parts per million (ppm) with reference to tetramethylsilane (TMS). The coupling constants (*J*) of the NMR spectra are given in hertz (Hz). MestReNova was the chemical software used to view the processed NMR spectra as well as for integration, multiplicity and obtaining coupling constants.

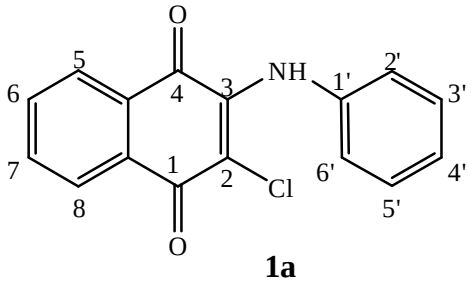
A Finnigan LCQ mass spectrometer (ESI, APCI) was used to determine the molecular weight of the synthesised products. A dual system of high pressure liquid chromatography coupled to electro-spray ionisation mass spectrometry (HPLC-ESI-MS) was utilised. The source of LCQ-MS detector for the mass spectrometry analysis was ESI and it was in the positive ion mode. Samples were dissolved in ethyl acetate and protonation of the analytes was performed in order to produce 'electron-spray active' species. Protonation of the samples was carried out by spiking the sample solution with one or two drops of acid.

Infrared spectra of the N-substituted 1,4-naphthoquinones derivatives (**compounds 1a-1f**) were recorded on a PerkinElmer Spectrum 100 FT-IR spectrometer with a potassium bromide (KBr) pellet.

2.2.2 Synthesis of 1,4-naphthoquinone derivatives

2.2.2.1 Coupling with anilines

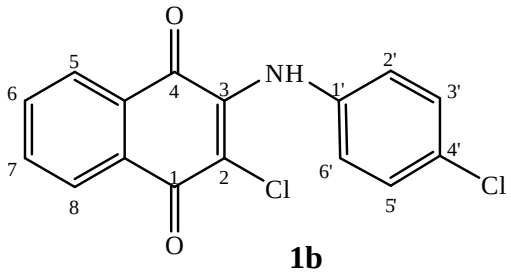
Synthesis of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a**



1a

To a suspension of 2,3-dichloro-1,4-naphthoquinone (2.27 g; 10 mmol) in absolute ethanol (50 mL) was pipetted aniline (0.9 mL; 12 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 6 hours. The reaction mixture was cooled to room temperature and the crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from chloroform and methanol (3:1) to afford 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a** as a maroon, needle-like solid (1.6392 g; 57.8%); mp 213 °C (lit.[2] 218-221 °C); ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.19 (d, *J* = 7.7 Hz, 1H, H8), 8.12 (d, *J* = 7.6 Hz, 1H, H5), 7.77 (td, *J* = 7.6, 1.1 Hz, 1H, H7), 7.70 – 7.68 (m, 2H, H6 overlapping with NH), 7.35 (t, *J* = 7.9 Hz, 2H, H3' and H5'), 7.22 (t, *J* = 7.4 Hz, 1H, H4'), 7.09 (d, *J* = 7.9 Hz, 2H, H2' and H6'); IR (KBr, cm^{-1}): 3233.81 (-NH), 1673.86 and 1636.37 (-C=O of the quinone); HPLC-ESI-MS (*m/z*): $[M+3H]^+$ (286.53).

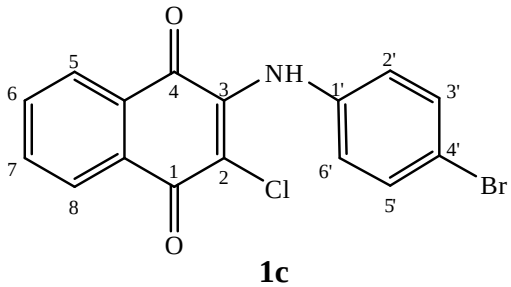
Synthesis of 2-chloro-3-(4-chlorophenylamino)-1,4-naphthoquinone **1b**



1b

To a solution of 4-chloroaniline (1.2757 g; 10 mmol) in absolute ethanol (50 mL) was added 2,3-dichloro-1,4-naphthoquinone (2.27 g; 10 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 6 hours. The reaction mixture was then cooled to room temperature and the crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from chloroform and methanol (3:1) and then further recrystallised from methanol to afford 2-chloro-3-(4-chlorophenylamino)-1,4-naphthoquinone **1b** as a maroon solid (2.2236 g; 69.9%); mp 265-269 °C (lit.[2] 263-265 °C); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.20 (d, 1H, H8), 8.12 (d, 1H, H5), 7.79 (t, 1H, H7), 7.71 (t, 1H, H6), 7.62 (s, 1H, -NH), 7.32 (d, *J* = 7.2 Hz, 2H, H3' and H5'), 7.01 (d, *J* = 7.2 Hz, 2H, H2' and H6'); IR (KBr, cm^{-1}): 3225.94 (-NH), 1673.71 and 1638.80 (-C=O of the quinone); HPLC-ESI-MS (*m/z*): $[M+3H]^+$ (320.67).

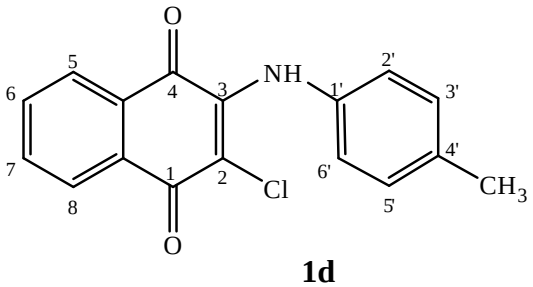
Synthesis of 2-chloro-3-(4-bromophenylamino)-1,4-naphthoquinone **1c**



1c

To a solution of 4-bromoaniline (0.8601 g; 5 mmol) in absolute ethanol (25 mL) was added 2,3-dichloro-1,4-naphthoquinone (1.135 g; 5 mmol). The reaction mixture was stirred and heated in a silicone oil bath which was maintained at 80 °C for 6 hours. The reaction mixture was cooled to room temperature and the crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from chloroform and methanol (3:1), twice, to afford 2-chloro-3-(4-bromophenylamino)-1,4-naphthoquinone as a maroon solid (1.1808 g; 65.1%); mp 276.1-277.9°C (lit.[2] 237-239 °C); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.20 (d, *J* = 7.7 Hz, 1H, H8), 8.13 (d, *J* = 7.5 Hz, 1H, H5), 7.79 (t, *J* = 7.3 Hz, 1H, H7), 7.71 (t, *J* = 7.6 Hz, 1H, H6), 7.60 (s, 1H, NH), 7.47 (d, *J* = 8.5 Hz, 2H, H3' and H5'), 6.95 (d, *J* = 8.4 Hz, 2H, H2' and H6'); IR (KBr, cm^{-1}): 3235.54 (-NH), and 1673.80 and 1635.52 (-C=O of the quinone); HPLC-ESI-MS (*m/z*): $[M+2H]^+$ (364.73).

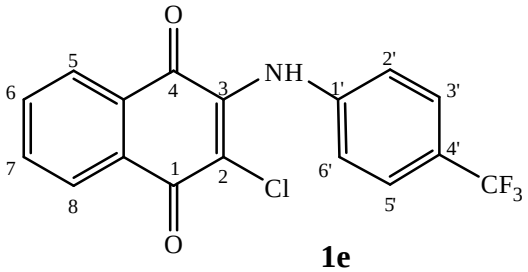
Synthesis of 2-chloro-3-(p-tolylamino)-1,4-naphthoquinone **1d**



1d

To a solution of p-toluidine, also known as 4-methylaniline, (1.0715 g; 10 mmol) in absolute ethanol (50 mL), was added 2,3-dichloro-1,4-naphthoquinone (2.27 g; 10 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 6 hours. The reaction mixture was cooled to room temperature and the crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from chloroform and methanol (3:1), twice, to afford 2-chloro-3-(p-tolylamino)-1,4-naphthoquinone **1d** as a maroon, needle-shaped solid (1.632 g; 54.8%); mp 193.3-196.7 °C (lit.[2] 184-186 °C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (d, *J* = 7.7 Hz, 1H, H8), 8.10 (d, *J* = 7.5 Hz, 1H, H5), 7.78 – 7.74 (m, 1H, H7), 7.69 – 7.65 (m, 2H, H6 overlapping with NH), 7.15 (d, *J* = 8.0 Hz, 2H, H3' and H5'), 6.98 (d, *J* = 8.1 Hz, 2H, H2' and H6'), 2.36 (s, 3H, -CH_3); IR (KBr, cm^{-1}): 3219.19 (-NH), 1674.93 and 1631.41 (-C=O of the quinone); HPLC-ESI-MS (*m/z*): $[M+3H]^+$ (300.60).

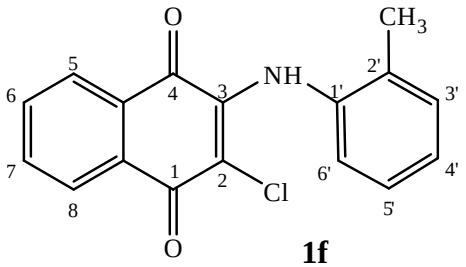
Synthesis of 2-chloro-3-(4-(trifluoromethyl)phenylamino)-1,4-naphthoquinone **1e**



1e

To a solution of 4-(trifluoromethyl)aniline (0.5mL; 4 mmol) in absolute ethanol (20 mL), was added triethylamine base (0.15 mL; 1 mmol) and then 2,3-dichloro-1,4-naphthoquinone (0.908 g; 4 mmol). The reaction mixture was stirred and heated in a silicone oil bath which was maintained between 80-100 °C for 10 hours. The reaction mixture was then cooled to room temperature and the crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from chloroform and methanol (3:1) to afford 2-chloro-3-(4-(trifluoromethyl)phenylamino)-1,4-naphthoquinone **1e** as orange, needle-shaped crystals (0.4925 g; 35.0%); mp 249.4-251.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, *J* = 7.6 Hz, 1H, H8), 8.15 (d, *J* = 7.5 Hz, 1H, H5), 7.80 (t, *J* = 7.5 Hz, 1H, H7), 7.73 (t, *J* = 7.6 Hz, 1H, H6), 7.68 (s, 1H, NH), 7.61 (d, *J* = 8.2 Hz, 2H, H3' and H5'), 7.12 (d, *J* = 8.1 Hz, 2H, H2' and H6'); IR (KBr, cm^{-1}): 3243.81 (-NH), 1673.30 and 1638.98 (-C=O of the quinone); HPLC-ESI-MS (*m/z*): $[M+1H]^+$ (352.73).

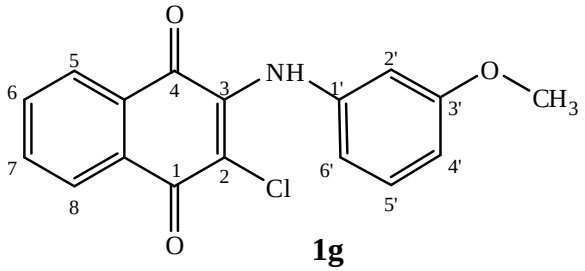
Synthesis of 2-chloro-3-(o-tolylamino)-1,4-naphthoquinone **1f**



1f

To a solution of 2-methylaniline, also known as o-toluidine, (1 mL; 10 mmol) in absolute ethanol (50 mL), was added 2,3-dichloro-1,4-naphthoquinone (2.27 g; 10 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 8 hours. The reaction mixture was cooled to room temperature and the crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from chloroform and methanol (3:1) to afford 2-chloro-3-(o-tolylamino)-1,4-naphthoquinone **1f** as a maroon powder (0.8441 g; 28.4%); mp 163.3-165.1 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.19 (d, *J* = 7.7 Hz, 1H, H8), 8.12 (d, *J* = 7.6 Hz, 1H, H5), 7.77 (t, *J* = 7.5 Hz, 1H, H7), 7.69 (t, *J* = 7.5 Hz, 1H, H6), 7.47 (s, 1H, -NH), 7.25 – 7.21 (m, 1H, phenyl-H), 7.21 – 7.17 (m, 2H, 2x phenyl-H), 7.04 (dd, *J* = 8.8, 4.2 Hz, 1H, phenyl-H), 2.30 (s, 3H, $-CH_3$); IR (KBr, cm^{-1}): 3243.81 (-NH), 1673.30 and 1638.98 (-C=O of the quinone); HPLC-ESI-MS (*m/z*): $[M+1H]^+$ (298.60).

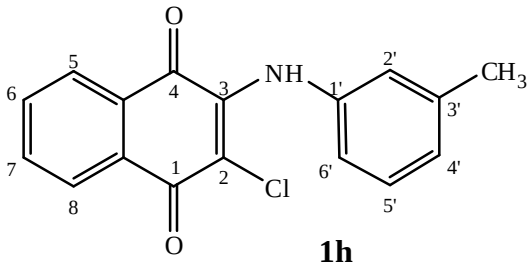
Synthesis of 2-chloro-3-(3-methoxyphenylamino)-1,4-naphthoquinone **1g**



1g

To a solution of 3-methoxyaniline, also called m-anisidine, (0.6 mL; 5 mmol) in absolute ethanol (25 mL) was added 2,3-dichloro-1,4-naphthoquinone (1.135 g; 5 mmol). The reaction mixture was stirred and heated in a silicone oil bath which was maintained at 80 °C for 6 hours. The reaction mixture was then cooled to room temperature and the crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from methanol, twice, and then in hexane to afford 2-chloro-3-(3-methoxyphenylamino)-1,4-naphthoquinone **1g** as a maroon solid (0.8922 g; 56.9%); mp 147.4-149.7 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.19 (dd, *J* = 8.4, 3.5 Hz, 1H, H8), 8.11 (d, *J* = 7.6 Hz, 1H, H5), 7.76 (t, *J* = 7.3 Hz, 1H, H7), 7.69 (t, *J* = 7.4 Hz, 1H, H6), 7.66 (s, 1H, -NH), 7.24 (t, *J* = 8.1 Hz, 1H, H5'), 6.76 (dd, *J* = 8.3, 2.3 Hz, 1H, H6'), 6.68 (dd, *J* = 7.9, 1.3 Hz, 1H, H4'), 6.61 (s, 1H, H2'), 3.81 (s, 3H, $-OCH_3$); IR (KBr,cm^{-1}): 3219.32 (-NH), 1673.67 and 1643.68 (-C=O of the quinone); HPLC-ESI-MS (*m/z*): $[M+3H]^+$ (316.73).

Synthesis of 2-chloro-3-(m-tolylamino)-1,4-naphthoquinone **1h**

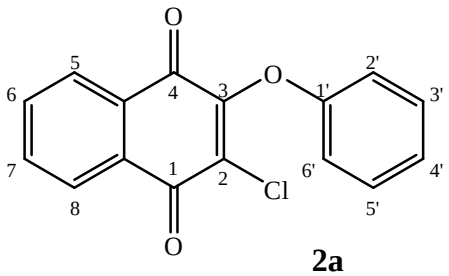


1h

To a solution of 3-methylaniline, also known as m-toluidine, (0.55 mL; 5 mmol) in absolute ethanol (25 mL) was added 2,3-dichloro-1,4-naphthoquinone (1.135 g; 5 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 9 hours. The reaction mixture was then cooled to room temperature and the crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from methanol, twice, and then from hexane to afford 2-chloro-3-(m-tolylamino)-1,4-naphthoquinone **1h** as a maroon solid (1.0763 g; 72.3%); mp 177.2-181.5 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.19 (d, *J* = 7.7 Hz, 1H, H8), 8.12 (d, *J* = 7.6 Hz, 1H, H5), 7.77 (t, *J* = 7.5 Hz, 1H, H7), 7.69 (t, *J* = 7.6 Hz, 1H, H6), 7.66 (s, 1H, -NH), 7.23 (t, *J* = 8.0 Hz, 1H, H5'), 7.03 (d, *J* = 7.6 Hz, 1H, H4'/H6'), 6.89 (d, *J* = 5.0 Hz, 1H, H6'/H4'), 6.88 (s, 1H, H2'), 2.36 (s, 3H, -CH_3); IR (KBr, cm^{-1}): 3226.17 (-NH), 1676.83 and 1630.92 (-C=O of the quinone); HPLC-ESI-MS (*m/z*): $[M+3H]^+$ (300.73).

2.2.2.2 Coupling with phenols

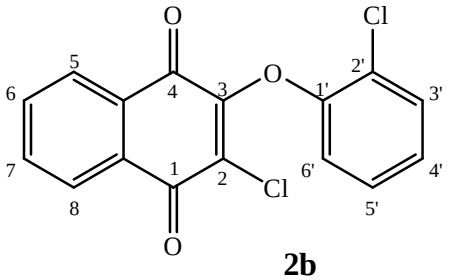
Synthesis of 2-chloro-3-phenoxy-1,4-naphthoquinone **2a**



2a

To a solution of phenol (1.8822 g; 20 mmol) in absolute ethanol (40 mL) was added triethylamine base (0.45 mL; 3mmol) and then 2,3-dichloro-1,4-naphthoquinone (2.27 g; 10 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 6 hours. The reaction mixture was then cooled to room temperature and the precipitated crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from hexane to afford 2-chloro-3-phenoxy-1,4-naphthoquinone **2a** as a yellow, fluffy solid (1.596 g; 56.1%); mp 133.1-135.0 °C (lit.[8] 138-139 °C); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.22 (dd, *J* = 7.5, 1.2 Hz, 1H, H8), 8.06 (dd, *J* = 7.5, 1.2 Hz, 1H, H5), 7.83 – 7.74 (m, 2H, H6 and H7), 7.34 (t, *J* = 7.8 Hz, 2H, H3' and H5'), 7.15 (t, *J* = 7.4 Hz, 1H, H4'), 7.02 (d, *J* = 7.8 Hz, 2H, H2' and H6'); HPLC-ESI-MS (*m/z*): $[M+2H]^+$ (286.67).

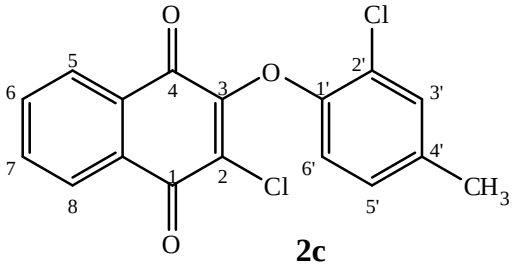
Synthesis of 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b**



2b

To a solution of 2-chlorophenol (1 mL; 10 mmol) in absolute ethanol (25 mL) was added triethylamine base (0.45 mL; 3 mmol) and then 2,3-dichloro-1,4-naphthoquinone (1.135 g; 5 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 24 hours. The reaction mixture was gravity filtered whilst hot, so as to remove the orange solid sediment at the bottom of the round bottomed flask. The orange sediment was removed from the flask by dissolving it in ethyl acetate and air dried in a fume hood to give orange, shinny, needle-shaped crystals. The needle-shaped crystals were obtained without purification to afford 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b**. The hot filtrate from the gravity filtration of the reaction mixture was then cooled to room temperature and the precipitated crude product was collected on a Büchner funnel by vacuum filtration. The crude product was recrystallised from methanol and then from hexane to 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b** as a dark yellow, amorphous powder. The crystals and amorphous powder showed similar ^{1}H NMR spectra and R_f value of 0.72. Total yield (0.6512 g; 40.8%); mp of orange crystals of **2b** was 178.3-181.3 °C; mp of the dark yellow, amorphous powder of **2b** was 172.1-175.7°C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.21 (dd, *J* = 7.6, 1.3 Hz, 1H, H8), 8.03 (dd, *J* = 7.6, 1.3 Hz, 1H, H5), 7.82 – 7.73 (m, 2H, H6 and H7), 7.45 (dd, *J* = 8.0, 1.6 Hz, 1H, H3'), 7.22(td, *J* = 7.8, 1.4 Hz, 1H, H5'), 7.12 (td, *J* = 7.8, 1.4 Hz, 1H, H4'), 6.98 (dd, *J* = 8.1, 1.4 Hz, 1H, H6'); HPLC-ESI-MS (*m/z*): $[M+1H]^+$ (320.92).

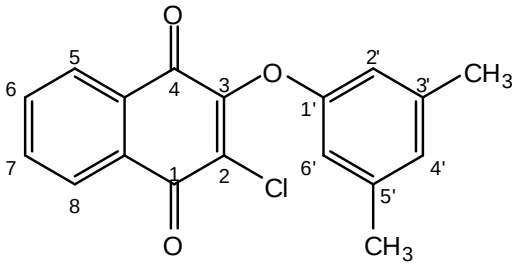
Synthesis of 2-chloro-3-(2-chloro-4-methylphenoxy)-1,4-naphthoquinone **2c**



2c

To a solution of 2-chloro-4-methylphenol (1.18 ml; 10 mmol) in absolute ethanol (25 ml) was added triethylamine base (0.45 ml; 3 mmol) and then 2,3-dichloro-1,4-naphthoquinone (1.135 g; 5 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 24 hours. The reaction mixture was then cooled to room temperature and the precipitated crude was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from hexane, and then further from methanol, to afford 2-chloro-3-(2-chloro-4-methylphenoxy)-1,4-naphthoquinone **2c** as orange, needle-shaped crystals (0.8649 g; 51.9%); mp 143.9-146.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (dd, *J* = 7.5, 1.3 Hz, 1H, H8), 8.02 (dd, *J* = 7.4, 1.3 Hz, 1H, H5), 7.84 – 7.71 (m, 2H, H6 and H7), 7.25 (s, 1H, H3'), 7.01 (dd, *J* = 8.3, 1.4 Hz, 1H, H5'/H6'), 6.89 (d, *J* = 8.3 Hz, 1H, H6'/H5'), 2.33 (s, 3H, -CH_3); HPLC-ESI-MS (*m/z*): $[M+1H]^+$ (334.88).

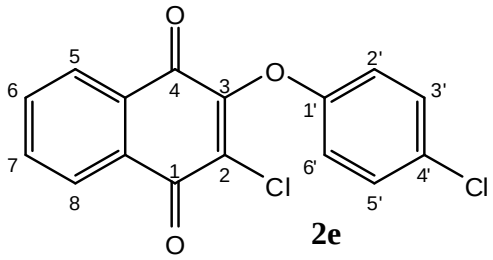
Synthesis of 2-chloro-3-(3,5-dimethylphenoxy)-1,4-naphthoquinone **2d**



2d

To a solution of 3,5-dimethylphenol (1.2216 g; 10 mmol) in absolute ethanol (25 mL) was added triethylamine base (0.45 mL; 3 mmol) and then 2,3-dichloro-1,4-naphthoquinone (1.135 g; 5 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 6 hours. The reaction mixture was then cooled to room temperature and the precipitated crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from hexane and further from methanol to afford 2-chloro-3-(3,5-dimethylphenoxy)-1,4-naphthoquinone **2d** as a dark yellow, needle-shaped, fluffy solid (0.8383 g; 53.6%); mp 155.9-157.4 °C (lit[3] no mention of the melting point); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (d, J = 6.8 Hz, 1H, H8), 8.12 – 8.00 (m, 1H, H5), 7.87 – 7.70 (m, 2H, H6 and H7), 6.77 (s, 1H, H4'), 6.62 (s, 2H, H2' and H6'), 2.29 (s, 6H, 2x -CH_3); HPLC-ESI-MS (*m/z*): $[M]^+$ (313.06).

Synthesis of 2-chloro-3-(4-chlorophenoxy)-1,4-naphthoquinone **2e**

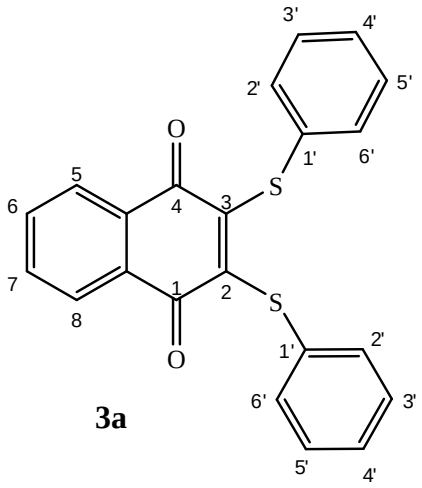


2e

To a solution of 4-chlorophenol (1.2856 g; 10 mmol) in absolute ethanol (25 mL) was added triethylamine base (0.45 mL; 3 mmol) and then 2,3-dichloro-1,4-naphthoquinone (1.135 g; 5 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 6 hours. The reaction mixture was then cooled to room temperature and the precipitated crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from hexane and then further from methanol to afford 2-chloro-3-(4-chlorophenoxy)-1,4-naphthoquinone **2e** as a yellow powder; (0.6834 g; 42.8%); mp 129.7-132.1 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (dd, *J* = 7.3, 1.5 Hz, 1H, H8), 8.06 (dd, *J* = 7.2, 1.5 Hz, 1H, H5), 7.85 – 7.75 (m, 2H, H6 and H7), 7.30 (d, *J* = 8.9 Hz, 2H, H3' and H5'), 6.96 (d, *J* = 8.9 Hz, 2H, H2' and H6').

2.2.2.3 Coupling with thiophenols

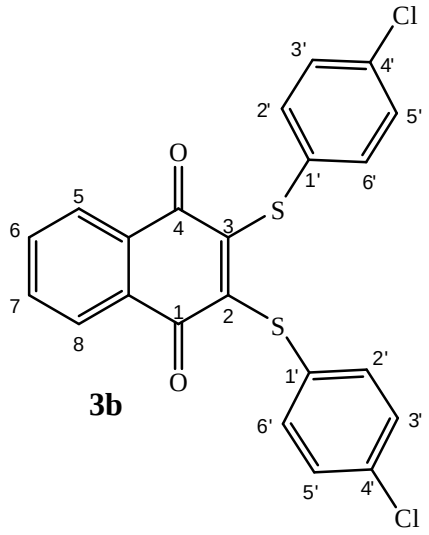
Synthesis of 2,3-bis(phenylthio)-1,4-naphthoquinone **3a**



3a

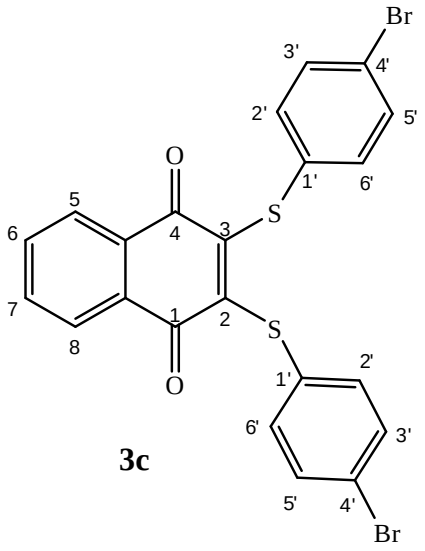
To a solution of thiophenol (2 mL; 20 mmol) in absolute ethanol (50 mL) was added 2,3-dichloro-1,4-naphthoquinone (2.27 g; 10 mmol). The reaction mixture was stirred and heated under in a silicone oil bath, maintained at 40 °C for 6 hours. The reaction mixture was cooled to room temperature and left in a fume hood overnight to get rid of the sulphur-like smell. The precipitated crude product was collected on a Büchner funnel by vacuum filtration. The residue was recrystallised from a mixture of hexane and methanol to afford 2,3-bis(phenylthio)-1,4-naphthoquinone **3a** as dark orange, needle-shaped crystals; (3.2677 g; 87.3%); mp 150.8-152.8 °C (lit[1] 150 °C) ;^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00 – 7.96 (m, 2H, H5 and H8), 7.69 – 7.65 (m, 2H, H6 and H7), 7.39 – 7.37 (m, 4H, 2x H2' and H6'), 7.32 – 7.28 (m, 6H, 2x H3', H4' and H5'); HPLC-ESI-MS (*m/z*): $[M]^+$ (374.95).

Synthesis of 2,3-bis(4-chlorophenylthio)-1,4-naphthoquinone **3b**



To a solution of 4-chlorothiophenol (0.7231 g; 5 mmol) in absolute ethanol (25 mL) was added 2,3-dichloro-1,4-naphthoquinone (1.135 g; 5 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 6 hours. The reaction mixture was cooled to room temperature and the precipitated crude product was collected on a Büchner funnel by vacuum filtration. The residue was first recrystallised from methanol and then further from a methanol/hexane (3:2) mixture to afford 2,3-bis(4-chlorophenylthio)-1,4-naphthoquinone **3b** as a mud brown, fluffy solid; (1.1155 g; 50.3%); mp 178.6-179.9 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00 – 7.96 (m, 2H, H5 and H8), 7.72 – 7.67 (m, 2H, H6 and H7), 7.32 (d, *J* = 8.5 Hz, 4H, 2x H3' and H5'), 7.28 (d, *J* = 8.5 Hz, 4H, 2x H2' and H6').

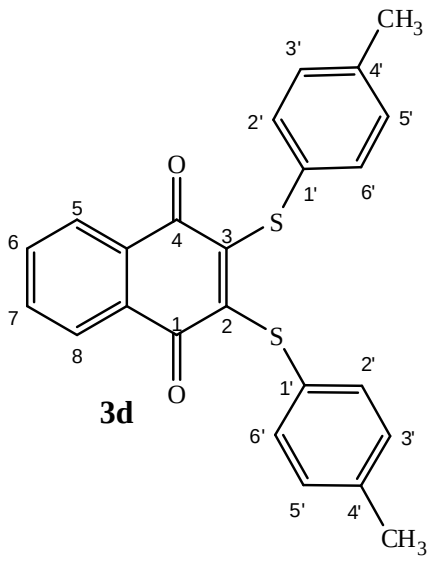
Synthesis of 2,3-bis(4-bromophenylthio)-1,4-naphthoquinone **3c**



3c

To a solution of 4-bromothiophenol (0.9454 g; 5 mmol) in absolute ethanol (25 mL) was added triethylamine base (1 mmol; 0.15 mL) and 2,3-dichloro-1,4-naphthoquinone (0.5675 g; 2.5 mmol). The reaction mixture was stirred and heated in a silicone oil bath, maintained at 80 °C for 6 hours. The product could be observed precipitating a few minutes after setting up the reaction. After 6 hours, the reaction mixture was cooled to room temperature and the precipitated crude product was collected on a Büchner funnel by vacuum filtration. The residue was first recrystallised from hexane and then from methanol to afford 2,3-bis(4-bromophenylthio)-1,4-naphthoquinone **3c** as a mud brown, fluffy solid; (0.9970 g; 74.9%); mp 196.9-198.1 °C (lit[9] 207 °C); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00 – 7.96 (m, 2H, H5 and H8), 7.72 – 7.67 (m, 2H, H6 and H7), 7.43 (d, *J* = 8.4 Hz, 4H, 2x H3' and H5'), 7.24 (d, *J* = 8.4 Hz, 4H, 2x H2' and H6').

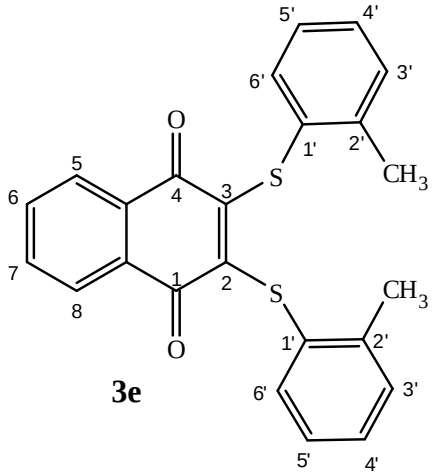
Synthesis of 2,3-bis(p-tolylthio)-1,4-naphthoquinone **3d**



3d

To a solution of 4-methylthiophenol (0.621 g; 5 mmol) in absolute ethanol (25 mL) was added triethylamine base (2 mmol; 0.3mL) and 2,3-dichloro-1,4-naphthoquinone (0.5675 g; 2.5 mmol). The reaction mixture was stirred at room temperature for 12 hours. The crude product was collected on a Büchner funnel by vacuum filtration. The residue was first recrystallised from hexane in a silicone oil bath set to 80 ℃ and a maroon product with traces of brown was obtained. Further recrystallisation from methanol afforded 2,3-bis(p-tolylthio)-1,4-naphthoquinone **3d** as a maroon powder; (0.5867 g; 58.3%); mp 174.8-176.8 ℃ (lit.[9] 169 ℃); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00 – 7.94 (m, 2H, H5 and H8), 7.69 – 7.62 (m, 2H, H6 and H7), 7.29 (d, *J* = 8.1 Hz, 4H, 2x H2' and H6'), 7.11 (d, *J* = 8.0 Hz, 4H, 2x H3' and H5'), 2.34 (s, 6H, 2x -CH_3).

Synthesis of 2,3-bis(o-tolylthio)-1,4-naphthoquinone **3e**



3e

To a solution of 2-methylthiophenol (1.18 mL; 10mmol) in absolute ethanol (25 mL) was added triethylamine base (3 mmol; 0.45mL) and 2,3-dichloro-1,4-naphthoquinone (1.135 g; 5 mmol). The reaction mixture was stirred at room temperature for 24 hours. The crude product was collected on a Büchner funnel by vacuum filtration. The residue was first recrystallised from hexane in a silicone oil bath set to 80 °C and then further recrystallisation from methanol to afford 2,3-bis(o-tolylthio)-1,4-naphthoquinone **3e** as a yellow, fluffy solid; (1.4732 g; 73.2%); mp 189.7-192.5 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.98 – 7.92 (m, 2H, H5 and H8), 7.67 – 7.61 (m, 2H, H6 and H7), 7.29 (d, *J* = 7.8 Hz, 2H, 2x H6'), 7.24 – 7.18 (m, 4H, 2x H4' and H5'), 7.10 (td, *J* = 8.3, 4.2 Hz, 2H, 2x H3'), 2.46 (s, 6H, 2x -CH_3).

2.3 BIOLOGICAL ASSAY METHODS

The biological assay was conducted by Raffaella Grimaldi of the Drug Discovery Unit at the University of Dundee in Dundee, Scotland using a similar procedure to that stipulated in one of their publication.[10] The synthesised 1,4-naphthoquinone derivatives were given compound codes and packed into Eppendorf tubes for transportation. The compound codes used were denoted by my initials, the year and the page numbers of the laboratory note book. For example, compound **1a** was coded as CC13(3)-24 and chemical names were not revealed.

2.3.1 Chemicals and materials used for biological assays

Melarsoprol used as the reference standard was from Rhone-Poulenc (France), resazurin was from Sigma, the white bottom clear plates for *T. brucei* growth assays where obtained from Greiner and the echo plates from LabCyte.

2.3.2 Trypanosome culture, preparation of compounds and growth assays

Culturing of bloodstream-form *T. b .brucei* (strain 427) was carried out at 37ºC in the presence of 5% carbon dioxide in HMI9-medium.[10] Cells were diluted to 2 x 10^4 mL^{-1} for 48 hour passages or diluted to 2 x 10^4 mL^{-1} for 72 hour passages. The cells were maintained in vented T75 flasks and the day before plating in 384-well plate, the cultures used for screening were split to 2 x 10^4 mL^{-1}.

To prepare the potency curves, 30 µL of compound in 10 mM in DMSO was dispensed into the 384-well compound-holding plate manually. Assay plate preparations were carried out by transferring the serial dilutions to LabCyte Echo certified plates and 250 nL of each concentration was dispensed into white clear bottom 384-assays plates.[10]

Standard growth assays were carried out by plating bloodstream form *T. brucei*, in fresh medium, into columns 1-22 of the 384-well assay plates using a Wellmate dispenser (50 µL/well). Thereafter, incubation for 68 hour at 37 ºC in the presence of 5% carbon dioxide was carried out. At 0.05 mM final concentration, resazurin was added and the plates were incubated

for another 4 hours. Fluorescence was then measured using a Perkin Elmer Victor 3 plate-reader (excitation 528 nm, emission 590 nm). The determination of growth rates was conducted by harvesting cells every 24 hours from 384-well plates and they were counted in a CASY Counter (Roche).[10]

2.3.3 Data analysis

All data were the result of one independent measurement. The obtained monophasic dose-response curves were fitted in Excel Add-in XL-fit (IDBS) following the 4 parametric equation equation:

$$y = A + \frac{B-A}{1+(C/x)^D}$$

where A = % inhibition at bottom, B = % inhibition at top, C = EC_{50}, D = Hill slope, x = inhibition concentration and y = % inhibition.

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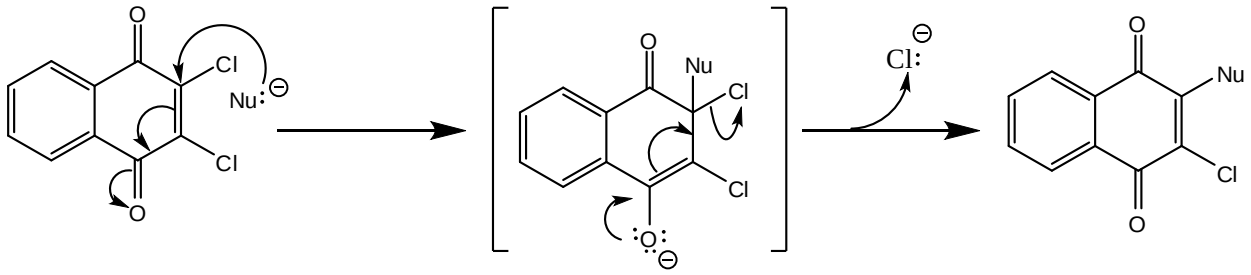
CHAPTER III: RESULTS AND DISCUSSION OF THE SYNTHESIS

3.1 NUCLEOPHILIC AROMATIC SUBSTITUTION REACTIONS

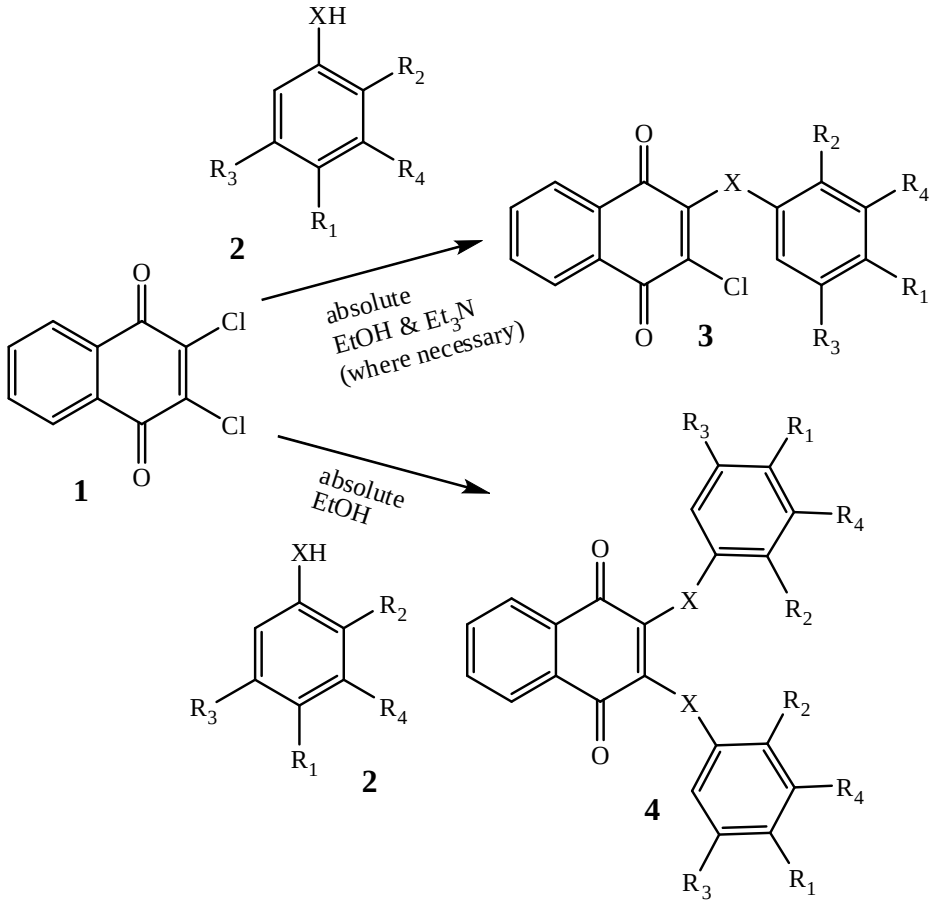


Scheme 3.1: Proposed mechanism of reaction of nucleophilic aromatic substitution of 2,3-dichloro-1,4-naphthoquinone with oxygen, nitrogen and sulphur nucleophiles, where Nu = nucleophile. The cycle is repeated in the case of disubstituted products to eliminate the second chlorine atom.

Naphthoquinone derivatives with amino and thioether linkages have been described as a component of the molecular framework of numerous biologically potent chemical entities.[1,2] This became the foundation of exploring the synthesis of naphthoquinone derivatives by nucleophilic substitution with oxygen, sulphur and nitrogen nucleophiles in our study. Naphthoquinones are deemed to be a privileged class of compounds, as they have been reported to possess a variety of biological activities, including anti-parasitic, anti-fungal, anti-cancer, anti-bacterial, anti-inflammatory, anti-molluscicidal and anti-viral activities.[3-5] This is why an electrophile with the naphthoquinone backbone was chosen for the coupling reactions with nitrogen, oxygen and sulphur nucleophiles. Synthesis of naphthoquinone derivatives is usually achieved from 2,3-dichloro-1,4-naphthoquinone – a commercially available substrate which is relatively affordable.

Various nitrogen, oxygen and sulphur nucleophiles with electron-withdrawing halogen substituents and electron-donating methyl substituents on para, meta and ortho positions were selected for coupling with the naphthoquinone (**Scheme 3.2** and **Table 3.1**). The N-, S- and O-

substituted 1,4-naphthoquinone derivatives had amino, thioether and ether linkages respectively. The purpose of using various nucleophiles was to investigate the effects of the structural features or functional group substituents of the naphthoquinone derivatives on biological activity. A structure-activity relationship study was conducted on the library of compounds to determine which structural features contribute or take away from the desired anti-trypanosomal activity.



where X= -O- , -S-, -NH-

Et_3N = triethylamine, EtOH = ethanol

R_1, R_2, $R_3$and R_4 are defined in **Table 3.1** below.

Scheme 3.2: General reaction scheme of the coupling of 2,3-dichloro-1,4-naphthoquinone **1** with a nucleophile **2** to give either a monosubstituted product **3** or a disubstituted product **4** in a nucleophilic aromatic substitution reaction. Reaction conditions: absolute ethanol as the solvent, triethylamine as a base catalyst where applicable, temperatures between 25 °C and 80 °C.

Table 3.1: Nucleophiles that were reacted with 2,3-dichloro,1-4-naphthoquinone in absolute ethanol.

NUCLEOPHILE	X	R_1	R_2	R_3	R_4	% Isolated yield
1. Anilines						
a. Aniline	-NH-	-H	-H	-H	-H	57.8
b. 4-Chloroaniline	-NH-	-Cl	-H	-H	-H	69.9
c. 4-Bromoaniline	-NH-	-Br	-H	-H	-H	65.1
d. 4-Methylaniline	-NH-	$-CH_3$	-H	-H	-H	54.8
e. 4-(Trifluoromethyl)aniline	-NH-	$-CF_3$	-H	-H	-H	35.0
f. 2-Methylaniline	-NH-	-H	$-CH_3$	-H	-H	28.4
g. 3-Methoxyaniline	-NH-	-H	-H	-H	$-OCH_3$	56.9
h. 3-Methylaniline	-NH-	-H	-H	-H	$-CH_3$	72.3
2-(Trifluoromethyl)aniline	-NH-	-H	$-CF_3$	-H	-H	0
2. Phenols						
a. Phenol	-O-	-H	-H	-H	-H	56.1
b. 2-Chlorophenol	-O-	-H	-Cl	-H	-H	40.8
c. 2-Chloro-4-methylphenol	-O-	$-CH_3$	-Cl	-H	-H	51.9
d. 3,5-Dimethylphenol	-O-	-H	-H	$-CH_3$	$-CH_3$	53.6
e. 4-Chlorophenol	-O-	-Cl	-H	-H	-H	42.8
Thymol	-O-	-H	$-C(CH_3)_2$	$-CH_3$	-H	0
3. Thiophenols						
a. Thiophenol	-S-	-H	-H	-H	-H	87.3
b. 4-Chlorothiophenol	-S-	-Cl	-H	-H	-H	50.3
c. 4-Bromothiophenol	-S-	-Br	-H	-H	-H	74.9
d. 4-Methylthiophenol	-S-	$-CH_3$	-H	-H	-H	58.3
e. 2-Methylthiophenol	-S-	-H	$-CH_3$	-H	-H	73.2

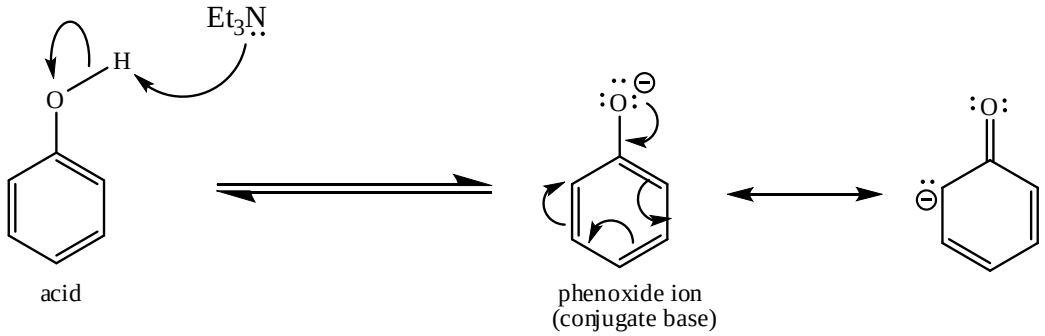
A number of reactions suffered from low yields as can be seen in **Table 3.1**. According to Furniss *et al*.(1989)[6], "the success of a reagent is judged from the percentage yield, being excellent (>90%), very good (>80%), good (>70%), fair (>50%) and poor (<40%)." Based on this statement, the obtained isolated percentage yields ranged between poor, fair, good and very good. Various reasons may be postulated as to why such average yields were possible. First, it is likely the reactions may have reached their equilibrium before completion because un-reacted substrate (naphthoquinone) was isolated after the recrystallisation procedures. The naphthoquinone was either a soluble impurity that precipitated from the filtrate of the recrystallisation mixture, and the desired product as the residue. The naphthoquinone could also be an insoluble impurity that became the residue from filtration of the recrystallisation mixture with the desired product precipitating from the filtrate. The ^{1}H NMR analysis of the un-reacted substrate confirmed that it was indeed the naphthoquinone.

Second, protic solvents used such as ethanol may possibly slow down the rate of reaction by solvating the reactant nucleophile. The -NH_2, -OH and -SH groups of the nucleophiles may form hydrogen bonds with the solvent (ethanol) molecules, which then cluster around the nucleophile and reduce its reactivity.[7,8] A strongly solvated nucleophile must shed off some of the solvent molecules, so as to attack the carbon of the naphthoquinone bearing the chloride leaving group.[8] In addition, sulphur atoms are bigger than oxygen atoms and are not as strongly solvated in protic solvents such as ethanol. As a result, aryl thiols (R-SH) are stronger and more powerful nucleophiles than phenols (R-OH); **Table 3.2**.[8-10] This was somewhat evinced by the higher yields obtained in reactions with thiol nucleophiles compared to the reactions involving phenols. Furthermore, greater reactivity of nucleophiles with large nucleophilic atoms may also be attributed to their greater polarisability. A bigger nucleophilic atom has the ability to donate a large electron density to the substrate compared to a smaller nucleophilic atom, whose electrons are more tightly held.[8]

Electron-withdrawing substituents on the nucleophiles reduce electron density from a conjugated pi (π) system through the inductive effects, thereby making them less nucleophilic. This has a negative effect on the rate of nucleophilic substitution reactions. On the other hand, electron donating substituents are deemed to be activating groups as they donate electrons, thereby increasing the electron density in the conjugated π system by making the nucleophile more

nucleophilic. Consequently, this has a positive effect on the rate of reaction, possibly resulting in higher yields.[8] The aforementioned, however, was generally not reflected in the results obtained for the isolated yields, as some of the nucleophiles with electron withdrawing groups had better yields than those with electron donating groups. For instance, the 4-chloroaniline and 4-bromoaniline reactions gave better yields of 69.9% and 65.1% respectively, compared to their para substituted analogue of 4-methylaniline with a 54.8% isolated yield. The theory, however, holds true for the 4-(trifluoromethyl)-aniline reaction, which had the most electronegative substituent and this, was reflected by the low percentage isolated yield (35%).

Critical parameters affecting the reaction progress such as time and temperature were investigated in an attempt to improve the yields. Increasing the reaction time did not result in complete substrate conversion in most cases, as the un-reacted naphthoquinone was still recovered during reaction work-up. A detailed optimisation study of the reaction conditions and parameters may have been the best option to improve the percentage isolated yields. At this stage, optimisation and improvement of the yields were not the primary objectives of the project.

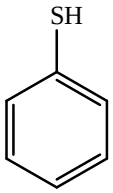
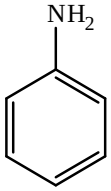
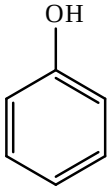


Scheme 3.3: The deprotonation of phenol using a triethylamine base to form the phenoxide ion.

Given that phenol is a weak acidic nucleophile[9,10], a base catalyst had to be added to the reaction mixture in order to deprotonate the phenol and activate it (**Scheme 3.3**). The phenoxide ion has a more stabilised resonance compared to phenol because the negative charge can be delocalised into the aromatic ring.[11] Without the addition of a base, phenol was not reactive and hence could

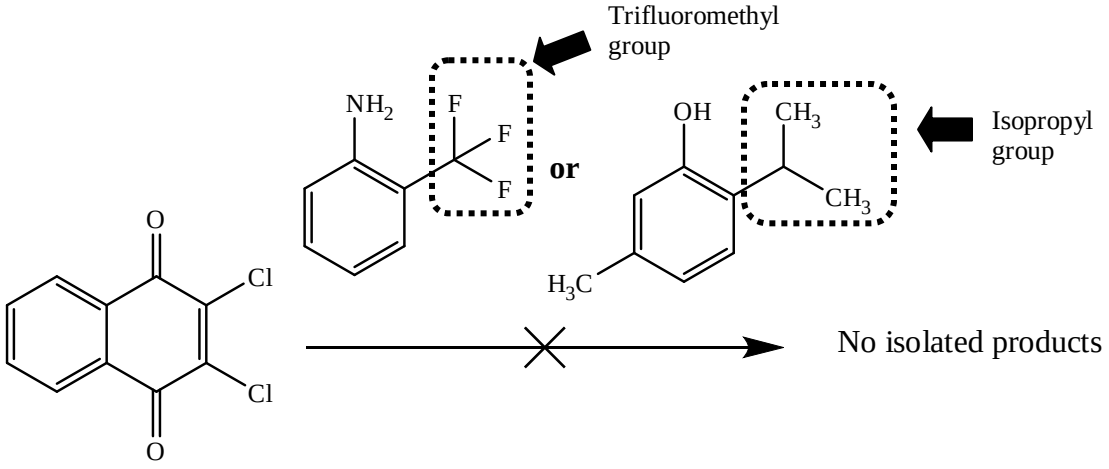
not couple with 2,3-dichloro-1,4-naphthoquinone. Activation of anilines and thiols was not necessary, as these reactants were readily reactive (**Table 3.2**).

Table 3.2: Physicochemical properties of thiophenol, aniline and phenol.[10]

Nucleophile	Physicochemical properties[10]	Relative reactivity
	• Thiophenol • Acidic nucleophile • pKa = 9-10	More reactive
	• Aniline • Basic nucleophile • pKa = 4-5	Reactive
	• Phenol • Acidic nucleophile • pKa = 9-11	Less reactive

3.2 STERIC HINDERANCE

Nucleophilic aromatic substitution reactions involving 2-(trifluoro-methyl)-aniline and 2-isopropyl-5-methylphenol (thymol) failed to produce any product (**Table 3.1** and **Scheme 3.4**). Alterations to the reaction conditions did not yield the desired products, possibly due to steric hindrance by the bulky *ortho* substituents. Steric hindrance occurs when two atoms or functional groups are so close to each other that there is repulsive interaction between, them due to spatial crowding. This spatial arrangement of atoms or functional groups near the reacting site hinders the reaction from proceeding.[8] The ^{1}H NMR spectra obtained after each attempt only showed two multiplets of the un-reacted substrate (2,3-dichloro-1,4-naphthoquinone) that was recovered at the end of the reactions.



Scheme 3.4: Reaction scheme of the attempted synthesis of naphthoquinone derivatives with 2-(trifluoromethyl)-aniline and thymol (2-isopropyl-5-methylphenol) highlighting the bulky *ortho*-substituents on these nucleophiles.

Steric parameters, such as the Van der Waal's radii (Å), have been used to demonstrate that the trifluoromethyl group (2.20 Å) is much larger in steric bulk than the methyl group (1.80 Å).[12,13] This could explain why reactions with nucleophiles consisting of smaller ortho substituents yielded some product for example, the substitution reaction with 2-methylaniline. It could be that steric effects from the smaller *ortho* substituents possibly had a retarding effect on the relative reaction rate, as poor yields were obtained, whilst those from the bulky ortho substituents

hindered the reaction progress altogether.[8] The trifluoromethyl group is sterically as large as the isopropyl group; both functional groups have a similar Van der Waal's radius of 2.20 Å.[12]

3.3 RECRYSTALLISATION

Purification of the synthesised products was achieved by the process of recrystallisation from organic solvents. Suitable solvents were either obtained from literature or developed by a trial and error procedure.

The major impurity recovered from the recrystallisation procedures was the un-reacted substrate of 2,3-dichloro-1,4-naphthoquinone. Thin layer chromatography (TLC) and ^{1}H NMR analysis of almost all the recrystallised products showed traces of the naphthoquinone after the first recrystallisation procedure. Further purification was much needed; so the recrystallisation procedures were repeated in fresh solvent mixtures, or a single solvent, until traces of the naphthoquinone and other impurities could not be seen on the ^{1}H NMR spectrum. Melting point determinations also assisted in confirming whether a product had reached purity, as it would remain unchanged.

It is common knowledge that after a recrystallisation procedure a product may still remain impure and further recrystallisation from a fresh solvent will be required. The principal underlying the removal of impurities by recrystallisation is the difference in solubilities between the product and the impurities in any given solvent. In cases where the impurity is more soluble than the expected product, it would be apparent that several recrystallisation procedures will yield a pure sample of the product.[6]

Tandon and colleagues (2009)[5] reported a dual method of purification of naphthoquinone derivatives. Firstly, the products were subjected to column chromatography and then further purified by recrystallisation.[5] Use of column chromatography proved challenging as both the un-reacted naphthoquinone and the desired product eluted at the same rate with very close R_f values. This can be attributed to the similarity in their functionalities as well as the polarity of their structures, because the naphthoquinone backbone is retained in the products. A series of recrystallisation procedures therefore seemed to be the best way to get rid of the un-reacted naphthoquinone. The stacked ^{1}H NMR spectra (**Figure 3.1** below) illustrates that the

naphthoquinone substrate was the trace impurity as its NMR signals at δ 8.22 and δ 7.79 were overlapping with equivalent protons in the product. Furthermore, melting point determinations of the isolated impurities were typically those of 2,3-dichloro-1,4-naphthoquinone which melts at 195 ºC. This trend was observed in all the synthesised products and recrystallisation was carried out until a 'clean' ^{1}H NMR spectrum was obtained.

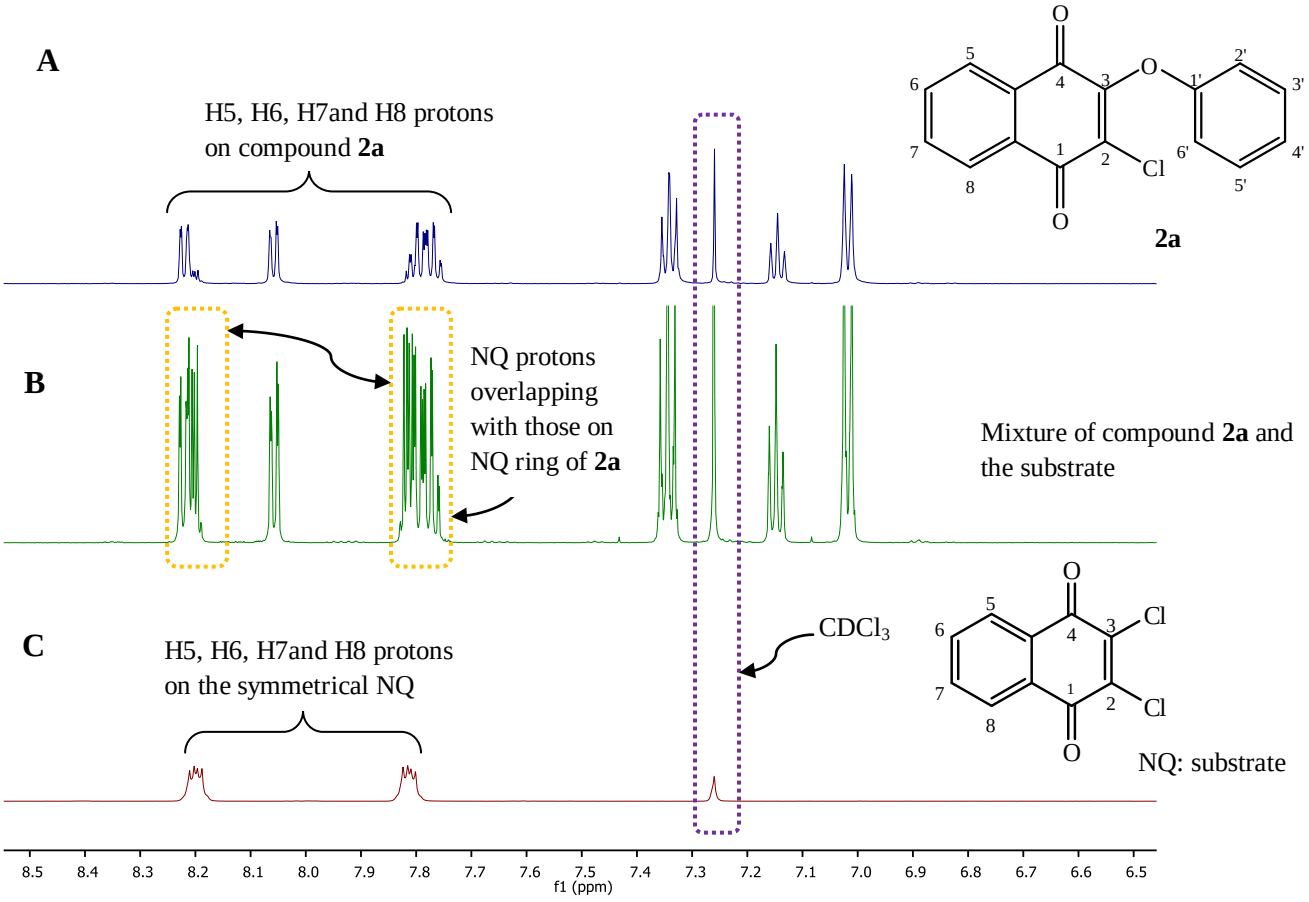


Figure 3.1: Stacked and expanded ^{1}H NMR spectra of: **a)** isolated 2-chloro-3-phenoxy-1,4-naphthoquinone **2a** after the second recrystallisation procedure; **b)** a mixture of compound **2a** and the un-reacted 2,3-dichloro-1,4-naphthoquinone after the first recrystallisation procedure; **c)** pure substrate: 2,3-dichloro-1,4-naphthoquinone; NQ = naphthoquinone. The signals highlighted in orange in **b)** are intensified compared to the same signals in **a)** and this is indicative of the trace impurity, which is the un-reacted substrate.

3.4 MONOSUBSTITUTED 1,4-NAPHTHOQUINONE DERIVATIVES

The aims of the synthetic procedures were to obtain both mono- and disubstituted products by nucleophilic displacement of the chlorine atom(s) on the quinone with nitrogen, oxygen and sulphur heteroatoms of the reactants. The task proved to be a challenging one, as only monosubstituted products were obtained from substitution with aniline and phenol nucleophiles; whilst only disubstituted products were obtained from substitution with thiol nucleophiles.

Coupling reactions of 2,3-dichloro-1,4-naphthoquione with anilines and phenols selectively yielded monosubstituted products (compounds **1a**-**1h** and **2a**-**2e**), despite the addition of two molar equivalents of the above mentioned nucleophiles. **Figure 3.2** illustrates an expansion of the super-imposed ^{1}H NMR spectra of the products obtained from coupling 2,3-dichloro-1,4-naphthoquinone and aniline (1:2 ratios respectively; red spectrum) and the same reactants in a 1:1 ratio; the blue spectrum. A similar spectrum, indicative of 2-chloro-3-(phenylamino)-1,4-naphthoquinone (compound **1a**) was obtained, despite coupling with two molar equivalents of the aniline. Integration of the signals gave intensity ratios corresponding to a total of ten protons in both spectra. The full ^{1}H NMR spectra can be found in the supplementary data.

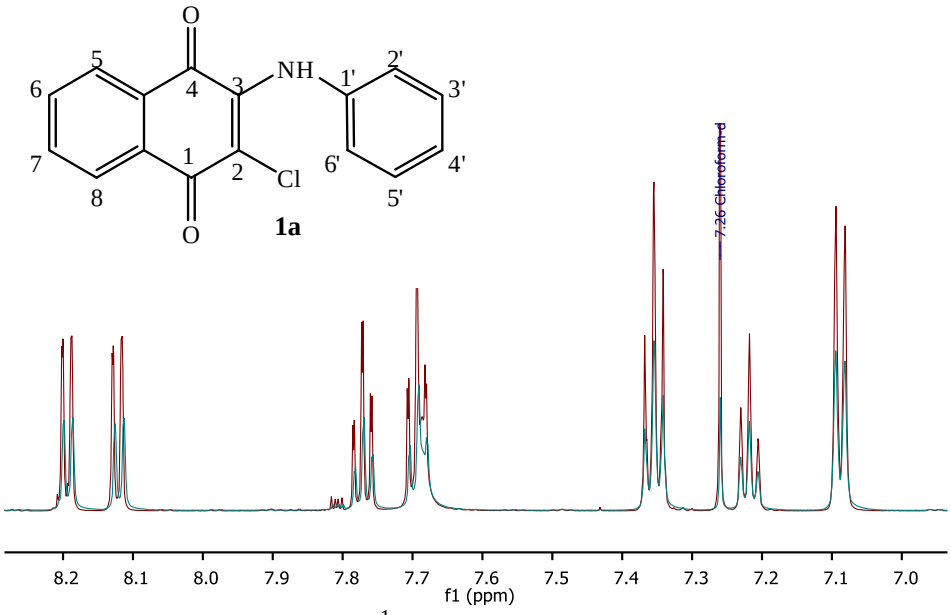


Figure 3.2: Expansion of the super-imposed ^{1}H NMR (600MHz, $CDCl_3$) spectra of the product obtained from coupling 2,3-dichloro-1,4-naphthoquinone and aniline (1:2 ratios respectively; red spectrum) and the same reactants in a 1:1 ratio; blue spectrum.

Intensity ratios obtained from the integration of the respective ^{1}H NMR spectra was the first evidence obtained to substantiate the formation of only monosubstituted products with respect to the number of protons. Furthermore, ^{13}C NMR spectra of these products obtained from anilines and phenols all showed two quaternary carbon peaks in the region between δ 178 and δ 180 which, is indicative of the non-equivalent carbonyl carbons present on the naphthoquinone backbone. 2,3-Dichloro-1,4-naphthoquinone is a symmetrical substrate and the structural asymmetry of the monosubstituted products can assist in explaining the occurrence of two carbonyl peaks.[14,15] The ^{13}C NMR spectrum of 2-chloro-3-(phenylamino)-1,4-naphthoquinone, compound **1a**, shows the two quaternary carbon signals corresponding to the non-equivalent carbonyl groups (**Figure 3.3**). The two quaternary carbons peaks are seen in all the ^{13}C NMR spectra of compounds **1a-1h** and **2a-2e**.

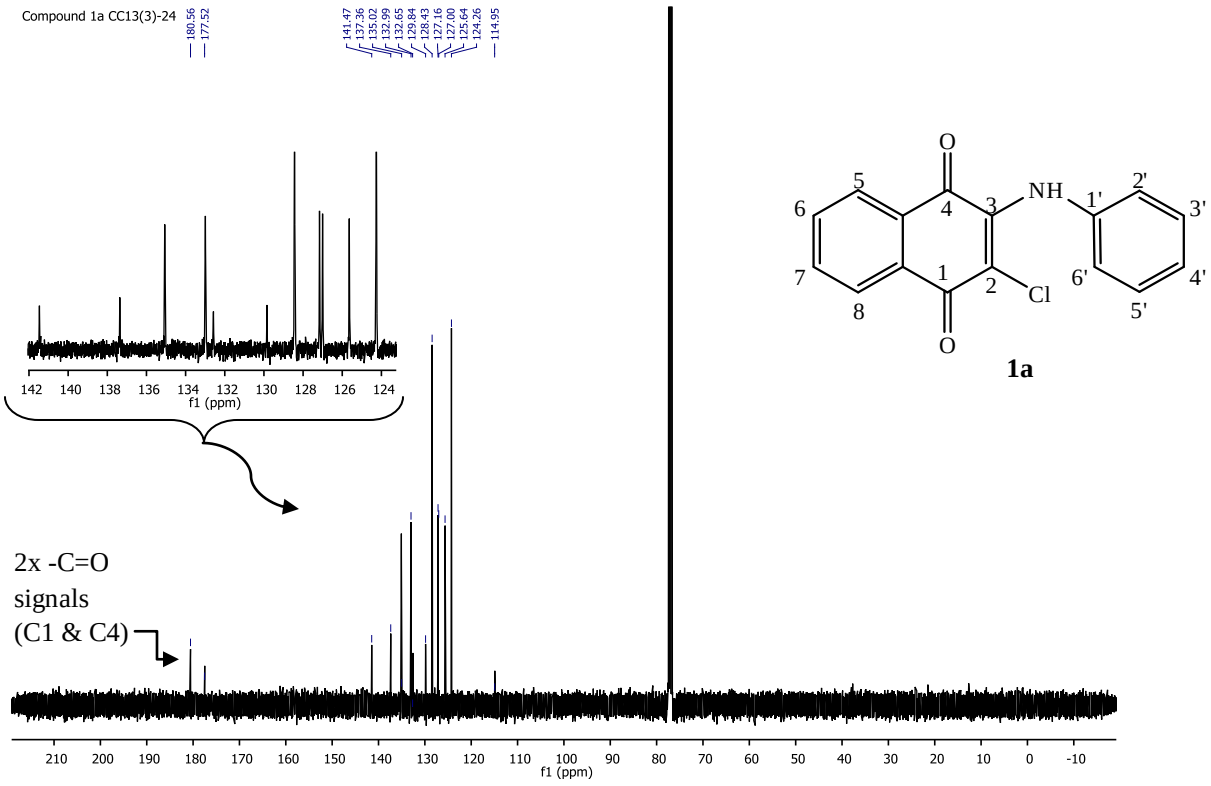


Figure 3.3: ^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2-chloro-3-(phenylamino)-1,4-naphtho-quinone **1a** showing the two quaternary carbon signals corresponding to the non-equivalent carbonyl groups. Expansion of the signals between 124 ppm and 142 ppm are shown in the off-set plot.

The HPLC-ESI-MS provided molecular weights which further substantiated the formation of monosubstituted products from phenols and anilines.

Electron density of the quinone backbone becomes uneven when the anilines and phenols only displace the chlorine atom on position three, leaving out the other on position two. Similar findings have been reported in literature, confirming the ease of formation of monosubstituted products in nucleophilic aromatic substitution reactions of naphthoquinones with anilines and phenols.[14,16] Nucleophilicity of substrates has been described as one of the determining factors of substitution of one or both chlorine atoms of 2,3-dichloro-1,4-naphthoquinone.[3] It has been reported that aryl amines of enhanced nucleophilicity substitute only one chlorine atom of 2,3-dichloro-1,4-naphthoquinone as a result of electronic enrichment of the quinone system. An electron withdrawing effect ought to be imposed on the quinone ring in order to substitute the second chlorine atom or a catalyst be employed.[3,14]

3.5 DISUBSTITUTED 1,4-NAPHTHOQUINONE DERIVATIVES

Contrary to anilines and phenols, thiophenols selectively produced disubstituted products when coupled with 2,3-dichloro-1,4-naphthoquinone. It has been noted in literature that aryl thiols spontaneously react with 2,3-dichloro-1,4-naphthoquinone, also known as dichlone, to obtain 2,3-bis(thio)-1,4-naphthoquinones (dithiothers)[3,17] compared to aryl amines that yield mono-arylamino-1,4-naphthoquinone as major products.[3]

Use of one molar equivalent of thiol nucleophiles produced disubstituted products and attempts to obtain monosubstituted products using a procedure stipulated by Tandon and colleagues (2009)[5] were not fruitful. Each attempt using one molar equivalent yielded a mixture of the disubstituted product and un-reacted 2,3-dichloro-1,4-naphthoquinone, after which the product was isolated by recrystallisation. Thiophenol and its aryl thiol analogues were so reactive that they displaced the two chlorine atoms on position two and three of the quinone backbone despite being the limiting reagent.

The ^{1}H NMR spectra of the products obtained from coupling with thiophenols (compounds **3a-3e**) all typically showed two multiplets, corresponding to the four protons on the quinone backbone. This meant protons **H5** and **H8** are in the same electronic environment hence they

resonate at the same frequency and can observed as one multiplet at δ 7.98. The same applies to protons **H6** and **H7** which were observed as a multiplet at δ 7.67 (**Figure 3.4** below).

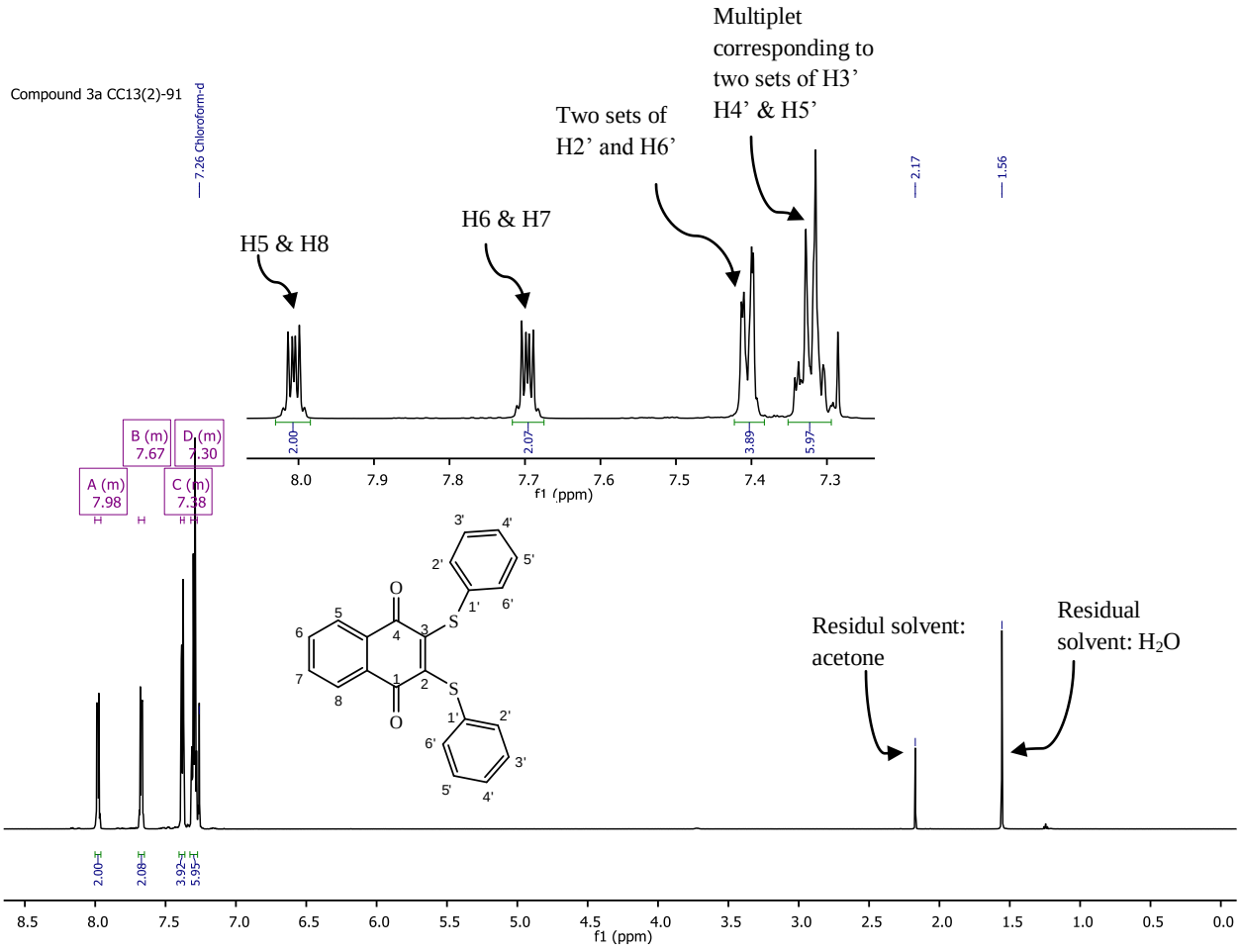


Figure 3.4: ^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2,3-bis(phenylthio)-1,4-naphthoquinone **3a**. The product was obtained by coupling 2,3-dichloro-1,4-naphthoquinone with either two molar equivalents or one molar equivalent of thiophenol. Expansion of the signals in the aromatic region is shown in the off-set plot.

In the ^{13}C NMR spectra of the disubstituted products, compounds **3a**-**3e**, carbon atom signals of the carbonyl groups were observed as one peak around δ 178. Due to the symmetrical structures, the two carbonyl groups are present in the same electronic environment and are equivalent; therefore they resonate at the same frequency and are observed as one carbonyl peak. This data

supports the disubstituted structural assignment of compounds **3a**-**3e**. Recently, Ibis and colleagues (2013)[15] reported the coupling of 2,3-dichloro-1,4-naphthoquinone with several other sulphur nucleophiles and a similar pattern was observed. These researchers also observed the two carbonyl groups on the naphthoquinone as one peak/signal in symmetrical structures but as two peaks in asymmetrical naphthoquinone derivatives.[15] Wellington and colleagues (2012)[17] synthesised compound **3a** via a one-pot laccase enzyme catalysed reaction and the ^{13}C NMR spectrum of this disubstituted product also revealed one peak at δ 178.7 corresponding to the two equivalent carbon atoms of the quinone carbonyl groups.[17] All the aforementioned literature reports further support our structural assignments of the naphthoquinone derivatives.

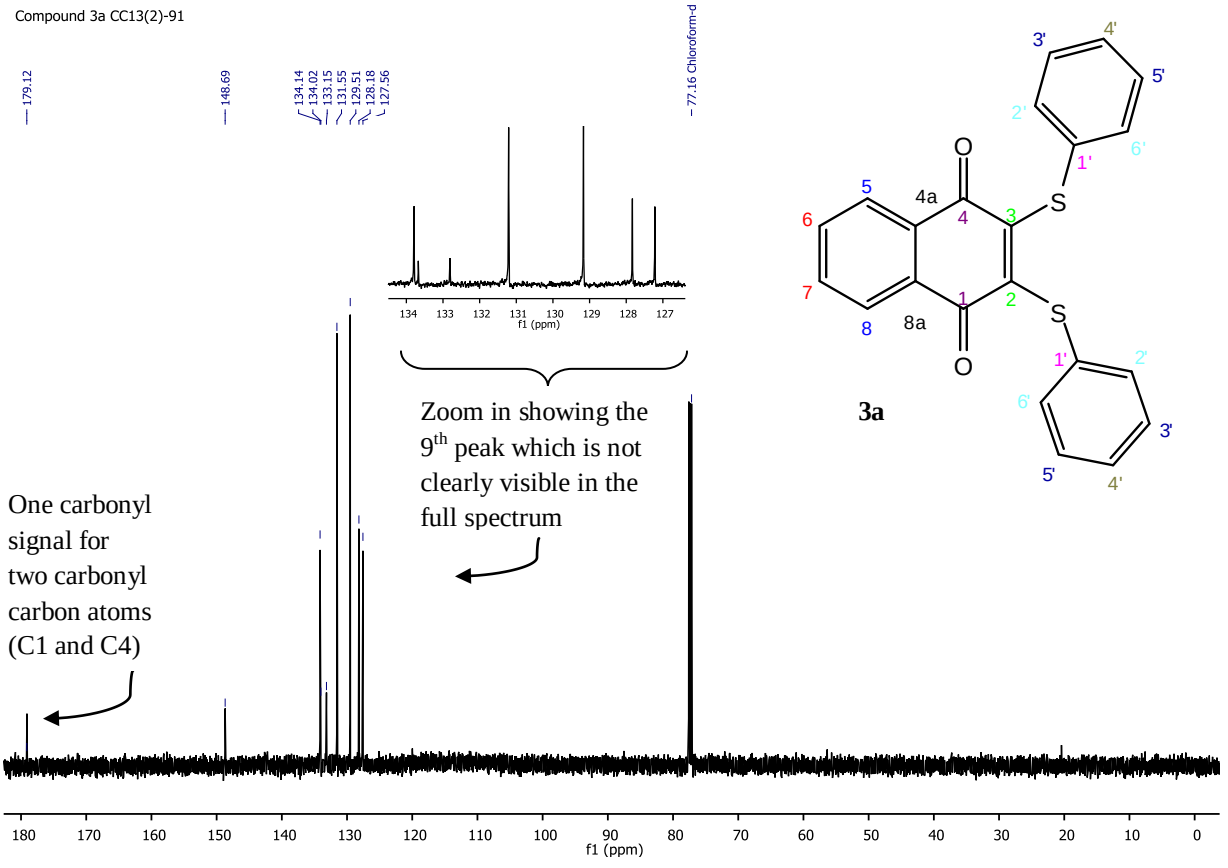


Figure 3.5: ^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2,3-bis(phenylthio)-1,4-naphthoquinone **3a**. One peak corresponding to the two equivalent carbonyl groups on the quinone was observed at δ 179. Refer to the appendix for the ^{13}C NMR spectra of compounds **3b**-**3e**.

The ^{13}C NMR spectrum of compound **3a** - illustrated in **Figure 3.5 -** revealed a total of nine peaks, corresponding to 22 carbon atoms. As a result of the symmetrical nature of the structure, carbon atoms in the same electronic environment resonate at the same frequency and share a similar chemical shift. Equivalent carbon atoms of compound **3a** have been colour coded in its structure (**Figure 3.5** above) and there are a total of nine colours, with each one denoting a carbon peak.

3.6 SIMILAR 1,4-NAPHTHOQUINONES PREVIOUSLY SYNTHESISED

Compound **1a** was previously synthesised and tested for anti-fungal activity by several researchers, including Tandon *et al.*[1] and Tran *et al.*[18] The latter group of researchers also reported the synthesis of compounds **1b**, **1c** and **1d** with the meta trifluoromethyl substituted analogue of compound **1e** also being reported by this group.[18] Benites *et al.*[2] synthesised and evaluated compound **1f** for anti-tumour activity. Compounds **2a**, **2d** and **2e** were also synthesised by Tandon *et al.*[19] and were evaluated for anti-fungal activity. Compound **2a** was also reported by Lee *et al.*[16] Compound **3a** was obtained by Tandon *et al.*[5,20] through a similar synthetic procedure and was evaluated for anti-fungal, anti-proliferative and anti-cancer activities. Wellington *et al.*[17] also synthesised compound **3a** using a one-pot laccase method, but no biological assays are mentioned in their publication. Singh *et al.*[21] synthesised compounds **3a**, **3c** and **3d** from oxidation of their respective 1,4-diols to produce quinones, and there was also no mention of any biological assays. Compound **1h**, the meta substituted analogue of compound **1d**, could not be found in the literature, nor could compounds **2b**, **2c**, **3b** and **3d**. Although some of the compounds have been previously synthesised and reported, none of these exact naphthoquinone derivatives been evaluated for anti-trypanosomal activity, to our best knowledge. Most researchers have focused on other biological activities, particularly anti-fungal activity.

3.7 SPECTROSCOPIC CHARACTERISATION OF THE 1,4-NAPHTHOQUINONES

Four NMR experiments (^{1}H, ^{13}C, COSY and HSQC) were conducted on each of the synthesised products. These were considered the most crucial, in this particular project, amongst many other NMR experiments for structural confirmation. (Refer to the appendix for the NMR spectra of the all compounds.) Both ^{1}H NMR and ^{13}C NMR are deemed to be the most important NMR experiments for an organic chemist as they provide information on the number and environment of protons and the carbon framework of a molecule respectively.[8]

Splitting or multiplicity in ^{1}H NMR arises from a phenomena called spin coupling which, in simplest terms, occurs when the nucleus undergoing resonance detects the spin of a nearby active nuclei.[22] Multiplicity of a resonance can be roughly predicted with the use of the n+1 rule, whereby a nucleus undergoing resonance which has two neighbouring/adjacent protons; will have its signal split into a triplet.[22] In the ^{1}H NMR spectrum, the intensity of a signal is deemed to be directly proportional to the number of protons that give rise to that signal.[22] Integration of the peaks to obtain intensity ratios was carried out and, if the total of the intensity ratios was equivalent to the number of expected protons, it would be a positive start to the structural assignment process and a further two dimensional NMR experiments would be conducted.

Compound **1g** 2-chloro-3-(3-methoxyphenylamino)-1,4-naphthoquinone which, was obtained by coupling 2,3-dichloro-1,4-naphthoquinone with 3-methoxyaniline, shall be used to demonstrate the procedure taken for characterisation of the synthesised products. Its ^{1}H NMR (**Figure 3.6**) revealed a singlet at δ 3.81, indicative of the methoxy (-OCH_3) protons which have been shifted downfield as a result of the deshielding effects of the electronegative oxygen atom compared to other methyl (-CH_3) containing products, whose signal resonated at δ 2.36. The rest of the ^{1}H NMR signals (**Figure 3.6**) were in the aromatic region as expected: the doublets at δ 8.19 and 8.11correspond to **H8** and **H5** protons respectively. The triplets at δ 7.76 and 7.69 are indicative of the other two protons of the naphthoquinone backbone which are **H7** and **H6** respectively; these assignments are consistent with literature.[3,18] The -NH singlet at δ 7.66 overlaps with a triplet; but in some of the amino-naphthoquinone derivatives, these two signals occurred separately. The remaining signals resonating at δ 7.24 (t), 6.76 (dd), 6.69 (dd) and 6.61 (s) correspond to the **H5'**, **H4'**, **H6'** and **H2'** protons of the 3-methoxyaniline backbone. Upon

integration of the ^{1}H NMR spectrum (**Figure 3.6**) relative to the signal at δ 8.19 which is responsible for only one proton, the intensity ratio indicated a total of twelve protons, supporting evidence that the product was monosubstituted. A disubstituted product would have been indicated by a total of twenty protons.

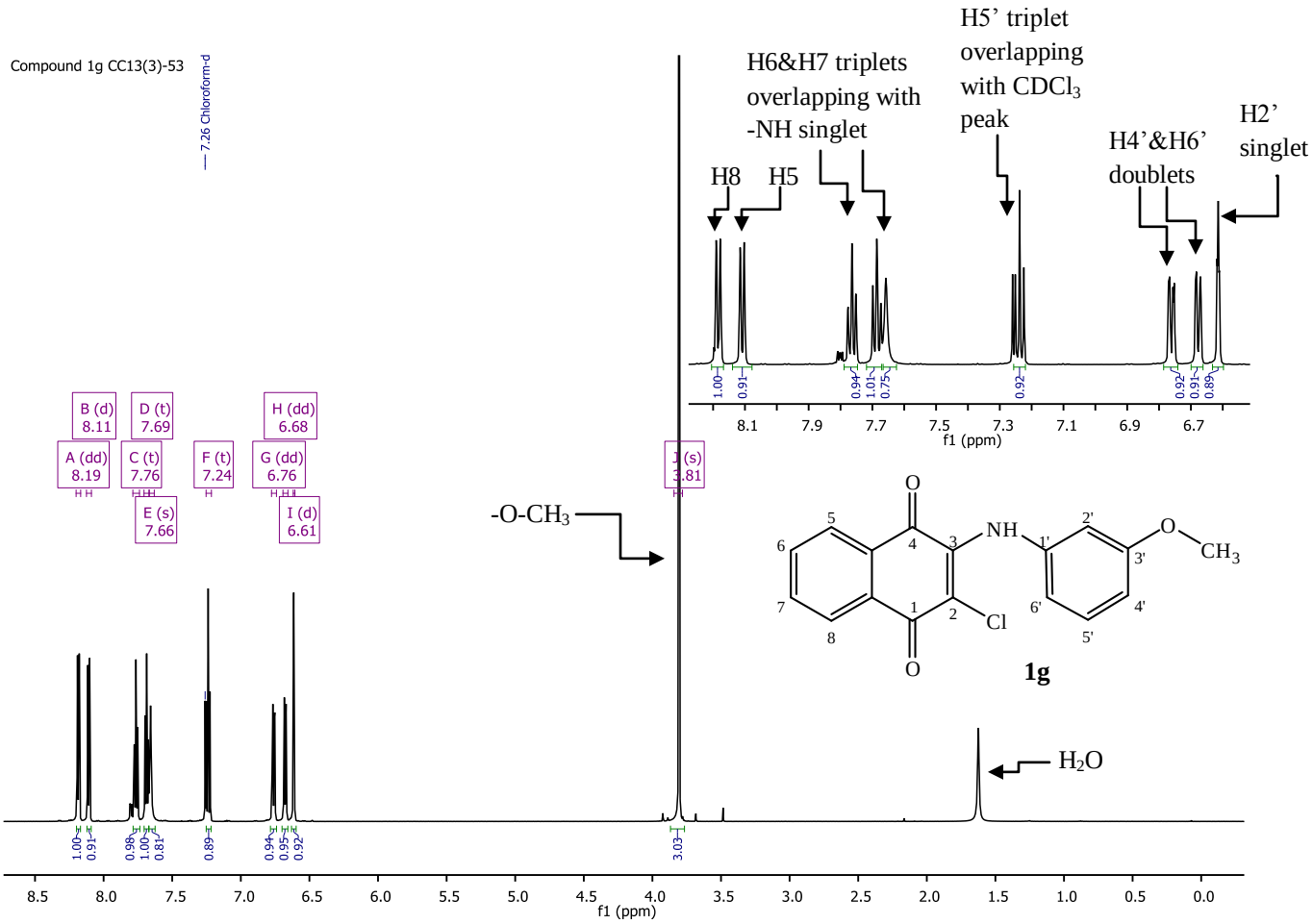


Figure 3.6: ^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2-chloro-3-(3-methoxy-phenylamino)-1,4-naphthoquinone **1g**. Expansions of signals in the aromatic region are shown in the off-set plot.

The ^{13}C NMR spectrum of compound **1g** has two quaternary carbon signals resonating at δ 180 and 177 (**Figure 3.7**), corresponding to the non-equivalent carbonyl carbon atoms which substantiates the formation of a monosubstituted product. A total of eight quaternary carbon

signals were observed, whilst the other nine carbon signals with greater peak height/intensity are directly attached to protons and their carbon-proton correlations can be seen in the HSQC NMR spectrum (**Figure 3.8**). Once again, the methoxy carbon signal is shifted downfield and resonates at δ 55, unlike the methyl carbons in other naphthoquinone derivatives, which resonated at δ 21. The HSQC NMR experiments were carried out to determine which carbon signals belonged to carbon atoms directly attached to protons. In the HSQC NMR spectrum of compound **1g** (**Figure 3.8**) there existed a cross-peak at the coordinates δ (3.81; 55.26), due to the methoxy protons and carbon atom respectively. Eight other HSQC cross-peaks were observed.

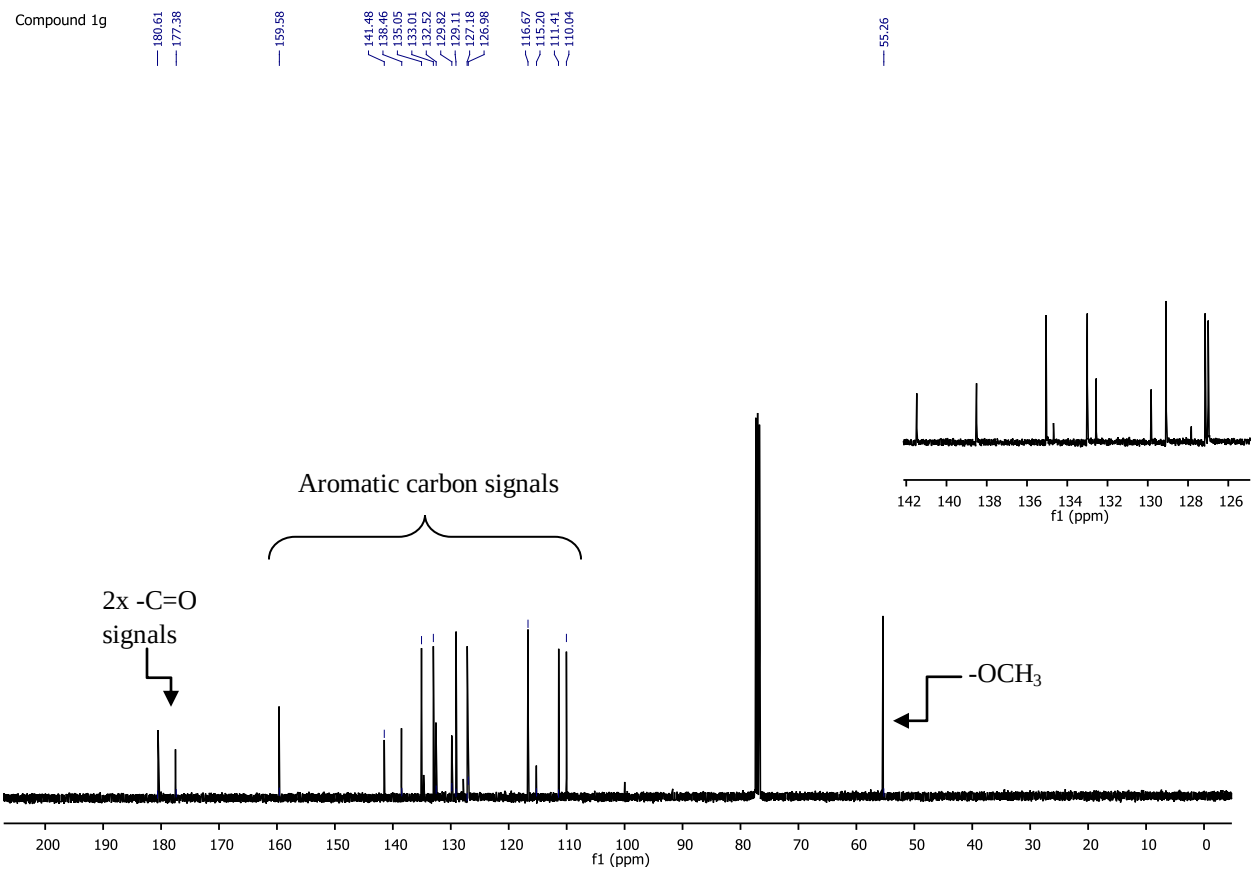


Figure 3.7: ^{13}C NMR (400MHz, $CDCl_3$) spectrum of 2-chloro-3-(3-methoxyphenylamino)-1,4-naphthoquinone **1g**. Expansion of the signals between 126 ppm and 142 ppm are shown in the off-set plot.

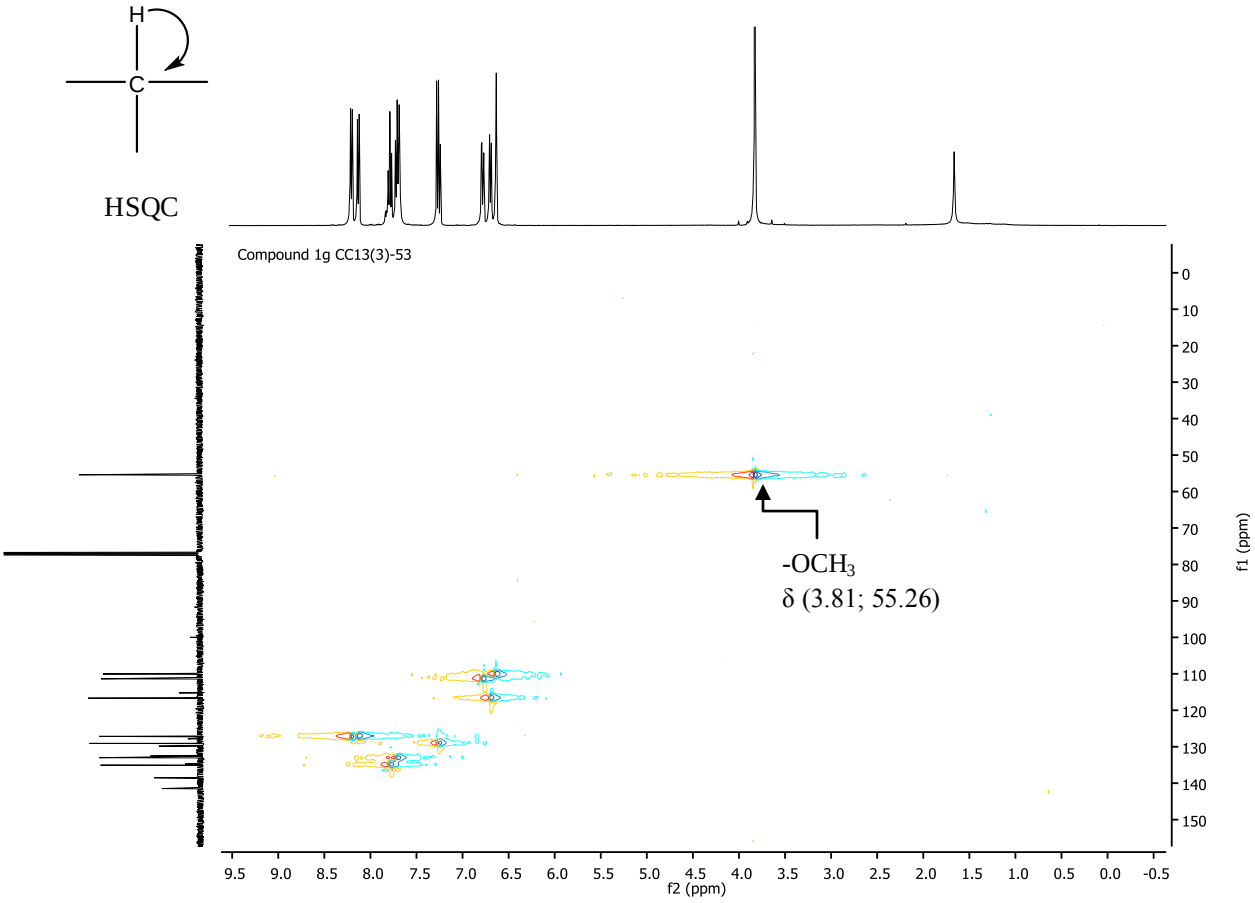


Figure 3.8: HSQC NMR spectrum of 2-chloro-3-(3-methoxyphenylamino)-1,4-naphtho-quinone **1g**.

The COSY NMR cross peaks, shown in **Figure 3.9** below, illustrated which protons were adjacent to each other and, in some cases, this may be confirmatory spectral data to the coupling (*J*) constants, as pairs of coupled protons always have the same *J* constants.[22] Therefore, COSY NMR spectra were used together with *J* constants to establish nearest-neighbour relationships in the structures of the synthesised products.

In the HPLC-ESI-MS spectra of compound **1g** (**Figure 3.10**) the molecular ion is observed as a multiple peak cluster with the base peak (most abundant ion) at *m/z* 316.73 $[M+3H]^+$ corresponding to the molecular weight of a monosubstituted product. The ^1H NMR, ^{13}C NMR, 2D NMR as well as the HPLC-ESI-MS data combined assisted in structural assignments of the synthesised naphthoquinone derivatives.

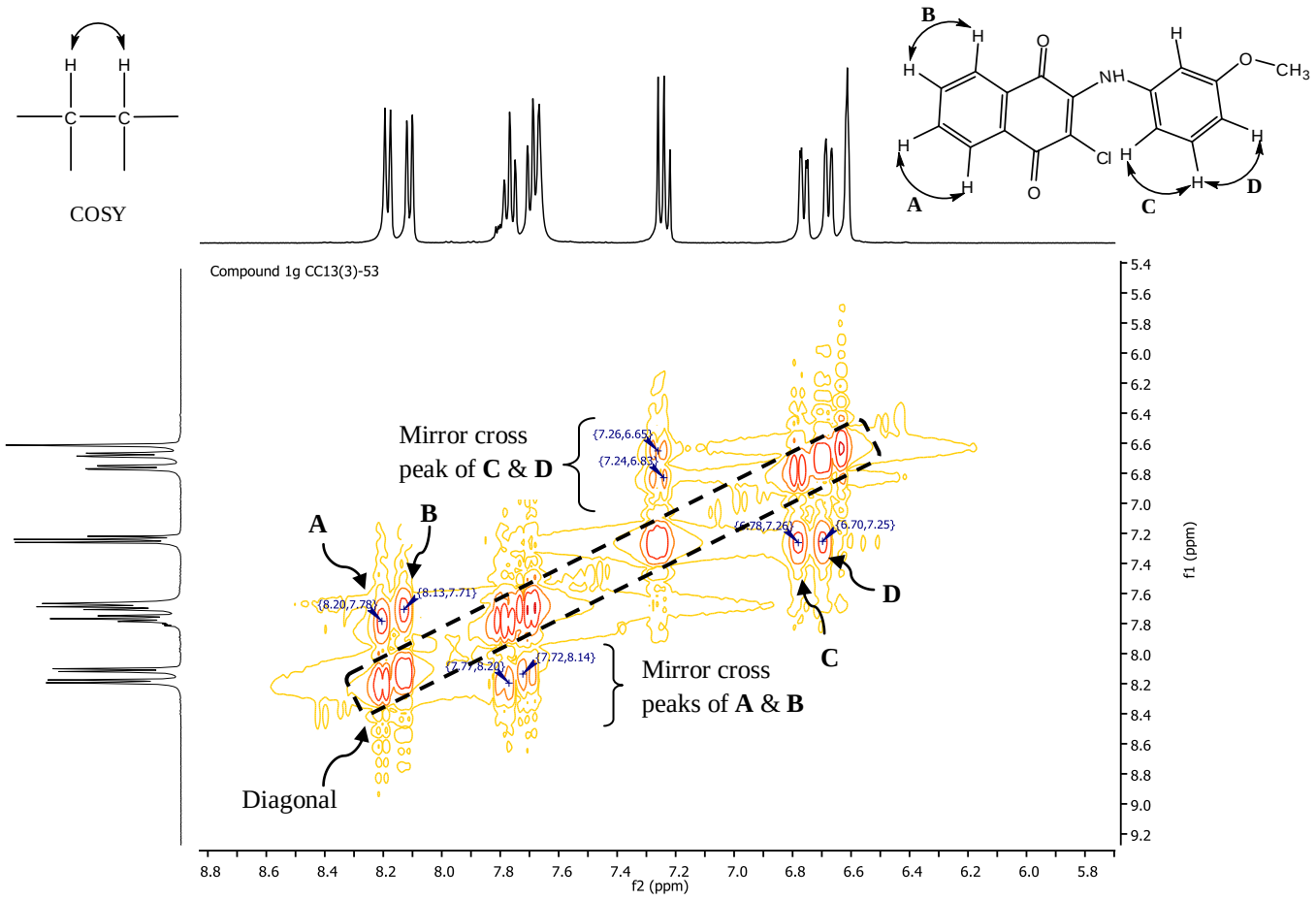


Figure 3.9: Expansion of the COSY NMR spectrum of 2-chloro-3-(3-methoxy-phenylamino)-1,4-naphthoquinone **1g**.

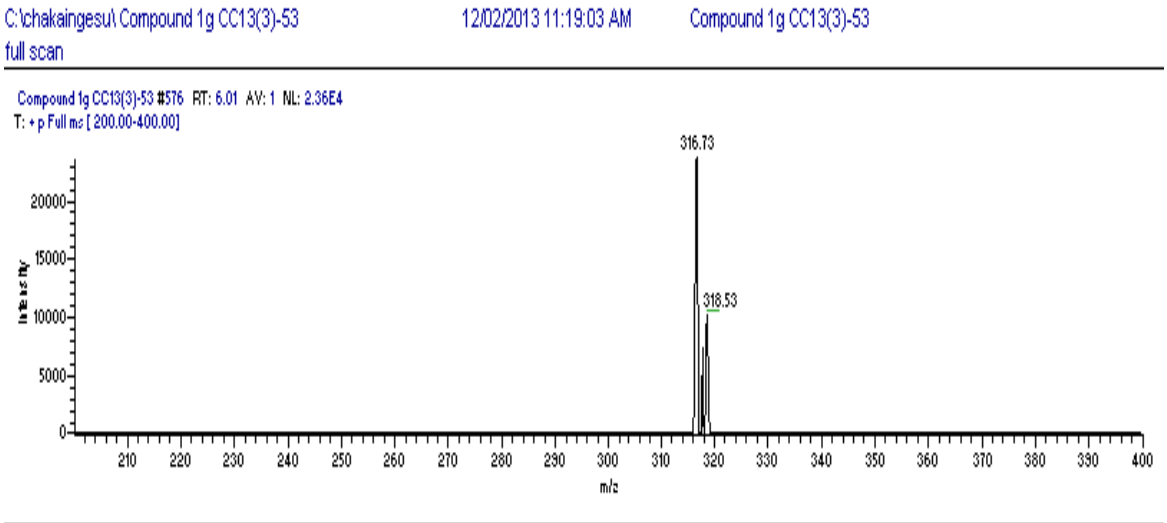


Figure 3.10: HPLC-ESI-MS spectrum of 2-chloro-3-(3-methoxy-phenylamino)-1,4-naphtho-quinone **1g**.

3.8 CRYSTALLINE AND AMORPHOUS STATES OF COMPOUND 2B

It was interesting that 2-Chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b** existed in two different solid physical states - needle-shaped crystals and amorphous powder. It is widely known that compounds can exist in more than one solid form with different spatial arrangements in the atoms or molecules.[23]

At the end of the reaction, an orange solid had crystallised out of the solution and was recovered by decanting the reaction mixture. The orange solid was removed from the reaction flask by solubilising in ethyl acetate and air dried in the fume hood, upon which pure needle-shaped crystals were obtained. The purity of these crystals was confirmed by ^{1}H NMR. A dark yellow amorphous powder also precipitated out of solution when the decanted reaction mixture was cooled to room temperature. Unlike the orange crystals, the yellow amorphous powder had to undergo purification by recrystallisation. The crystals and amorphous powder showed similar ^{1}H NMR and ^{13}C NMR spectra and similar R_f values, but had different melting points. The orange crystals had a melting point of 178.3-181.3°C, whilst the melting point of the dark yellow powder was 172.1-175.7°C. This slight difference may be attributed to the different spatial arrangement of atoms or molecules. Crystalline compounds have a lattice with a definite shape and geometry, requiring higher energy to break the bonds as compared to amorphous compounds, which have an irregular atomic arrangement and therefore lower bond strength. Atoms or molecules in a crystal lattice are held together tightly, unlike those of an amorphous. In general, amorphous solids have lower melting points when compared to the crystalline form of the same compound.[23]

Upon integration of the ^{1}H NMR spectra of both solid physical states of compound **2b**, intensity ratios of 1:1:1:2:1:1:1:1 were obtained, corresponding to a total of eight protons (**Figure 3.11**). Compound **2b** has sixteen non-equivalent carbon atoms, and half of these are quaternary carbons whose ^{13}C NMR signals are observed as short peaks as shown in **Figure 3.12** below. The other eight carbon atoms are directly attached to protons and were observed as the longer ^{13}C NMR peaks with similar height/intensities (**Figure 3.12**). These also produced cross-peaks in the HSQC NMR spectrum of compound **2b**. The spectral data provided evidence confirming the structural assignments and proving that the orange needle-shaped crystals and dark yellow amorphous powder were chemically identical, despite the difference in their physical

appearance. Further analytical techniques such as differential scanning calorimetry (DSC) and thermo-gravimetric analysis (TGA) could be conducted to assess whether these two solid physical forms are polymorphs of each other.

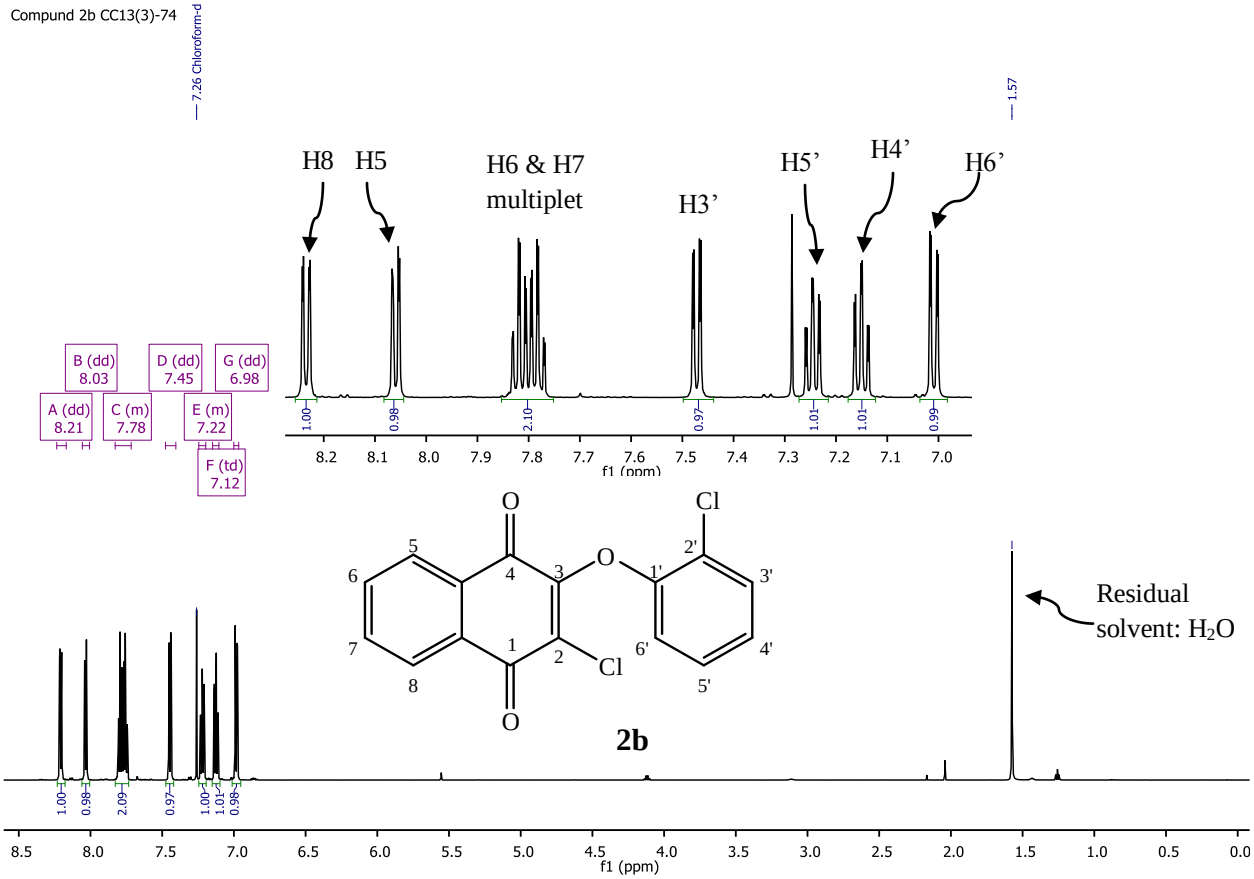


Figure 3.11: ^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b**. Expansion of the signals is shown in the off-set plot. The product was obtained by coupling 2,3-dichloro-1,4-naphthoquinone with 2-chlorophenol.

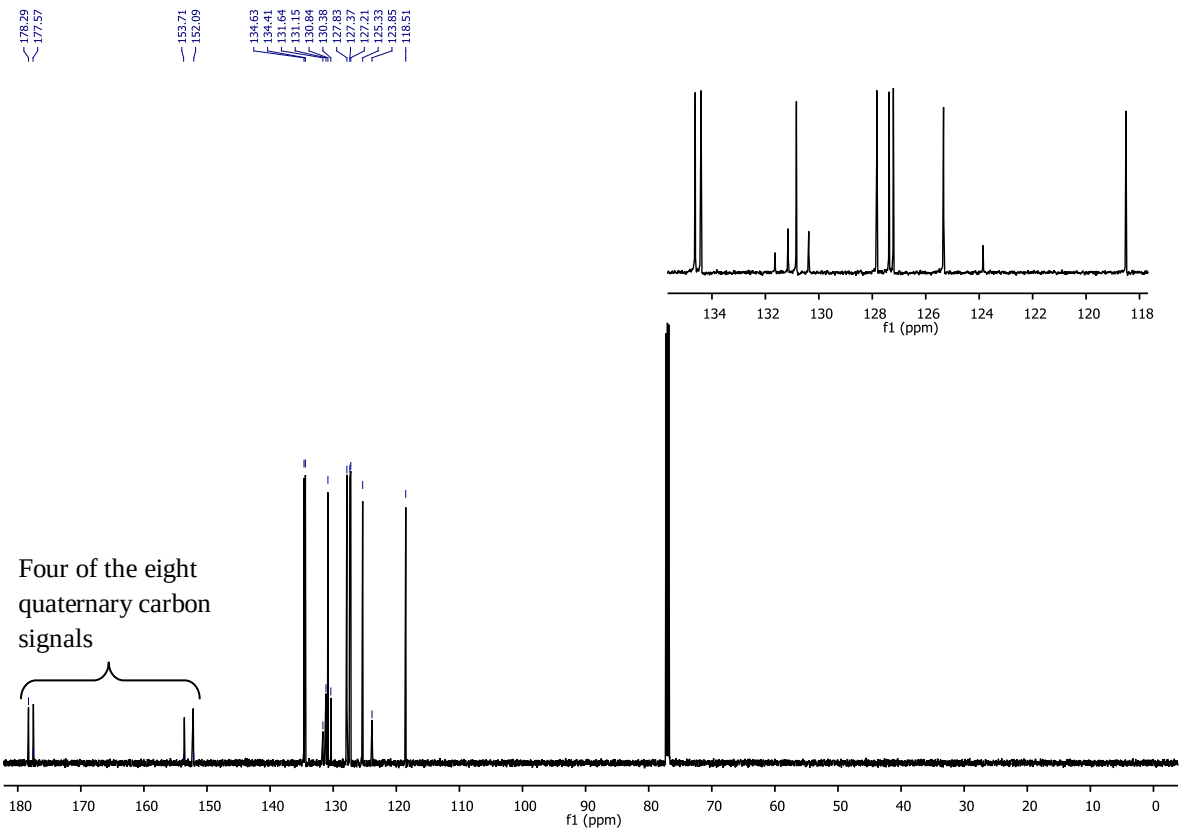


Figure 3.12: ^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b**. Expansion of the signals between 118 ppm and 134 ppm is shown in the off-set plot, which reveals the other four quaternary carbons and the eight signals of the carbon atoms directly attached to protons.

3.9 INFRARED (IR) SPECTROSCOPY

Infrared analysis was only carried out on the N-substituted 1,4-naphthoquinone derivatives (compounds **1a-1h**) to investigate the presence of a secondary amine (-NH) functionality. The anilines used to synthesise compounds **1a-1h** are primary amines ($-NH_2$) and, once each couples to the naphthoquinone, the -NH functional group is expected. ^1H NMR spectra of these compounds had a singlet around δ 7.60 which, in some cases, overlapped with a triplet signal. This singlet was not accounted for in the HSQC NMR spectra of compounds **1a-1h**, meaning that the proton responsible for this signal was not attached to a carbon atom. This confirmed that it was the proton attached to a nitrogen atom. Infrared spectroscopy was then employed to

determine the type of amine present. Applications of IR spectroscopy include the detection of functional groups amongst others.[24] For this reason, IR analysis was utilised, and it is known that the N-H stretch(s) occur in the region between 3500-3200 cm^{-1}, with primary amines giving two bands whilst secondary amines give only one band.[24,25]

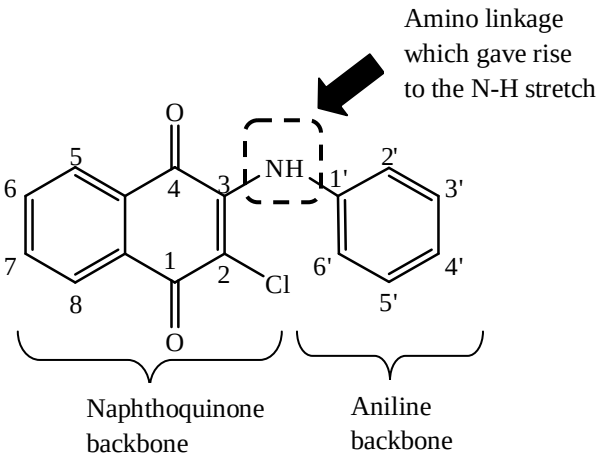


Figure 3.13: Structure of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a**.

Compounds **1a**-**1h** gave one N-H stretch, with a frequency falling between 3219 and 3243 cm^{-1} amongst these eight compounds. The IR and ^{1}H NMR spectral data confirmed the presence of a secondary amine in compounds **1a**-**1h** and that it formed the link between the naphthoquinone and aryl amine backbones. **Figure 3.14** illustrates the IR spectrum of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a**, highlighting the single N-H stretch at 3233 cm^{-1}.

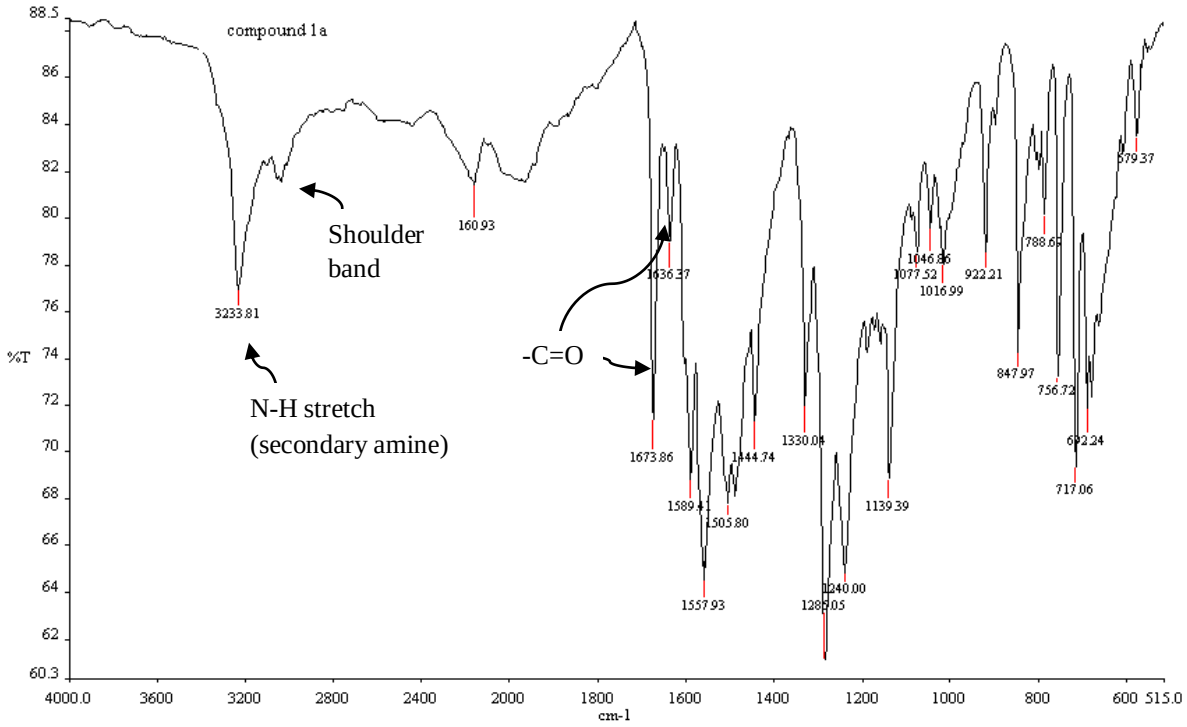


Figure 3.14: IR (KBr, cm^{-1}) spectrum of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a** highlighting the single N-H stretch at 3233 cm^{-1}.

In this study, IR spectroscopy was utilised for its application of functional group identification. Compounds **2a-2e** consist of an ether linkage comprised of an sp^3 hybridised oxygen atom attached to two carbon atoms: one carbon of the naphthoquinone and the other from the phenyl ring.[9] There is loss of the phenolic proton during the coupling of the two substrate moieties to form the naphthoquinone derivative and hence the O-H stretch can no longer be seen on the IR spectrum. The same applies to compounds **3a-3e**, which consist of thioether linkages, where an sp^3 hybridised sulphur atom is attached to two carbon atoms[9]. There is loss of a proton from the aryl thiols during the coupling reaction, and hence the S-H stretch is absent from the IR spectrum. For these reasons, IR was only performed on compounds **1a-1h** which had secondary amine linkages.

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CHAPTER IV: RESULTS AND DISCUSSION OF THE BIOLOGICAL ASSAYS

4.1 INTRODUCTION

Structure-activity relationship (SAR) is a paradigm relating the structure of a molecule to its biological activity. The concept is used by medicinal chemists in their quest to discover better drugs and it entails the modification of functional groups within a series of analogous compounds in search of more potent, selective and safer drugs.[1,2]

The relationship between the molecular structure of a compound and its biological activity has been studied since the nineteenth century. In 1869, Crum-Brown and Fraser demonstrated that some compounds, such as the pain-killer morphine, with tertiary amine groups acted as muscle relaxants when converted to quaternary ammonium compounds.[1,2]

Although there was much debate that followed with regards to this hypothesis, Crum-Brown and Fraser's discovery prompted other scientists to begin searching for organic functional groups that would produce specific biological activities. This became the foundation of structure-activity relationship studies.[1]

The hallmarks of SAR studies are said to be the synthesis of a number of analogues and their testing for activity to determine the effects of small changes in chemical structure on the potency.[2] In this case, an SAR study was conducted to assess the relationships between structure and anti-trypanosomal activity.

Quantitative structure-activity relationships (QSARs) pertain to the chemical and physical properties that describe the association between structure and property. QSARs are defined by biological, physical and intrinsic properties (**Figure 4.1**). These rely on the ability to investigate multiple relationships between biological and physical properties.[1] The historic development of QSARs involves contributions by Richet, Ferguson, Taft, Hammett, Hansch and Fujita, amongst others.[3] The Hansch-type analysis is a classic QSAR that affords an equation defining biological activities as a linear free energy relationship.[1]

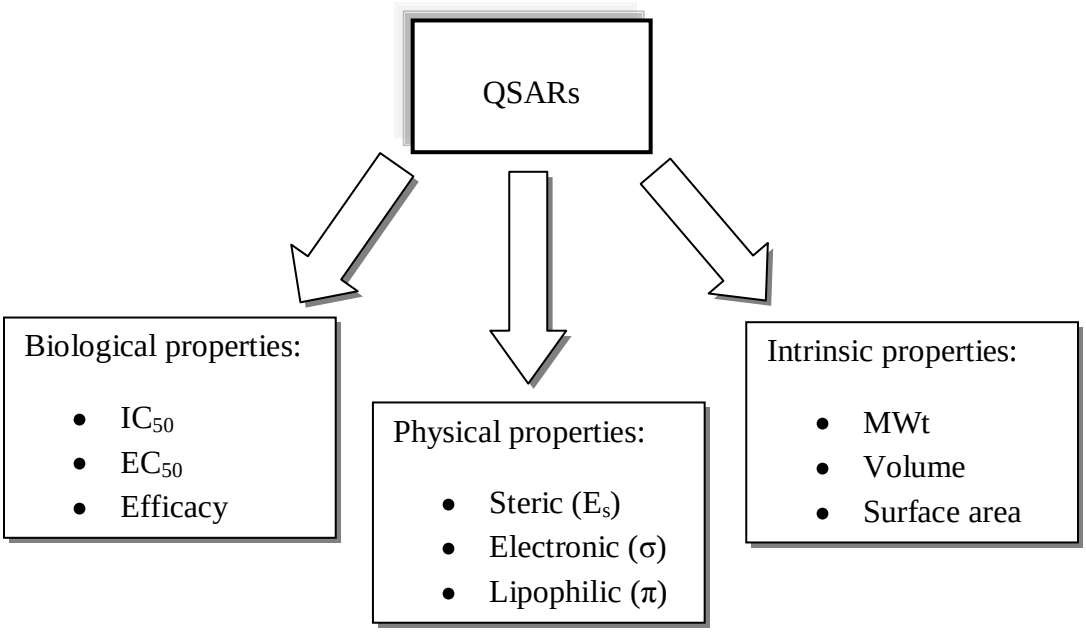


Figure 4.1: Properties that define quantitative structure activity relationships (QSARs): biological, physical and intrinsic properties.[1] MWt = molecular weight, IC_{50} = concentration required to produce 50% inhibition of cell growth and EC_{50} refers to 50% of the maximal effective concentration.

SARs and QSARs of a series of analogous 1,4-naphthoquinone derivatives shall be discussed in this chapter. The aim of this particular QSAR study was to investigate the electron-donating or withdrawing effects of the substituents on anti-trypanosomal activity. This study investigated the correlation between only two descriptors of QSARs, namely Hammett substituent constant (σ), an electronic property, and EC_{50}, a biological property. The σ constants are electronic descriptors which quantify the electron donating or withdrawal potency of substituents on aromatic rings and they were put forward by Hammett in 1940.[4,5] Values of σ constants are dimensionless and are described with respect to the meta or para position of the substituent on a benzene ring. The effects of substituents on the reaction centre differ in the *ortho*, *para* and *meta* positions as a result of variable inductive, resonance and steric effects. The σ values are generally deemed to be constant for a particular substituent and are relative to hydrogen whose value is, by definition, set to zero.[4] The σ values of the substituents investigated in this study are 4-Cl: 0.23, 4-Br: 0.23, 4-CH_3: -0.17, 3-CH_3: -0.07, 4-CF_3: 0.54 and 3-OCH_3: 0.12.[6]

Whilst Hammett constants have been used to quantify the electronic effects of *para* and *meta* substituents, the same is usually not applicable to ortho substituents. The initial Hammett equation does not hold for *ortho* substituents, as they are adjacent to the reaction centre (nucleophilic centre in this situation) and hence, a variety of proximity effects such as steric effects come into play. This does not give a true reflection of electronic effects as the *ortho* substituents are governed by both electronic and steric effects.[7] It is for this reason compounds with ortho substituents were left out during the QSAR analysis.

4.2 SAR & QSAR STUDY OF 1,4-NAPHTHOQUINONE DERIVATIVES

4.2.1 Phenylamino containing 1,4-naphthoquinones

Table 4.1: Hammett substituent constants (σ), 50% of the maximal effective concentration (EC_{50}) and the negative logarithm of one over EC_{50} values of naphthoquinone derivatives prepared from anilines.

Compound	**Phenylamino ring substituent**	**σ constant**	**EC_{50} (μM)**	**-log(1/EC_{50})**
1a	-H	0.00	12.59	1.10
1b	4-Cl	0.23	<50.12	---
1c	4-Br	0.23	10.96	1.04
1d	4-CH_3	-0.17	2.454	0.39
1e	4-CF_3	0.54	2.137	0.33
1f	2-CH_3	----	2.818	0.45
1g	3-OCH_3	0.12	2.511	0.40
1h	3-CH_3	-0.07	2.754	0.44

Hammett substituent constants (σ), 50% of the maximum effective concentration (EC_{50}) and the negative logarithm of one over EC_{50} values of 2-chloro-3-(substituted phenylamino)-1,4-

naphthoquinones (compounds **1a**-**1h**) are given in **Table 4.1** above. The lower the EC_{50} value of any given compound, the more potent it is.[8] The EC_{50} (µM) data set was plotted against the phenylamino substituent in order to present a graphical picture of the data (**Figure 4.2**).

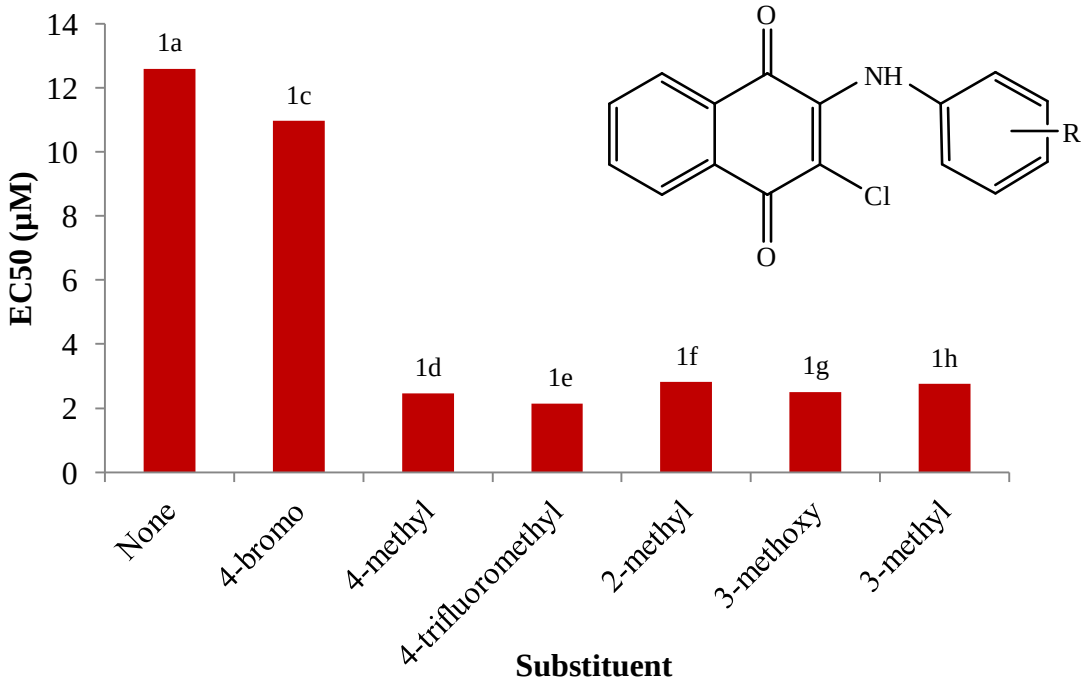


Figure 4.2: Graphic representation of the EC_{50} values of 2-chloro-3-(substituted phenylamino)-1,4-naphthoquinones (**1a**,**1c**-**1h**) where R is the phenylamino ring substituent. Compound **1b** has been excluded as a result of a lack of potency.

Figure 4.2 illustrates that substititution of the phenylamino ring increases potency, as the non-substituted ring gave the lowest potent 1,4-naphthoquinone derivative (compound **1a**, EC_{50} = 12.59 µM). Substitution of the phenyl amino ring with bromine on the para position (compound **1c**), increased potency slightly in comparison to the non-substituted phenyl amino ring. Substitution with a trifluoromethyl group on the para position gave the most potent 2-chloro-3-(substituted phenylamino)-1,4-naphthoquinone (compound **1e**), which is 6-fold more potent than the non-substituted phenylamino derivative (compound **1a**).

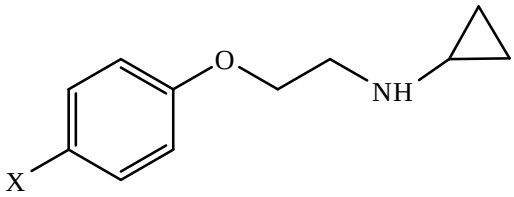
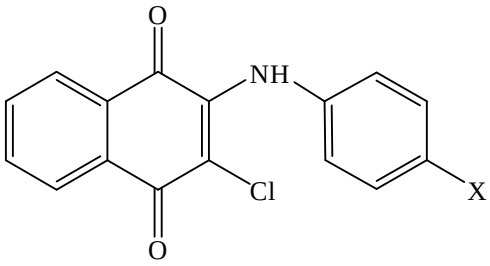
The position of methyl substituent on the phenylamino ring has no huge effect on potency as the *para* (**1d**), *ortho* (**1f**) and *meta* (**1h**) methyl substituted 2-chloro-3-(substituted phenylamino)-1,4-naphthoquinones gave almost similar EC_{50} values of 2.454, 2.818 and 2.754 µM respectively.

Substitition of the phenylamino ring with a methoxy or methyl groups on the *meta* position resulted in almost equipotent compunds **1g** and **1h**, with EC_{50} values of 2.511 and 2.754 µM respectively. Depending on the position, a methoxy substituent can either donate or withdraw electrons. In the meta position, electronegativity of the methoxy oxygen atom produces an inductive effect on the withdrawal of electrons.[6] However, this had no huge effect on potency because presence (*m*-OCH_3) or absence of the oxygen atom (*m*-CH3) on the phenylamino ring resulted in almost equipotent compounds **1g** and **1h** respectively.

Amongst the 2-chloro-3-(substituted phenylamino)-1,4-naphthoquinones, only the para chloro substituted compound **1b** had little or no potency, hence its absence in **Figure 4.2**. Electronegativity of the chlorine substituent (3.19) could not be employed to explain why compound **1b** lacked potency because the trifluoromethyl substituted compound **1e** showed the greatest potency in the series of analogous compounds, yet it is more electronegative (3.30).[9]

A similar trend of the effects of halogens on potency was obtained with an SAR study of monoamine oxidase (MAO) inhibitors.[10] A bromine substituent increased potency whilst a trifluoromethyl substituent made the MAO inhibitors even more potent (**Table 4.2** below).[10] This trend was also observed in this study whereby a bromine substituent on the phenylamino ring gave a better potency than a non-substituted phenylamino ring and a trifluoromethyl group enhanced the potency even further.

Table 4.2: The influence of halogen substituents on *in vitro* potency.

MAO inhibitor [10]		**2-chloro-3-(substituted phenylamino)-1,4-naphthoquinones from the current study**	
X	**IC_{50} (µM)**	**X**	**EC_{50} (µM)**
-H	1.20	-H (**1a**)	12.59
-Br	0.20	-Br (**1c**)	10.96
$-CF_3$	0.10	$-CF_3$ (**1e**)	2.137
			

A quantitative structure activity relationship (QSAR) was carried out to investigate if a correlation between the potency of 2-chloro-3-(substituted phenylamino)-1,4-naphthoquinones (**1a**,**1c-1h**) and electron-donating or withdrawing properties of substituents exists. A scatter graph of the negative logarithm of one over the EC_{50} against the σ constants showed that there is no linear correlationship between potency and the substituent effect (**Figure 4.3**).

A correlation coefficient (r^2) very close to one means that there is a linear correlation between any two variables; however, a value very close to zero indicates that there is no correlation.[11] The line of best fit from the plot illustrated in **Figure 4.3** gave an r^2 value of 0.0053, hence the regression equation does not fit the data. This goes to show that substituent effects on the phenylamino ring are not directly proportional to potency. The equation of the line can be given as $\log (1/EC_{50}) = 0.1009\sigma - 0.6276$ and, since the regression analysis suggests that there is a non-linear correlation, the equation therefore cannot be used to estimate the potency.

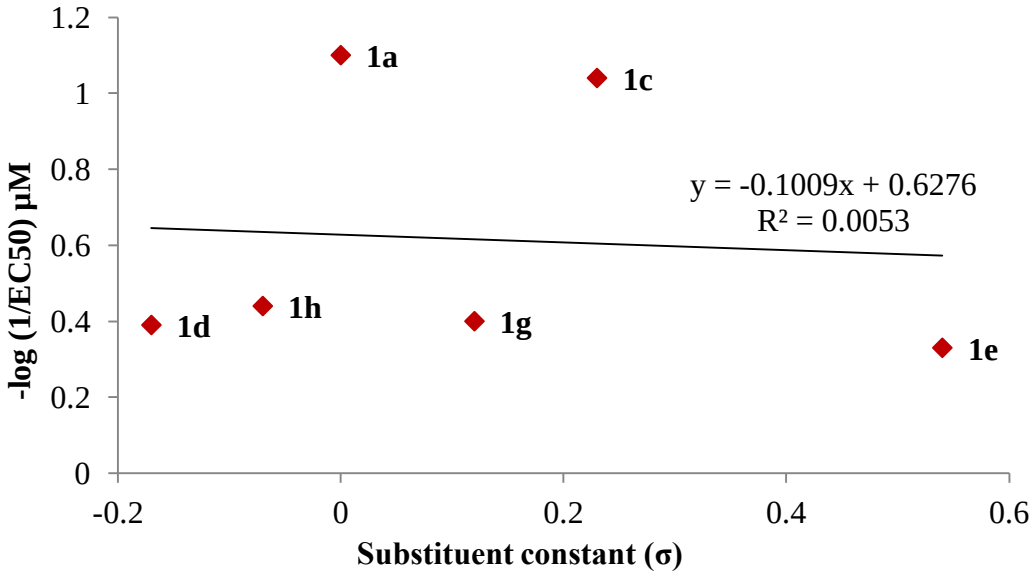


Figure 4.3: The effects of electronic properties (σ) of the substituent on the potency of 2-chloro-3-(substituted phenylamino)-1,4-naphthoquinones, compounds **1a**,**1c**-**1h**.

4.2.2 Phenoxy containing 1,4-naphthoquinones

EC_{50}, σ constants and the negative logarithm of one over EC_{50} values of 2-chloro-3-(substituted phenoxy)-1,4-naphthoquinone, compounds **2a**-**2e**, are given in **Table 4.3**. Compounds **2b** and **2c** have *ortho* substituents, and hence their σ constant values were not given, as ortho substituents have been said to have some steric effects on the reactive group or centre.[7]

Table 4.3: Hammett substituent constants (σ), EC_{50} and the negative logarithm of one over EC_{50} values of naphthoquinone derivatives prepared from phenols.

Compound	**Phenoxy ring substituent**	**σ constant**	**EC_{50} (μM)**	**-log(1/EC_{50})**
2a	-H	0.00	5.888	0.770
2b	2-Cl	---	6.025	0.780
2c	2-Cl,4-CH_3	---	3.89	0.590
2d	3,5-CH_3	---	5.370	0.730
2e	4-Cl	-0.17	6.760	0.829

Amongst the 2-chloro-3-(substituted phenoxy)-1,4-naphthoquinone series, compound **2c** was the most potent, with an EC_{50} of 3.89 μM. The structural difference between compounds **2b** and **2c** is the additional para substituted methyl group on the phenoxy ring of the latter compound. Presence of this methyl group increased potency by almost 2-fold (**Figure 4.4**). The 3,5-dimethyl substituted phenoxy ring of compound **2d** was slightly more potent than the non-substituted ring of compound **2a**.

Chloro-substitution of the phenoxy ring on the *ortho* position increased potency slightly when compared to substituting the same functional group on the para position, as compounds **2b** and **2e** had EC_{50} values of 6.025 and 6.760 μM respectively. Absence of a substituent in the phenoxy ring (compound **2a**) results in better potency compared to the chloro substitution of the same ring

(compounds **2b** and **2e**). The order of potency amongst the 2-chloro-3-(substituted phenoxy)-1,4-naphthoquinone series illustrated in **Figure 4.4** is as follows: compound **2c** > **2d** > **2a** > **2b** > **2e**.

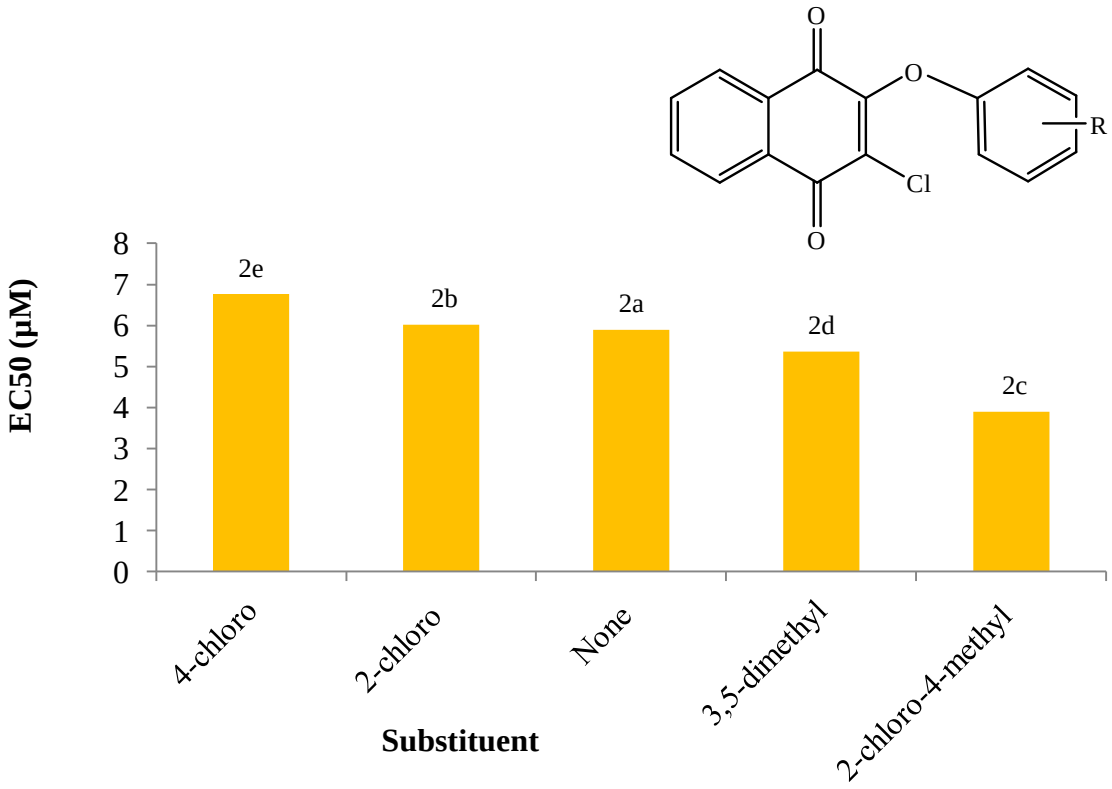


Figure 4.4: Graphic representation of the EC_{50} values of 2-chloro-3-(substituted phenoxy)-1,4-naphthoquinones (compounds **2a**-**2e**) where R is the phenoxy ring substituent.

4.2.3 Bis-thiophenyl containing 1,4-naphthoquinones

EC_{50}, σ constants and the negative logarithm of one over EC_{50} values of 2,3-bis(substituted thiophenyl)-1,4-naphthoquinones (disubstituted compounds **3a**, **3b** and **3d**) are given in **Table 4.4**. Compound **3e** has an *ortho* substituent and hence its σ constant value is not given due to the steric effects on the reactive group/centre.[7] The EC_{50} (µM) data set was plotted against the thiophenyl substituent in order to produce a graphical picture of the data (**Figure 4.5**).

Table 4.4: Hammett substituent constants (σ), EC_{50} and the negative logarithm of one over EC_{50} values of naphthoquinone derivatives prepared from thiophenols.

Compound	**Thiophenyl ring substituent**	**σ constant**	**EC_{50} (μM)**	**-log(1/EC_{50})**
3a	-H	0.00	2.884	0.46
3b	4-Cl	0.23	32.36	1.51
3c	4-Br	0.23	<50.12	---
3d	4-CH_3	-0.17	7.244	0.86
3e	2-CH_3	----	<50.12	---

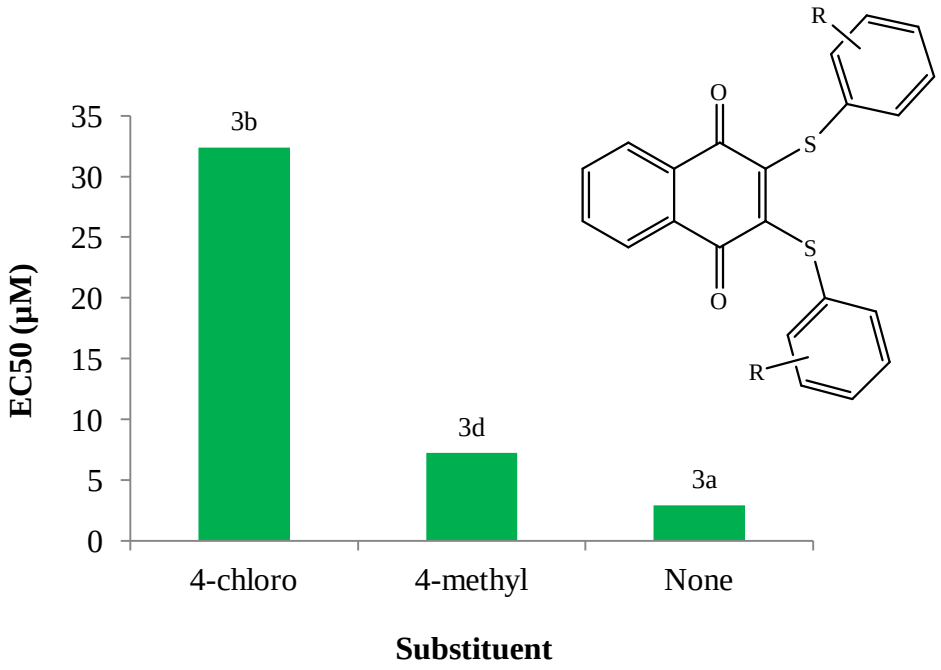


Figure 4.5: Graphical representation of the EC_{50} values of 2,3-bis(substituted thiophenyl)-1,4-naphthoquinones (compounds **3a**, **3b** and **3d**) where R is the thiophenyl ring substituent. Compounds **3c** and **3e** have been excluded as a result of their lack of potency.

Compound **3a** had the highest potency amongst the 2,3-bis(substituted thiophenyl)-1,4-naphthoquinones and it has a non-substituted thiophenyl ring. Substitution on the *para* position with methyl or chloro groups reduced the potency (**Figure 4.5**). Introduction of a halogen substituent

on the thiophenyl drastically reduced the potency as compound **3b** was 11-fold less potent than compound **3a**. Presence of the methyl substituent (compound **3d**) reduced the potency by almost 3-fold in comparison to compound **3a**, which has no thiophenyl substituent. However, substitution with the electron-donating methyl group resulted in better potency compared to substitution with the electron-withdrawing chlorine atom. Substitution of the thiophenyl ring with a bromine atom (compound **3c**) resulted in a lack of potency and the same was observed with substitution with a methyl group on the ortho position (compound **3e**), explaining their absence in **Figure 4.5** above.

A QSAR study was conducted to investigate if a correlation between the potency of 2,3-bis(substituted thiophenyl)-1,4-naphthoquinones (compounds **3a**, **3b** and **3d**) and the electron-donating or withdrawing properties of the substituents exists. A scatter plot of the negative logarithm of one over the EC_{50} against the σ constant revealed that the relationship between these two variables is non-linear as an r^2 value of 0.4612 was obtained (**Figure 4.6**). Therefore, potency was not directly proportional to the substituent effects. The equation of the line can be given as: $\log (1/EC_{50}) = 1.7928\sigma - 0.9075$, and since the regression analysis suggests that there is a non-linear correlation, the equation therefore cannot be used to predict the potency.

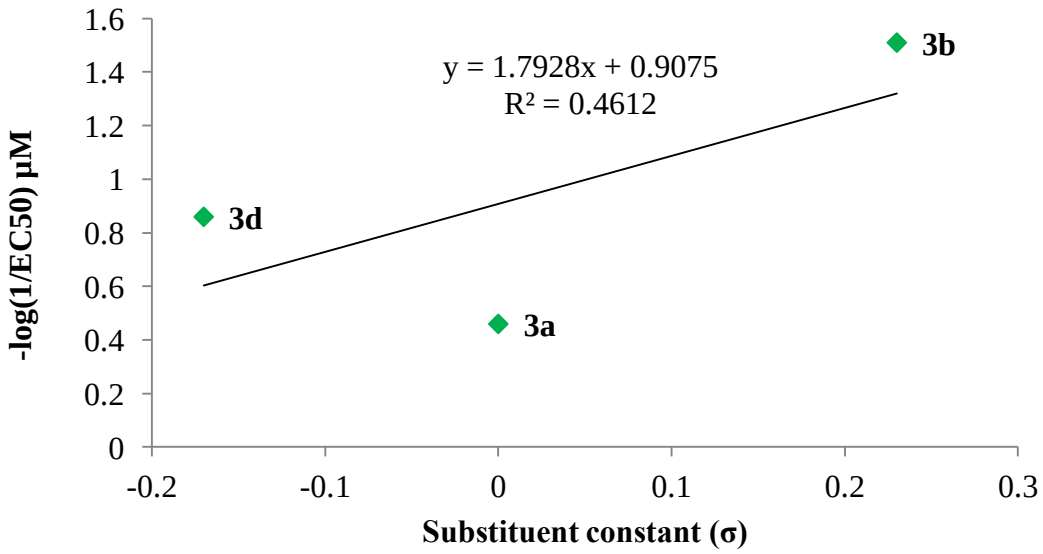


Figure 4.6: The effects of electronic properties (σ) of the substituent on the potency of 2,3-bis(substituted thiophenyl)-1,4-naphthoquinones (compounds **3a**,**3b** and **3d**).

4.2.4 General discussion

In this study, the reference standard used was melarsoprol, a melaminophenyl-based organic arsenical compound (**Figure 4.7**) currently used in the treatment of stage 2 of either the *T.b. gambiense* form or the *T. b. rhodesiense* form of sleeping sickness.[12] Melarsoprol was very potent compared to the synthesised 1,4-naphthoquinone derivatives. It was 142-fold more potent than 2-chloro-3-(4-(trifluoromethyl)phenylamino)-1,4-naphthoquinone **1e**, which was the most potent compound amongst the series of 1,4-naphthoquinone derivatives (**Figure 4.8**).

Although melarsoprol showed a high degree of potency, the drug is very toxic and results in treatment-related deaths in approximately 5% of patients treated with it.[13] The toxicity may possibly be conferred by the arsenic containing moiety.

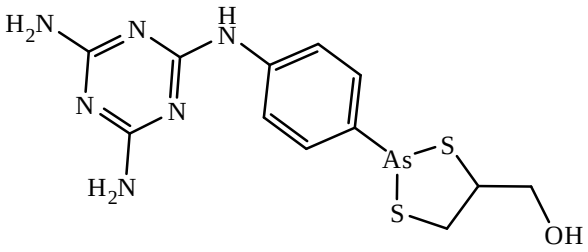


Figure 4.7: The structure of melarsoprol, a drug currently used for the treatment of sleeping sickness.

The substrate of 2,3-dichloro-1,4-naphthoquinone was also tested for potency in order to investigate the effects of coupling with aniline, phenol and thiophenol nucleophiles. **Figure 4.8** illustrates that 2,3-dichloro-1,4-naphthoquinone, denoted as NQ, is equipotent to compounds **2e** and **3d**. Furthermore, it is more potent than compounds **1a**, **1c** and 4-fold more than compound **3b**. This means that displacement of the chlorine atom on position three of 2,3-dichloro-1,4-naphthoquinone with a phenylamino ring (**1a**) or a 4-bromophenylamino ring (**1c**) results in the reduction of anti-trypanosomal activity. Compounds **1d-1h** and **3a** are twice as potent as the starting material (2,3-dichloro-1,4-naphthoquinone), as can be seen in **Figure 4.8**.

Replacement of an amino linkage (-NH-) with an ether linkage (-O-) increased the potency by 2-fold, as demonstrated by the EC_{50} values of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a** and 2-chloro-3-(phenoxy)-1,4-naphthoquinone **2a** of 12.39 and 5.888 µM respectively. Presence of a thioether linkage (-S-) and disubstitution of both chlorine atoms of 2,3-dichloro-1,4-naphthoquinone results in an increment of potency, as compound **3a** had an EC_{50} value of 2.888 µM compared to the starting material, 2,3-dichloro-1,4-naphthoquinone, with an EC_{50} value of 7.244 µM. Compounds with the best *in vitro* anti-trypanosomal potencies in the series of analogous 1,4-naphthoquinone derivatives had EC_{50} values in the range 2.137 to 2.884 µM (**Figure 4.8**).

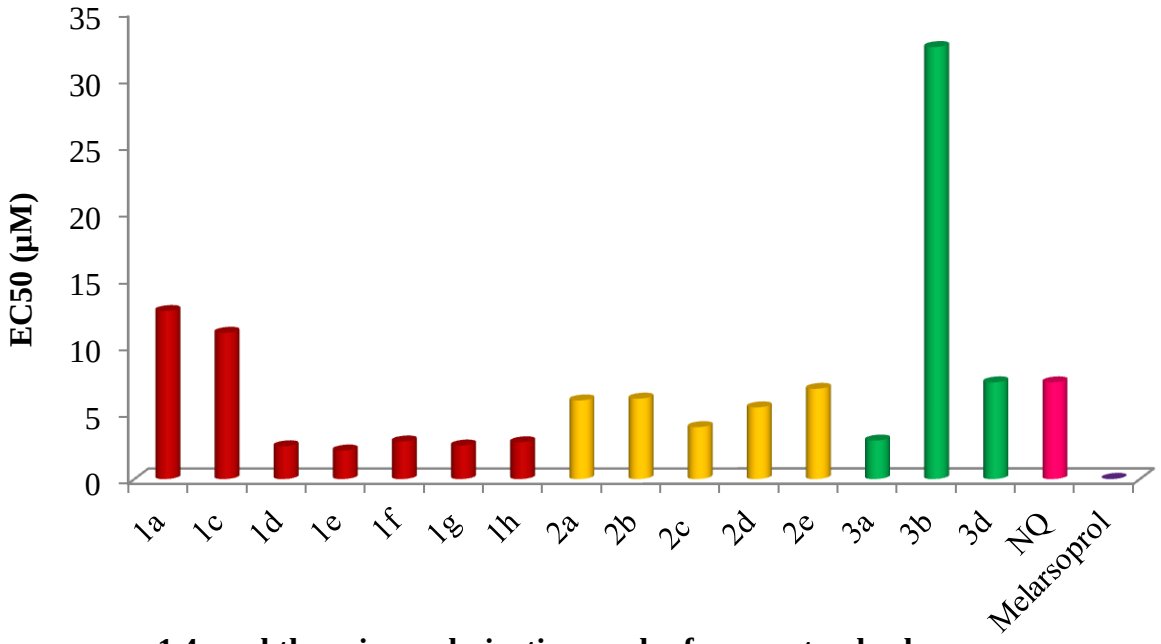


Figure 4.8: Comparison of the potency of 1,4-naphthoquinone derivatives as well as 2,3-dichloro-1,4-naphthoquinone (NQ) and melarsoprol (reference standard) against *T.brucei*. Compounds **1b**, **3c** and **3e** have been excluded as a result of a lack of potency.

Compounds **1b**, **3c** and **3e** had poor or lack of anti-trypanosomal activity and their EC_{50} values were reported as less than 50.12 µM and were therefore excluded from the graphs. Dose-response curves of these 1,4-naphthoquinone derivatives (**Figure 4.9**) are a graphical

representation illustrating their poor or lack of anti-trypanosomal activity. A standard dose-response curve is defined by four parameters which make up the four parametric equation given in section **2.3.3** of Chapter 2. The parameters are baseline response (bottom), maximum response (top), EC_{50} and Hill slope[14,15]; these could not be obtained for compounds **1b**, **3c** and **3e**. The rest of the compounds of the series of 1,4-naphthoquinone derivatives produced sigmoid-shaped dose-response curves, each with a single plateau (monophasic). For example, 2-chloro-3-(4-(trifluoromethyl)phenylamino)-1,4-naphtho-quinone **1e**, the most potent compound of the series, produced a sigmoidal dose-response curve with a Hill slope of 2.59 (**Figure 4.9**). The Hill slope refers to the steepness of a curve and a dose-response curve with a standard slope is said to have a Hill slope of 1.[15]

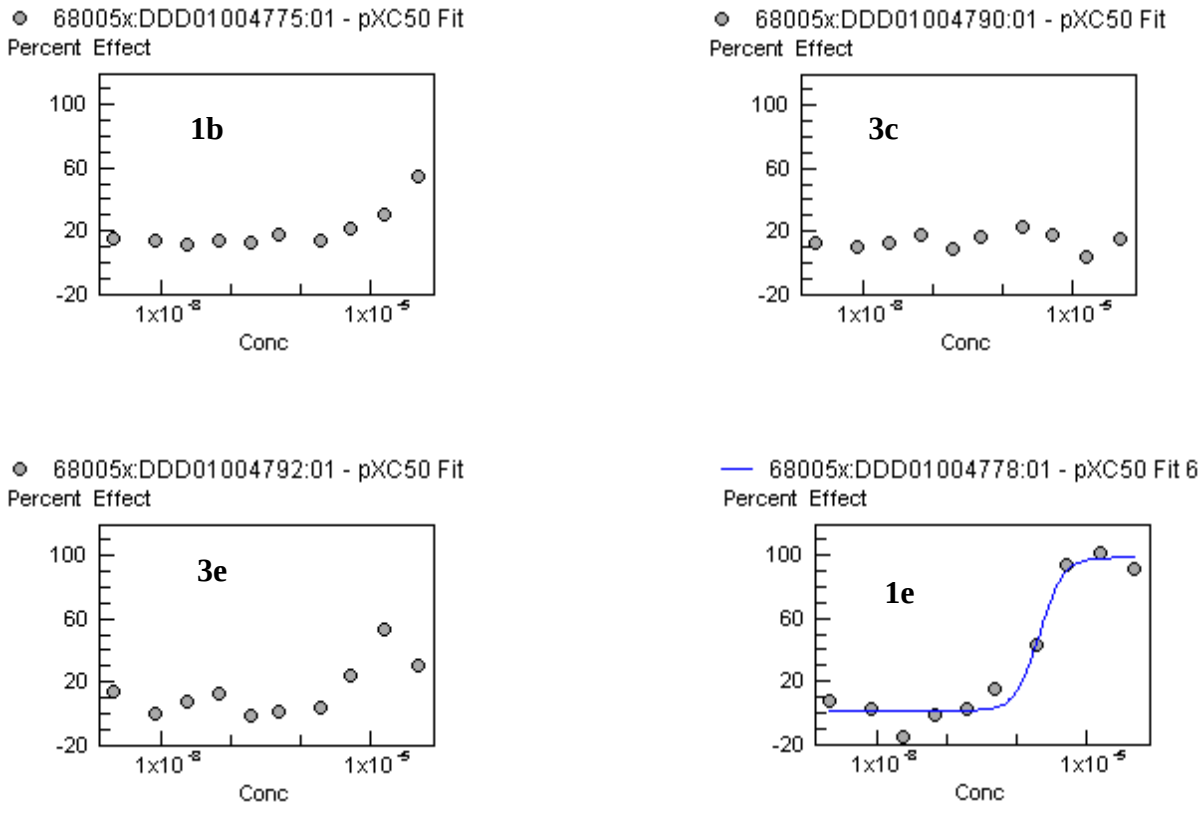


Figure 4.9: Dose-response curves of the 1,4-naphthoquinone derivatives that showed poor or lack of anti-trypanosomal activity (compounds **1b**, **3c** and **3e**) in compassion to the 1,4-naphtho-quinone derivative that showed the highest potency (compound **1e**).

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CHAPTER V: CONCLUSION

Eighteen 1,4-naphthoquinone derivatives were successfully synthesised using ethanolic solutions. Structural confirmation and purity of these compounds were confirmed using spectroscopic methods and melting point determination. Unfortunately, attempts to synthesise 1,4-naphthoquinones in reactions involving 2-(trifluoro-methyl)aniline and 2-isopropyl-5-methylphenol (thymol) were unsuccessful, presumably due to steric hindrance by the bulky *ortho*-substituents.

Although the aims of the synthetic procedures were to obtain both mono- and disubstituted products by nucleophilic displacement of the chlorine atom(s), only monosubstituted products were obtained from substitution with aniline and phenol nucleophiles. Thiol nucleophiles selectively yielded only disubstituted products.

Synthesised naphthoquinone derivatives were successfully tested against *T.brucei* and the calculation of EC_{50} values from the obtained dose-response curves was carried out using a four parametric equation. All the 1,4-naphthoquinones showed a degree of potency except compounds **1b**, **3c** and **3e** which had little or no potency.

An SAR study was successfully conducted to determine which structural features or substituents of the naphthoquinone derivatives contribute or detract from the desired anti-trypanosomal activity.

Three types of nucleophiles were used namely phenols, thiophenols as well as anilines, and the synthesised 1,4-naphthoquinone derivatives contained ether, thioether and amino linkages respectively. Presence of an ether linkage (-O-) increased the potency by 2-fold in comparison to the amino linkage (-NH-) as demonstrated by compounds **2a** and **1a** respectively. A thioether linkage (-S-) and disubstitution of both chlorine atoms of 2,3-dichloro-1,4-naphthoquinone (compound **3a**) resulted in further increment in potency compared to compounds **1a** and **2a**.

Electron-withdrawing chloro substituents on the phenoxy ring of 2-chloro-3-(substituted phenoxy)-1,4-naphthoquinone reduce potency whilst the presence of electron donating methyl substituents on the same ring increases potency. Position of methyl substituent on the

phenylamino ring had no huge effect on potency as the *para* (**1d**), *ortho* (**1f**) and *meta* (**1h**) methyl substituted 2-chloro-3-(substituted phenylamino)-1,4-naphthoquinones yielded almost similar EC_{50} values. In addition, *meta* substitition of the phenylamino ring with a methoxy or methyl groups also had no huge effect on the potency.

The starting material (2,3-dichloro-1,4-naphthoquinone) was found to be potent against *T.brucei* and this can be attributed to its 1,4-ketone motif. This substrate was equipotent to compound **3d** but more potent than compounds **1a**, **1c**, and **3b**.

Compounds with the best *in vitro* anti-trypanosomal potencies in the series of analogous 1,4-naphthoquinone derivatives had EC_{50} values in the range 2.137 to 2.884 µM. The most potent compound in the series was 2-chloro-3-(4-(trifluoromethyl)phenylamino)-1,4-naphthoquinone **1e**; but this was 142-fold less potent than reference standard of melarsoprol.

QSARs were also conducted to investigate the electron donating or withdrawing effects of the substituents on anti-trypanosomal activity, but these excluded *ortho*-substituent containing 1,4-naphthoquinones derivatives.

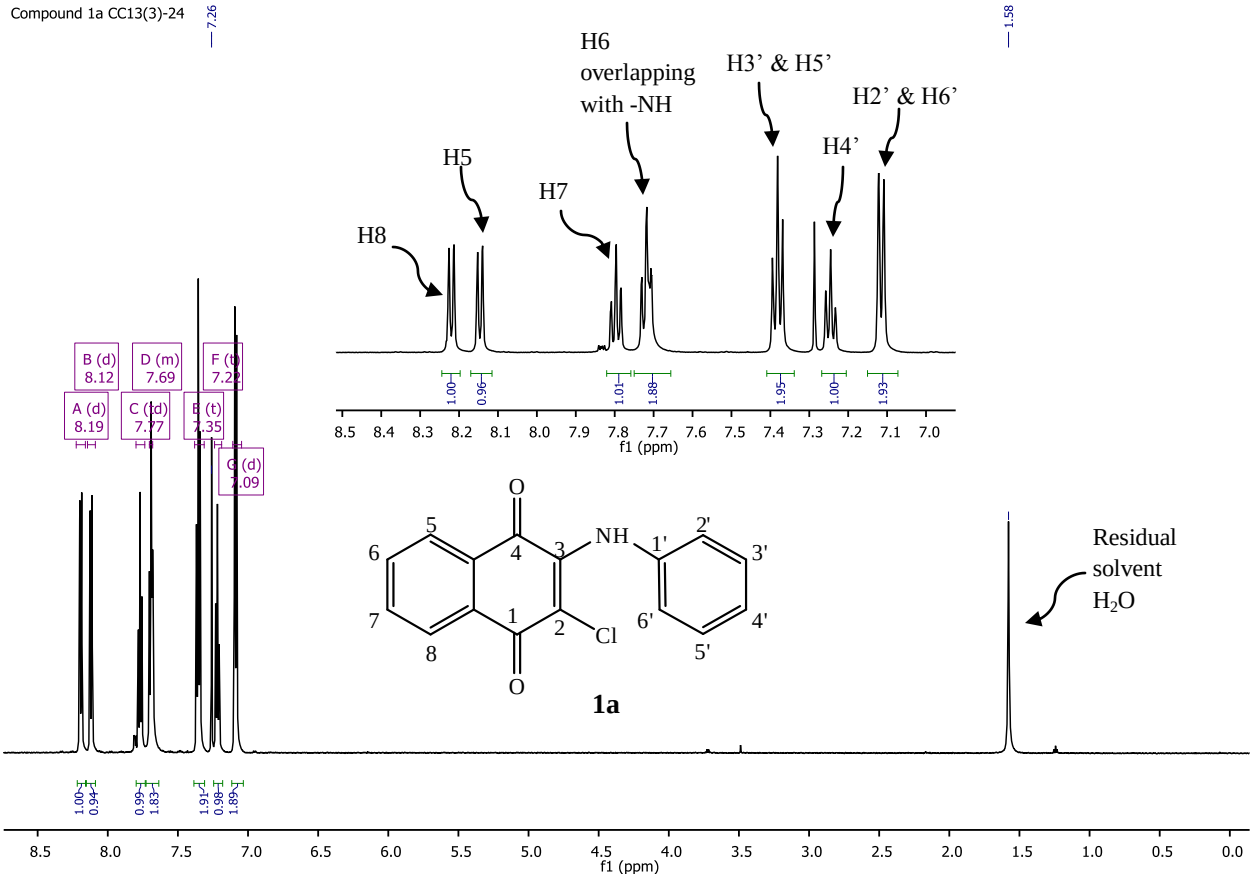
The relationship between electronic properties (σ) of the substituent effect and the potency of 2,3-bis(substituted thiophenyl)-1,4-naphthoquinones (compounds **3a**,**3b** and **3d**) and 2-chloro-3-(substituted phenyl-amino)-1,4-naphtho-quinones (compounds **1a**,**1c**-**1h**) were non-linear. This was demonstrated by r^2 values that were close to zero and their regression equations therefore cannot be used to predict the potency.

APPENDIX

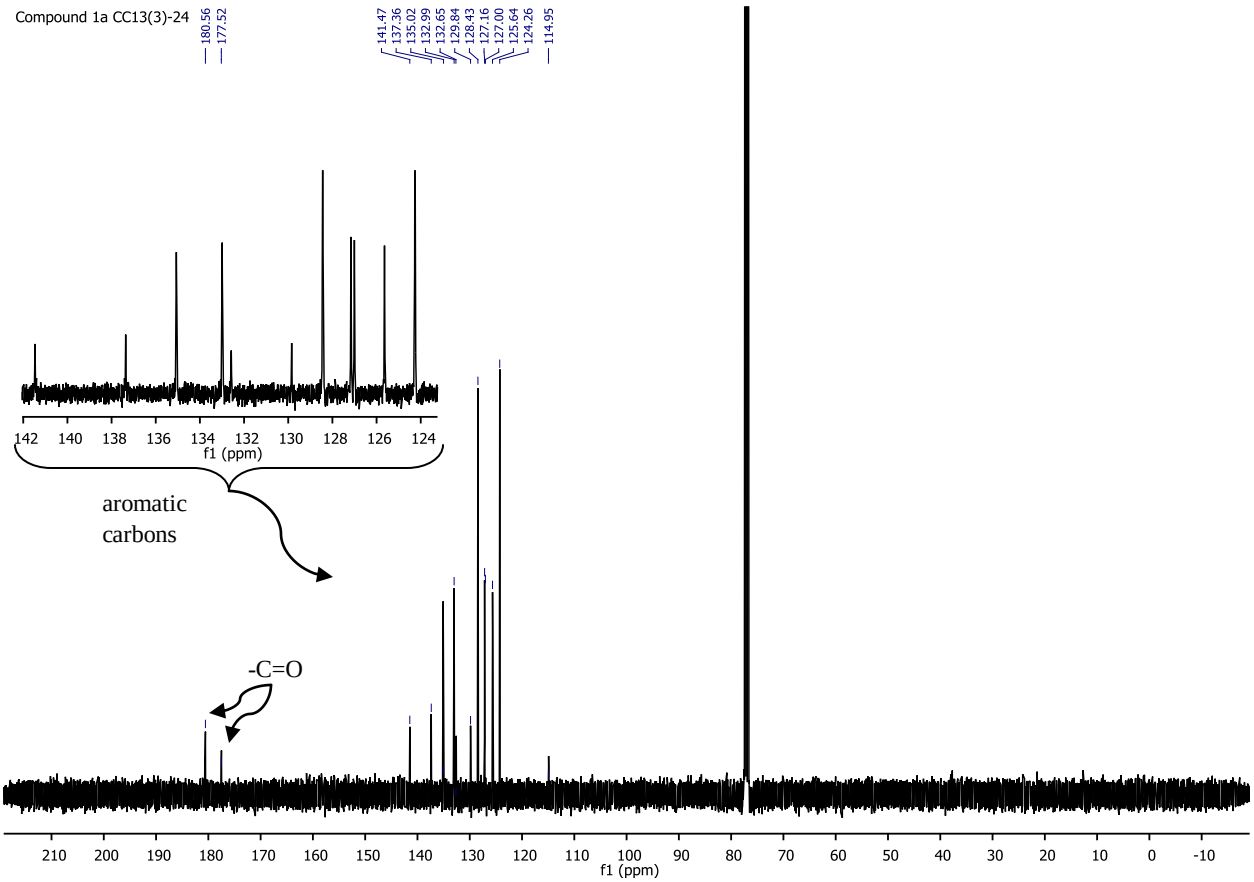
SPECTROSCOPIC DATA

BIOLOGICAL ACTIVITY DATA

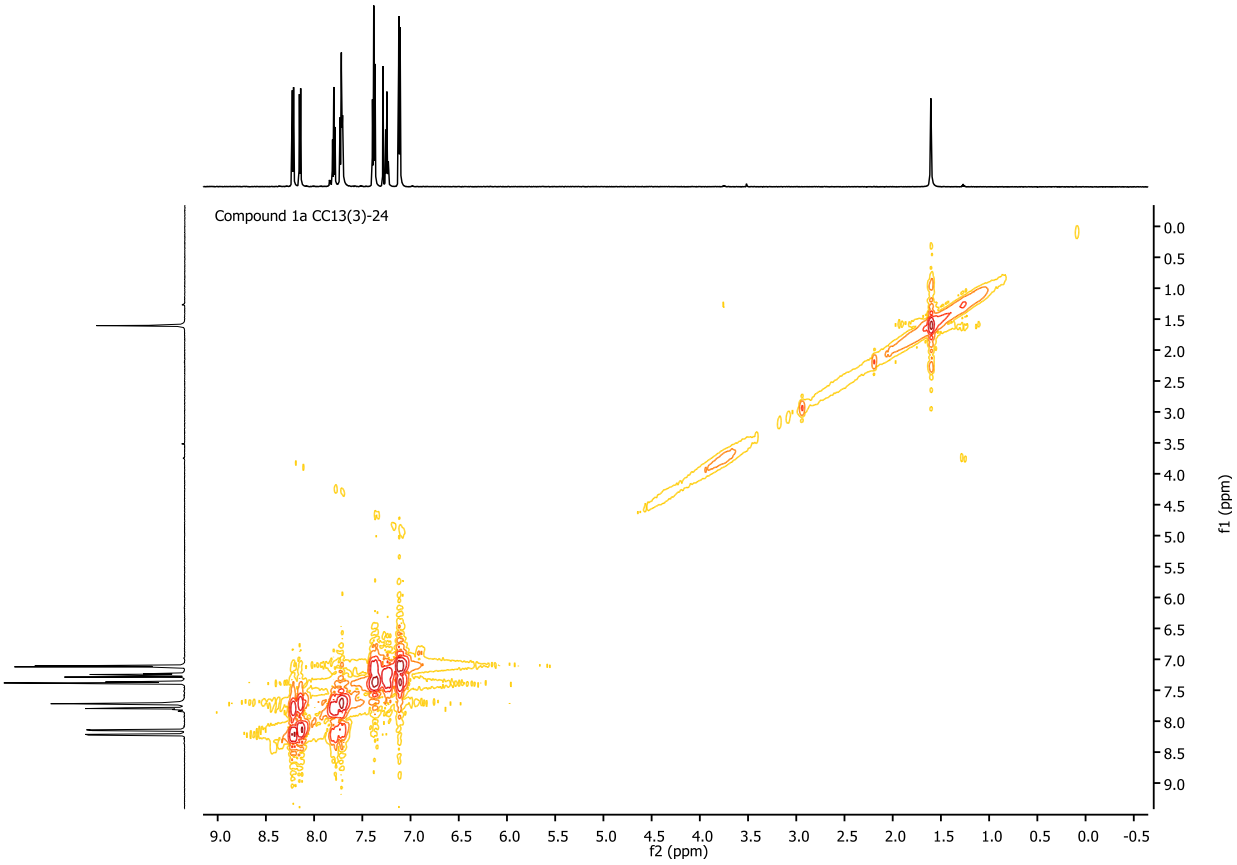
COMPOUND 1a



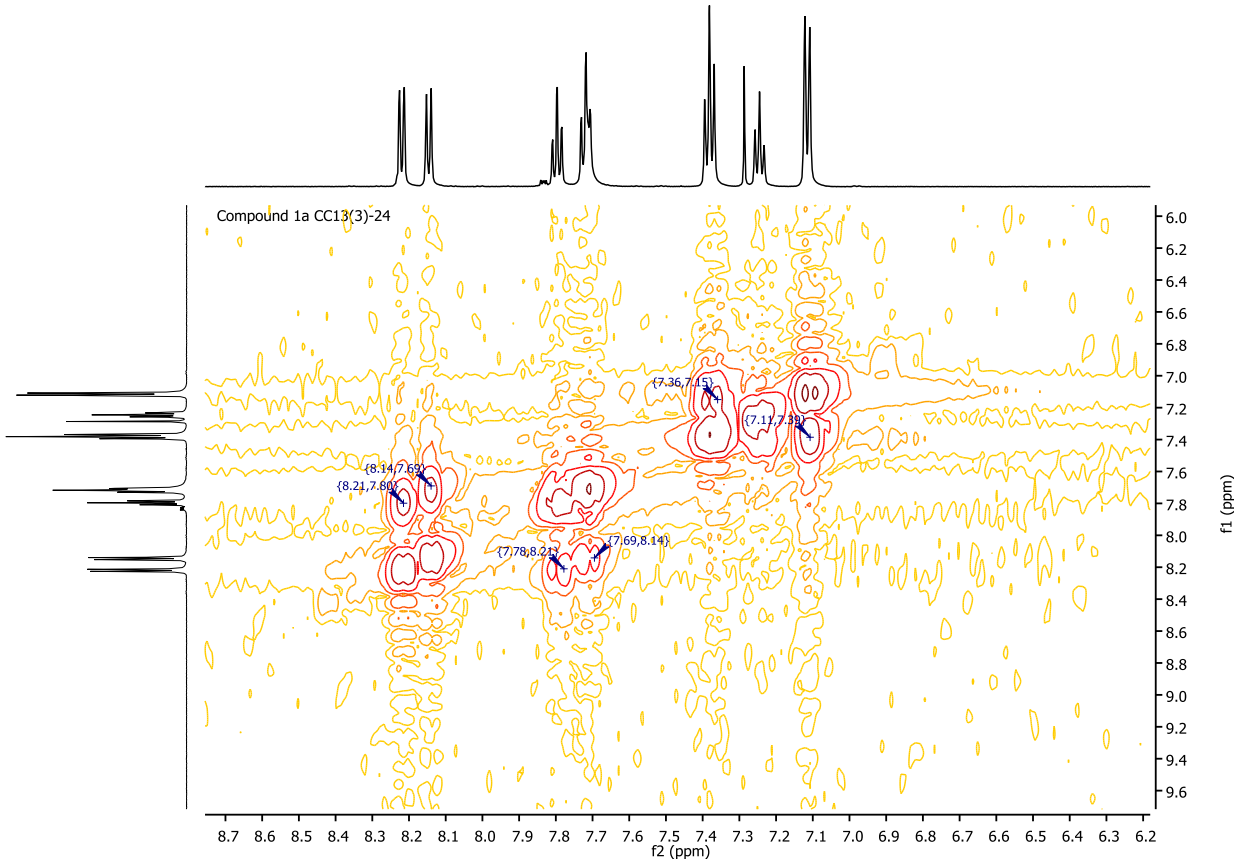
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with aniline. Expansion of the aromatic region is shown in the off-set plot.



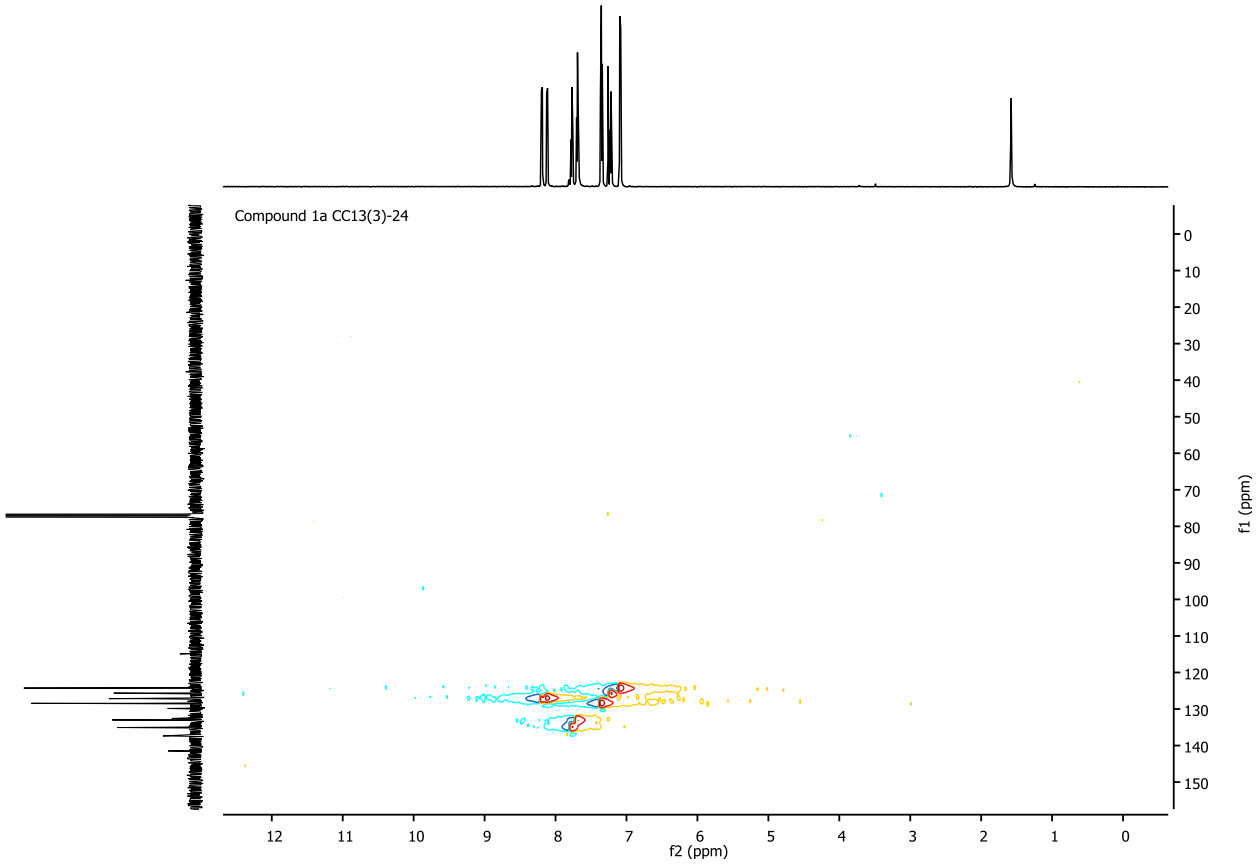
^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a**. Expansion of the signals between 124 ppm and 142 ppm are shown in the off-set plot.



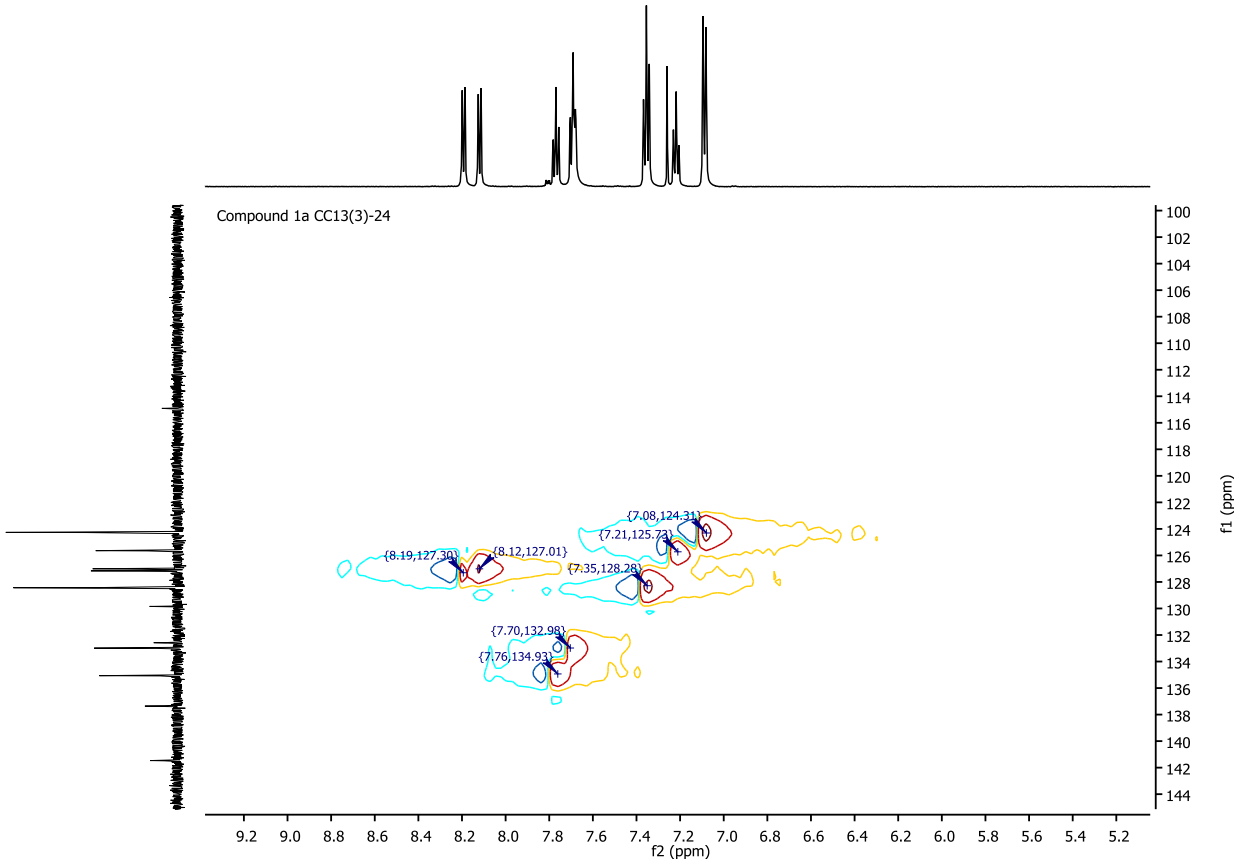
Full COSY NMR spectrum of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a**.



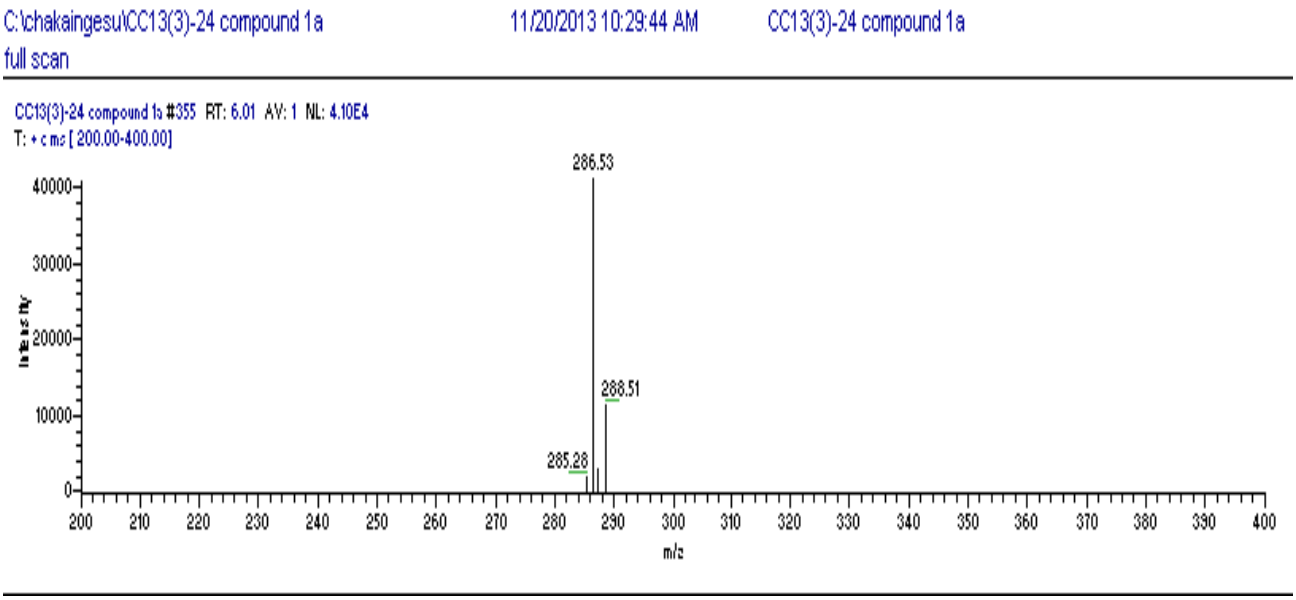
Expansion of the COSY NMR spectrum of 2-chloro-3-(phenylamino)-1,4-naphtho-quinone **1a** showing the aromatic region only.



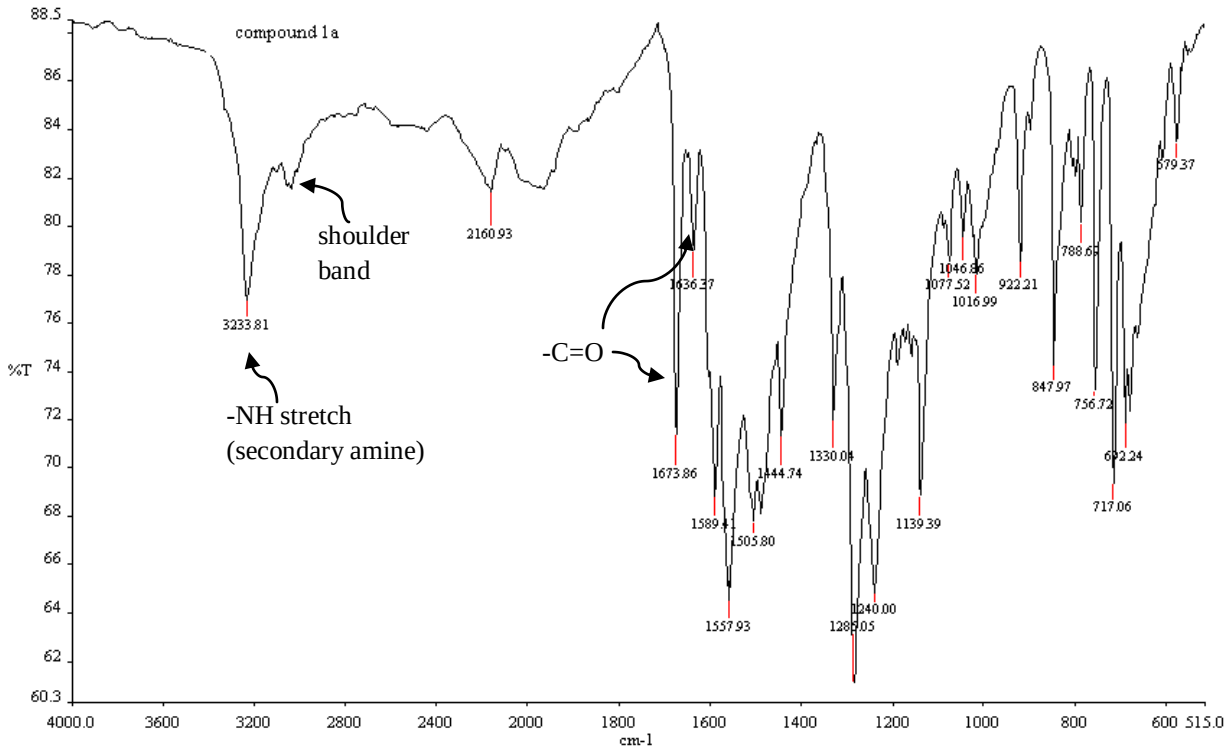
Full HSQC NMR spectrum of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a**.



Expansion of the HSQC NMR spectrum of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a** showing the aromatic region only.

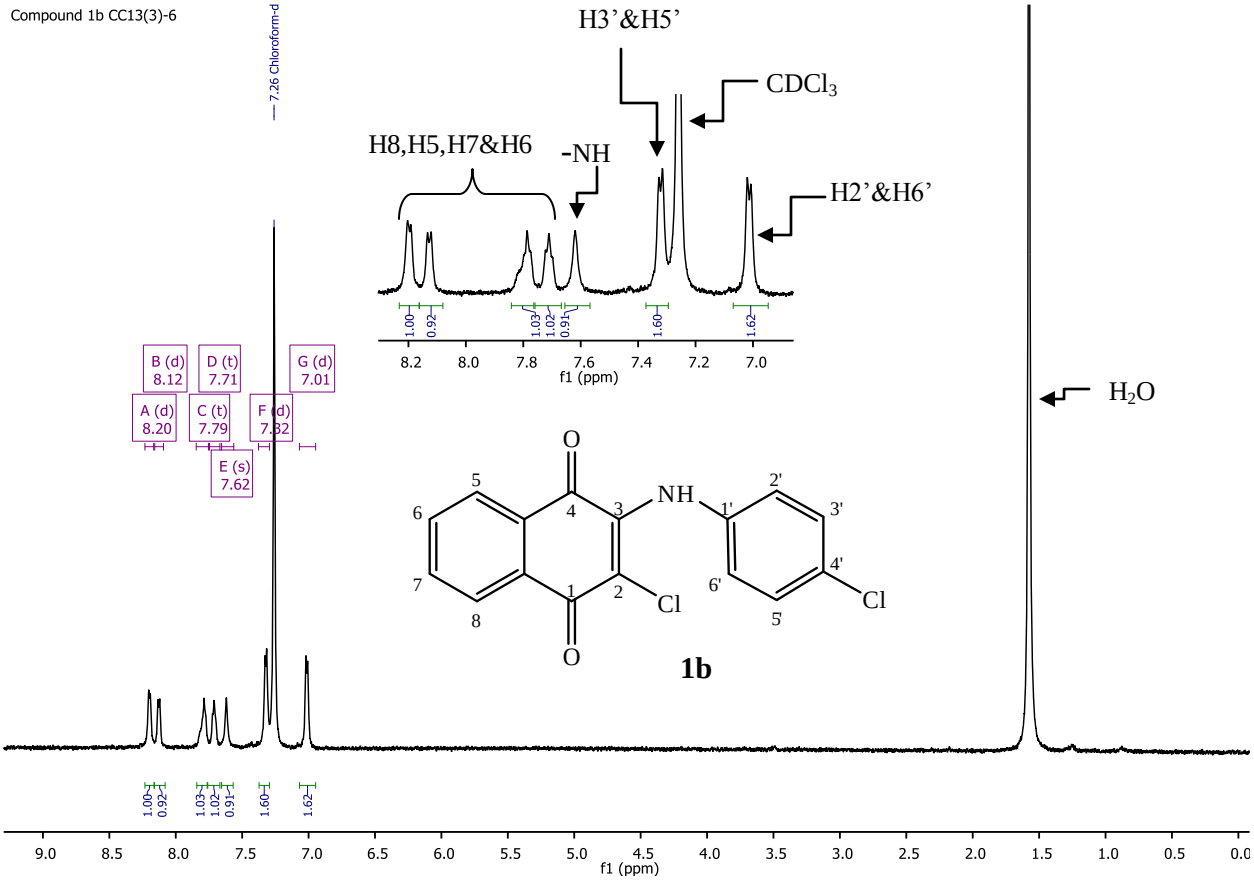


HPLC-ESI-MS spectrum of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a**.

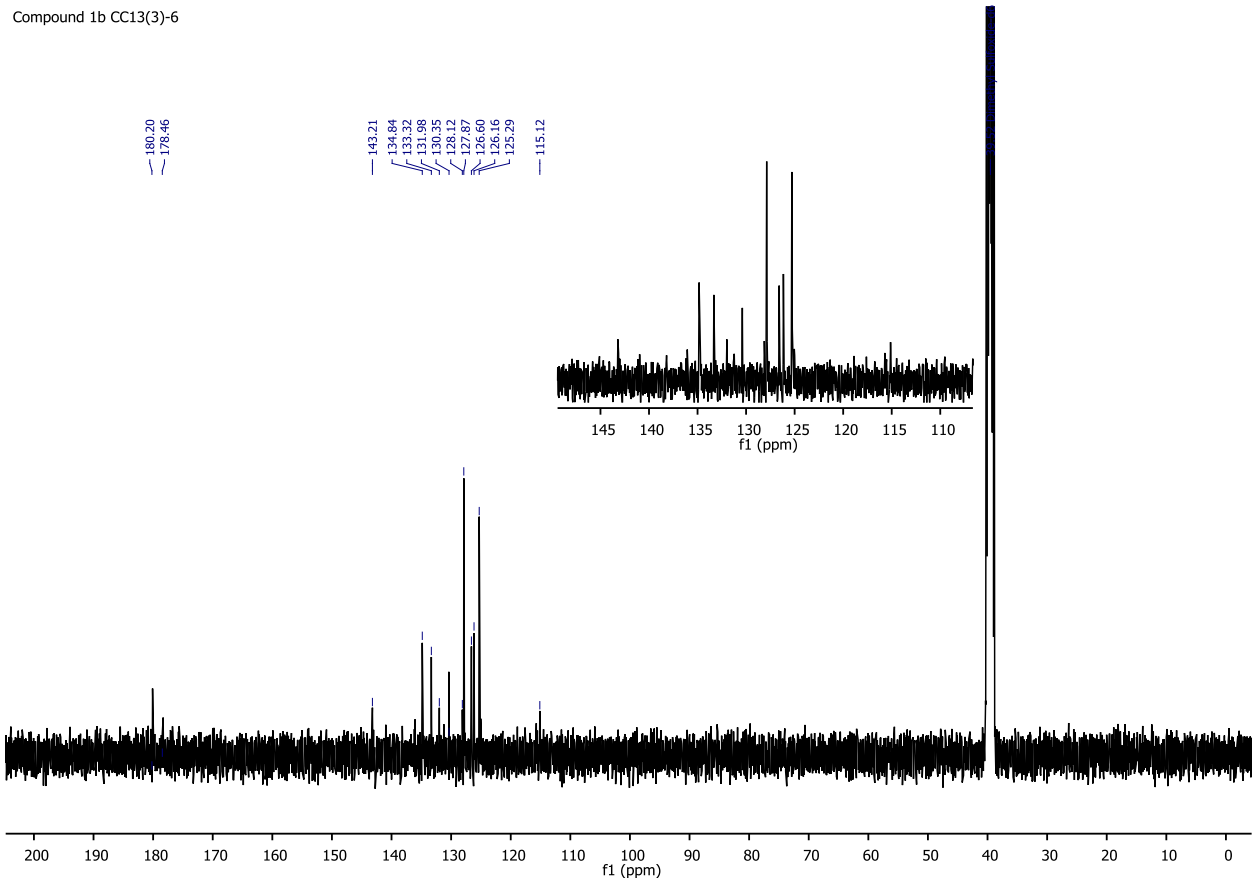


IR (KBr, cm^{-1}) spectrum of 2-chloro-3-(phenylamino)-1,4-naphthoquinone **1a**.

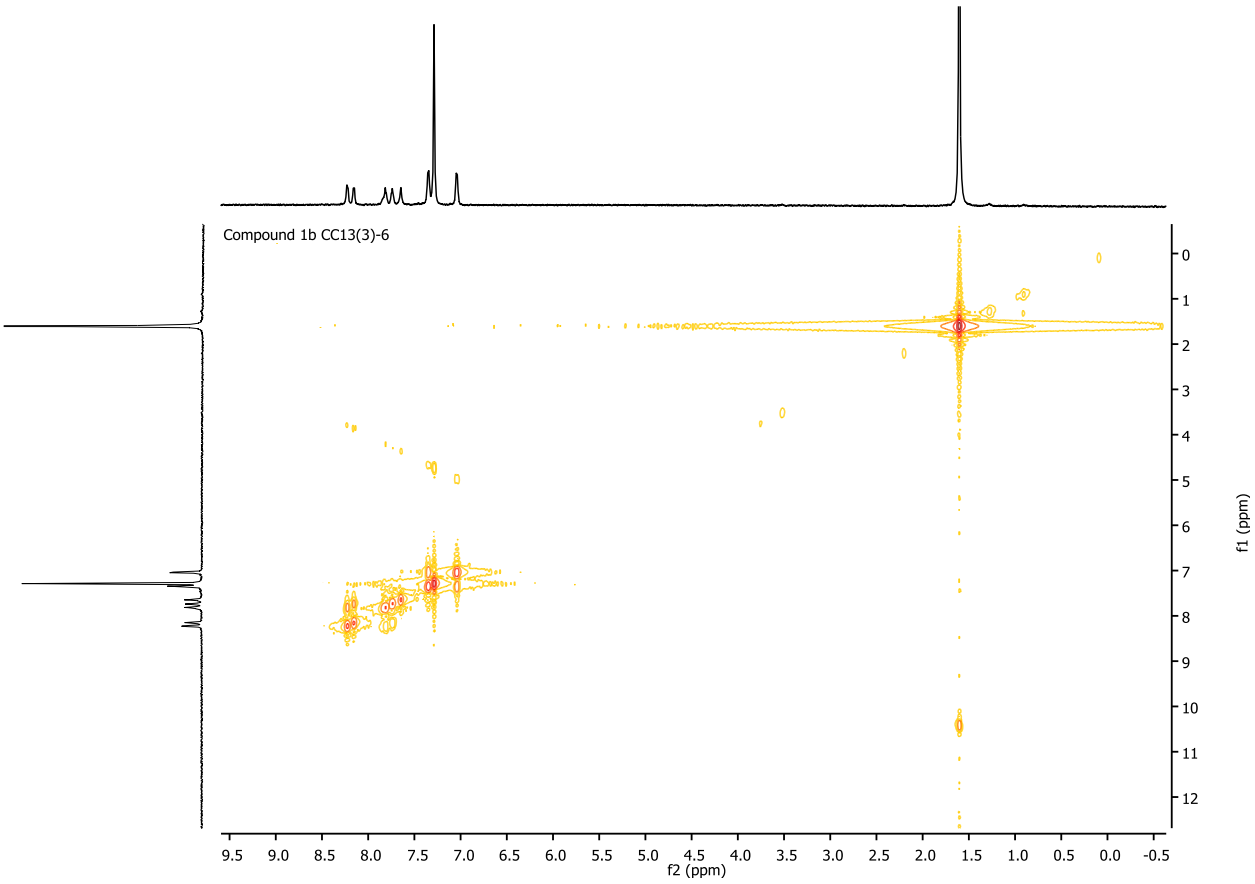
COMPOUND 1b



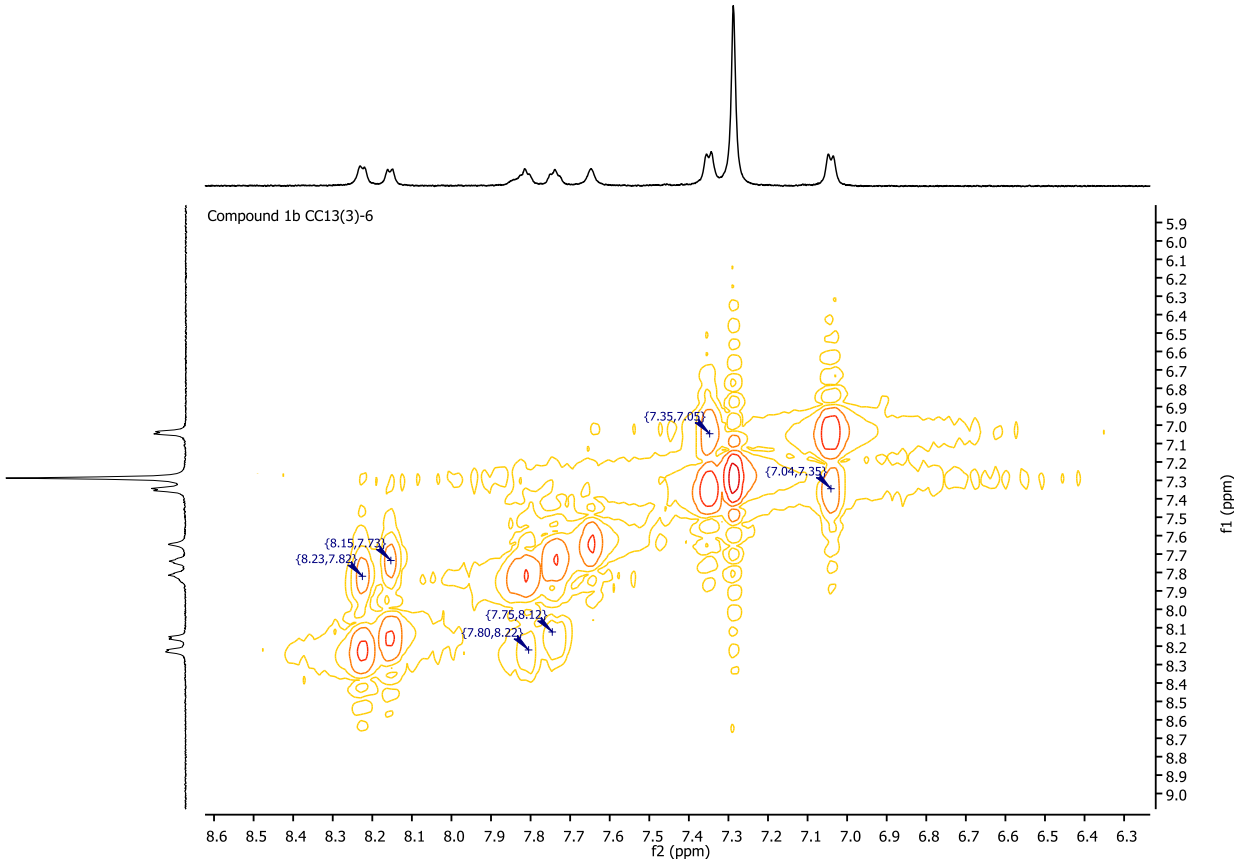
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2-chloro-3-(4-chloro-phenylamino)-1,4-naphthoquinone **1b**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with aniline. Expansions of signals in the aromatic region are shown in the off-set plot.



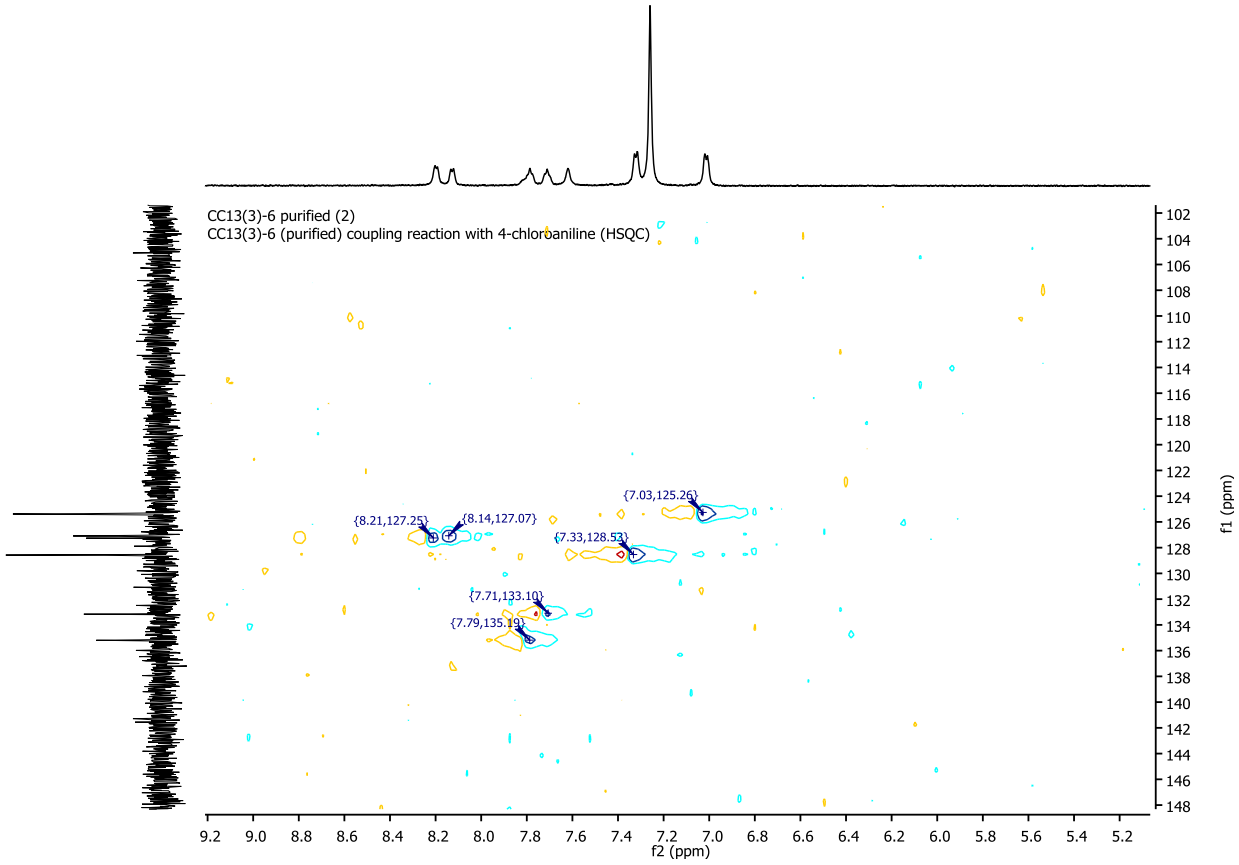
^{13}C NMR (100MHz, DMSO-d) spectrum of 2-chloro-3-(4-chloro-phenylamino)-1,4-naphthoquinone **1b**. Expansion of the signals between 110 ppm and 145 ppm are shown in the off-set plot.



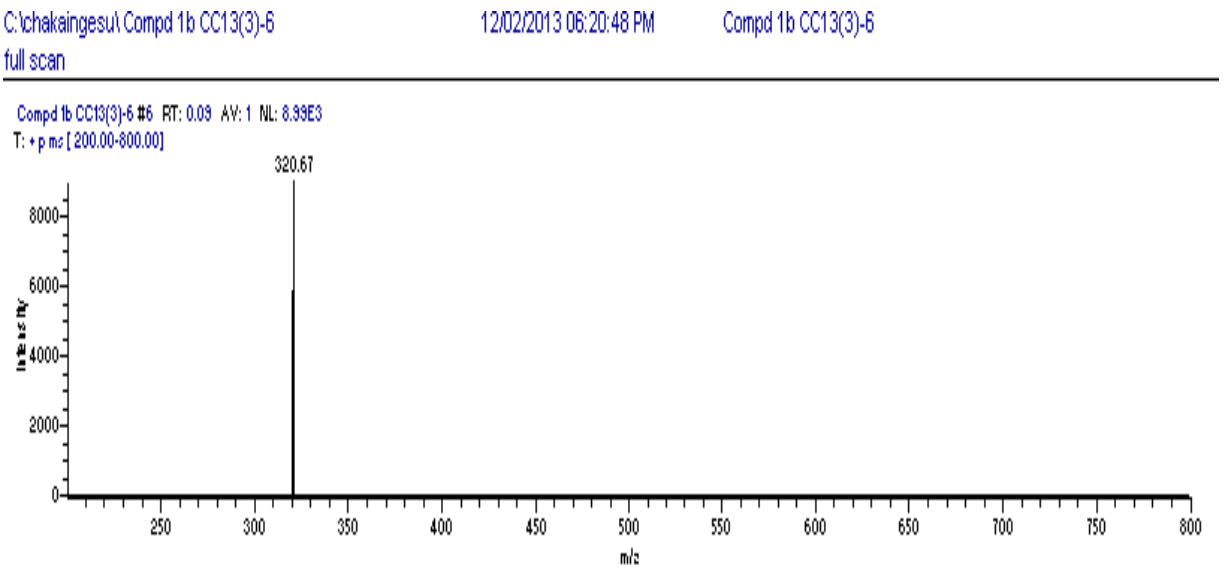
Full COSY NMR spectrum of 2-chloro-3-(4-chloro-phenylamino)-1,4-naphthoquinone **1b**.



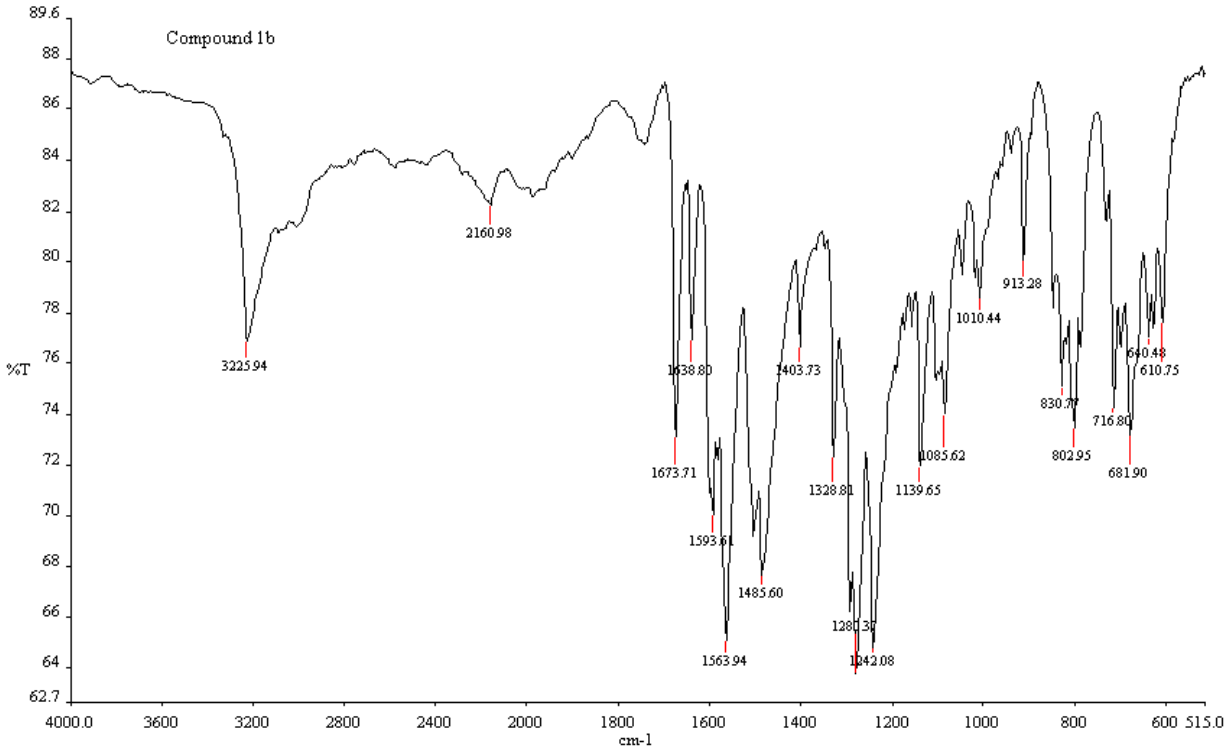
Expansion of the COSY NMR spectrum of 2-chloro-3-(4-chloro-phenylamino)-1,4-naphtho-quinone **1b**.



Expansion of the HSQC NMR spectrum of 2-chloro-3-(4-chloro-phenylamino)-1,4-naphtho-quinone **1b**.

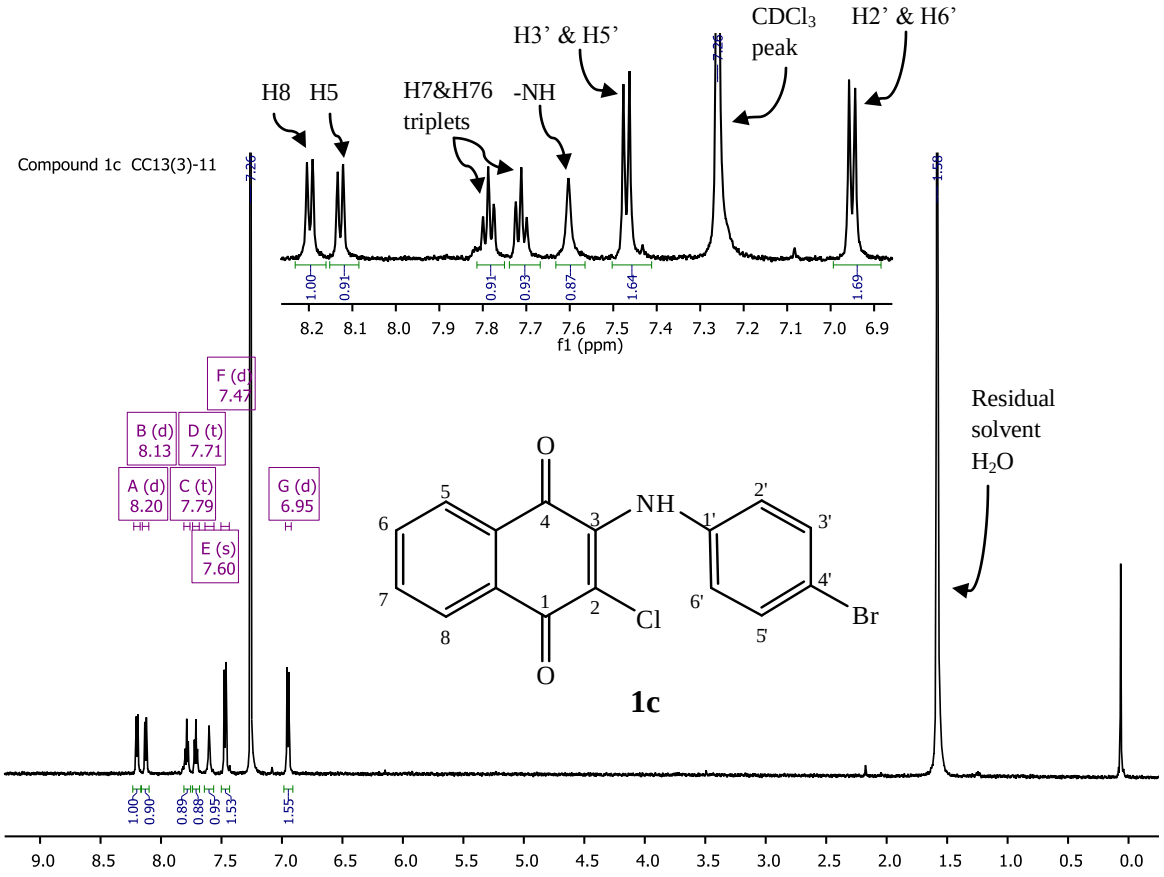


HPLC-ESI-MS spectrum of 2-chloro-3-(4-chloro-phenylamino)-1,4-naphthoquinone **1b**.

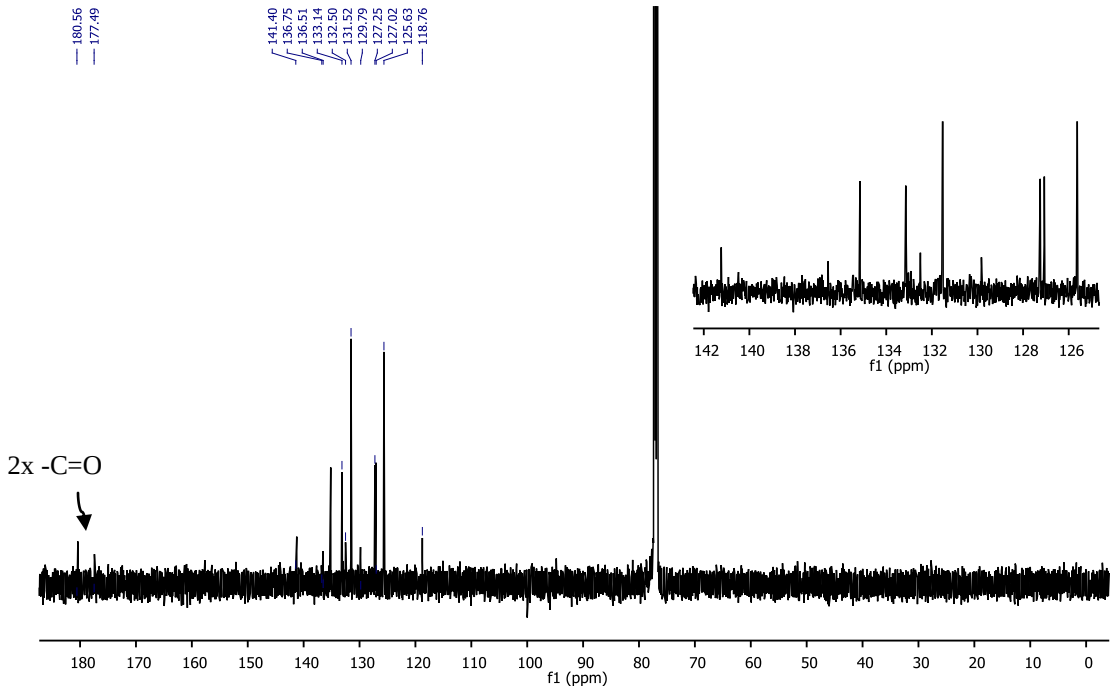


IR (KBr, cm^{-1}) spectrum of 2-chloro-3-(4-chloro-phenylamino)-1,4-naphthoquinone **1b**.

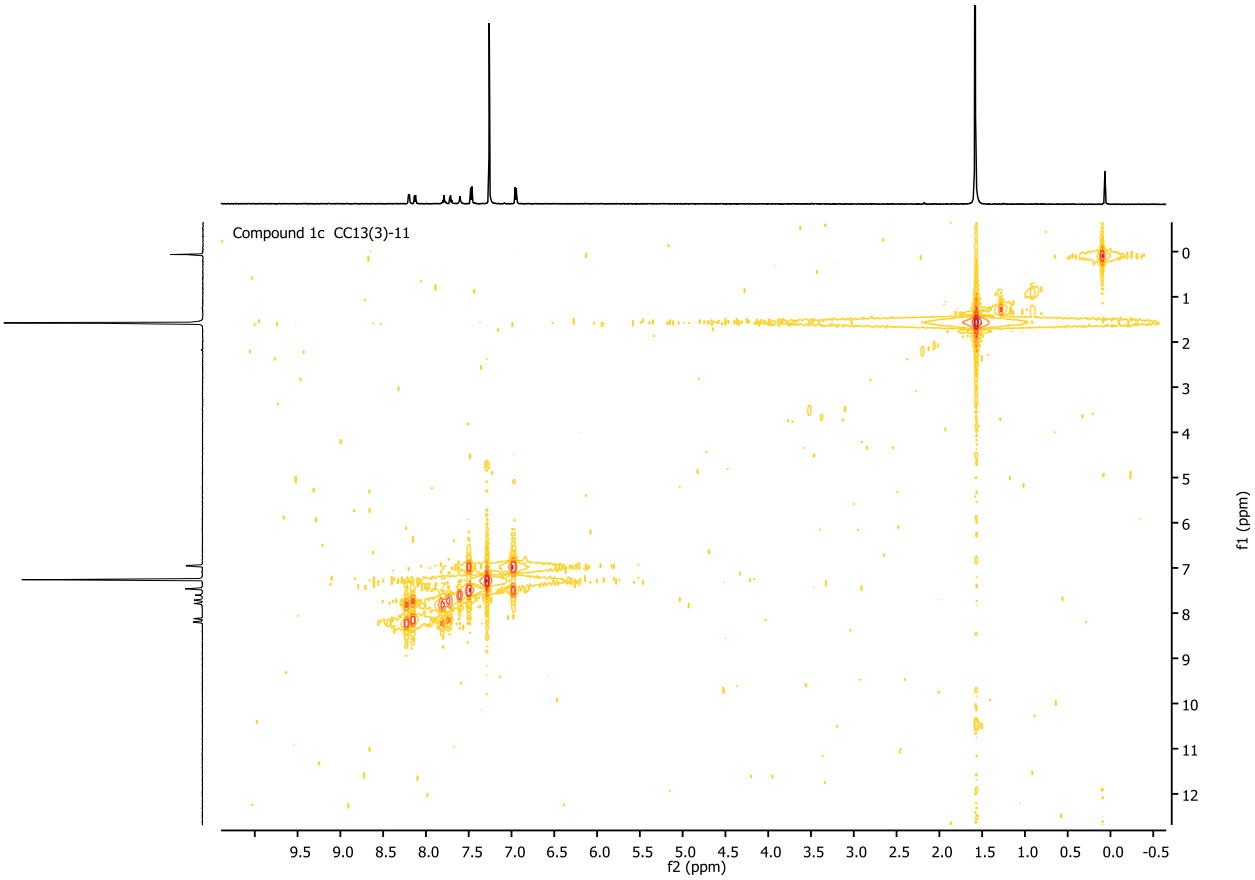
COMPOUND 1c



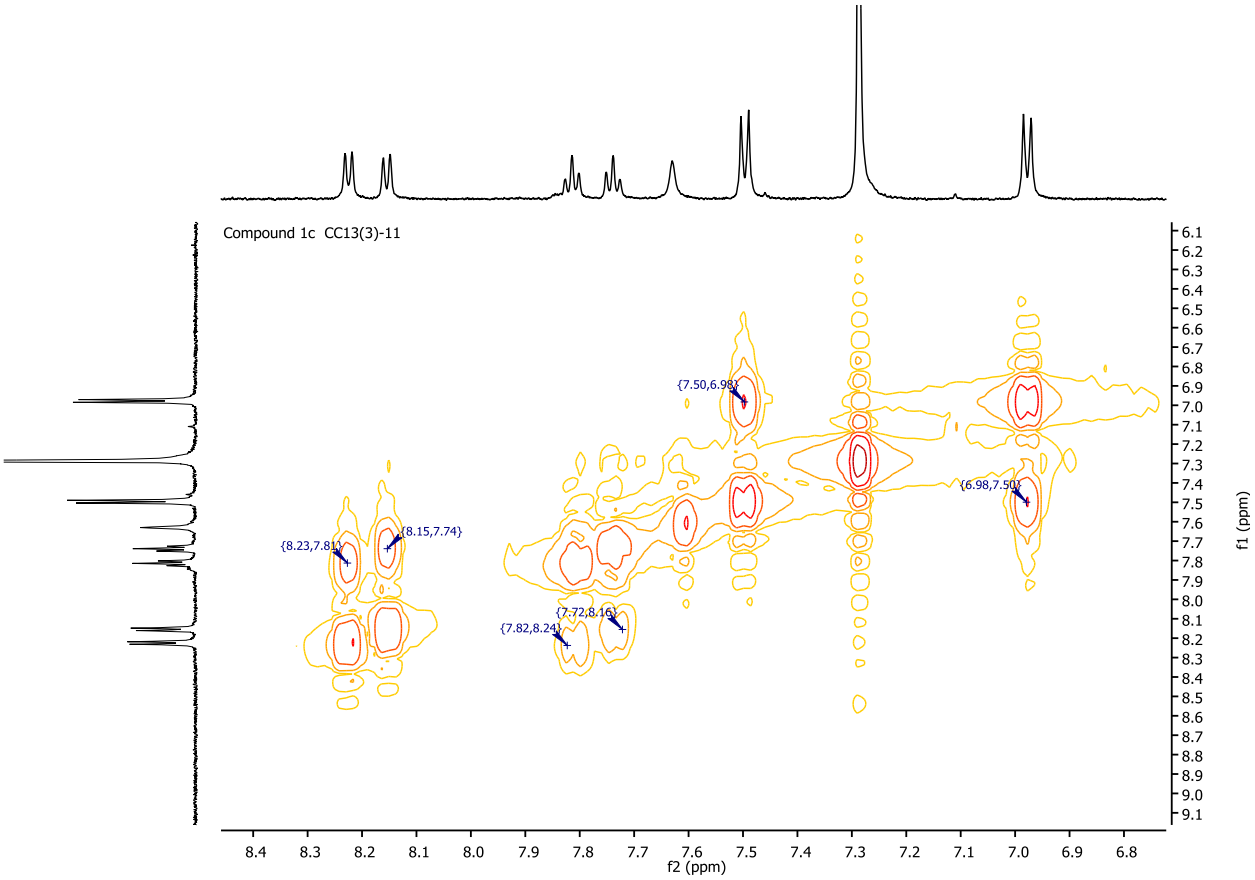
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2-chloro-3-(4-bromophenylamino)-1,4-naphthoquinone **1c**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone and 4-bromoaniline. Expansions of the signals in the aromatic region are shown in the off-set plot.



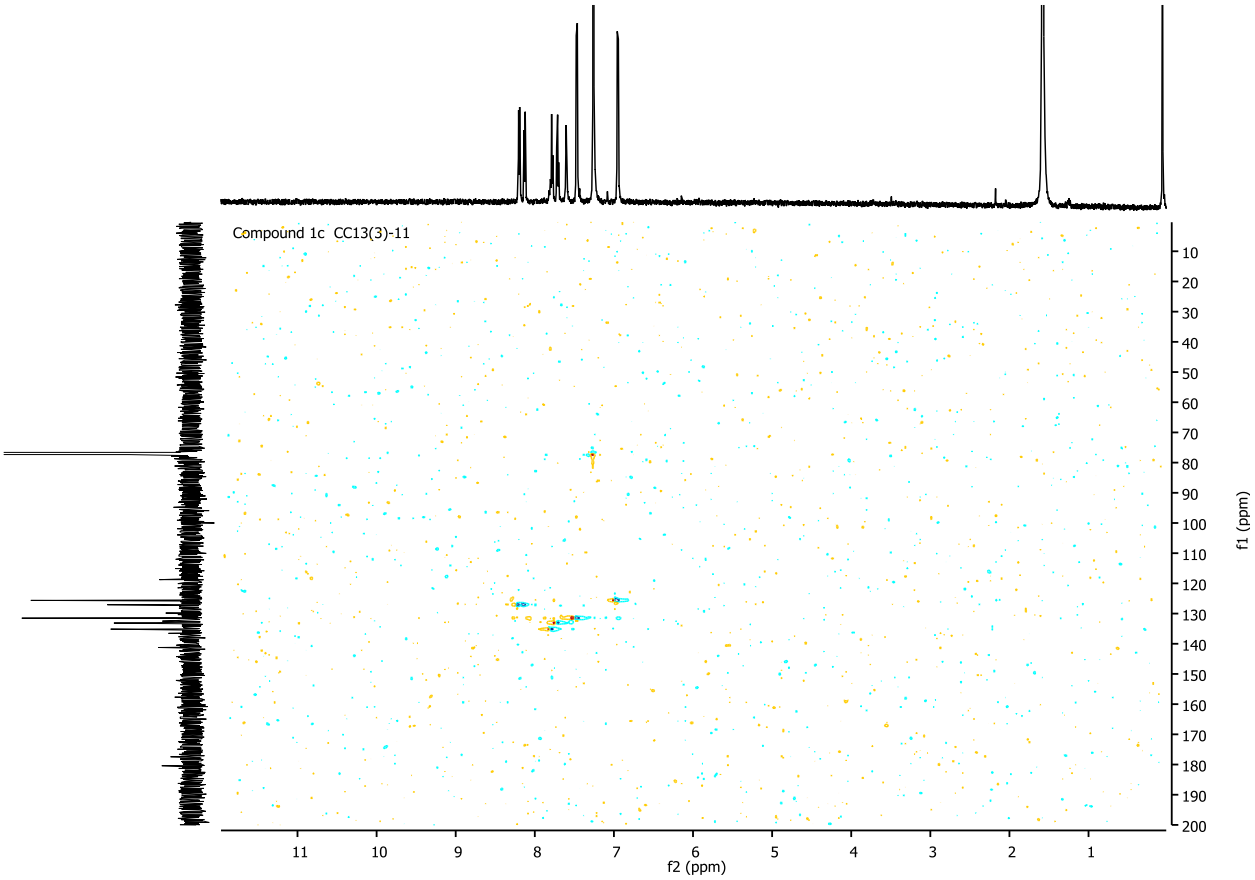
^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2-chloro-3-(4-bromo-phenylamino)-1,4-naphthoquinone **1c**. Expansion of the signals between 126 ppm and 142 ppm are shown in the off-set plot.



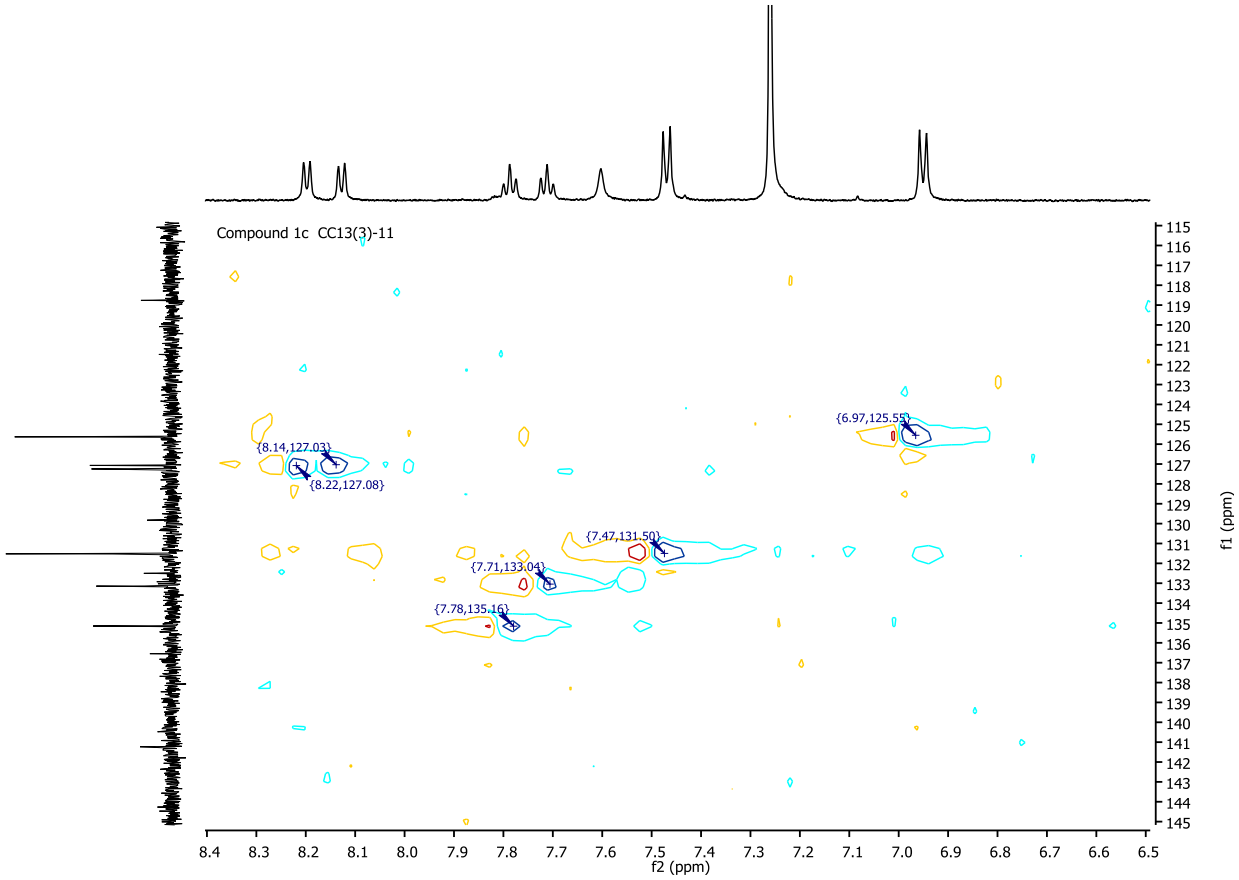
Full COSY NMR spectrum of 2-chloro-3-(4-bromo-phenylamino)-1,4-naphthoquinone **1c**.



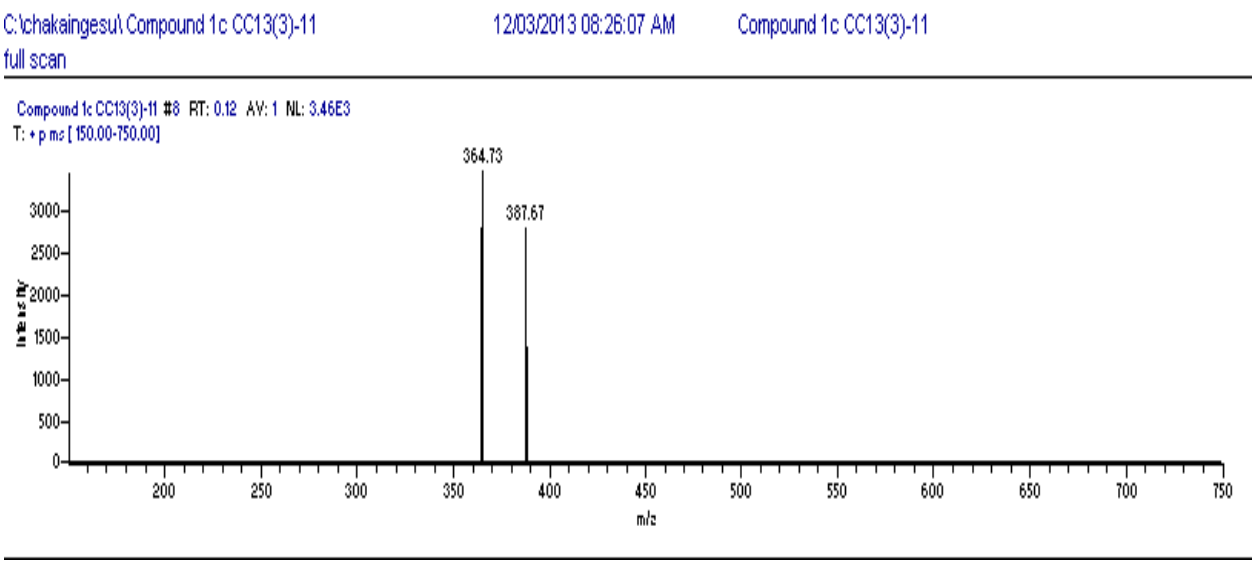
Expansion of the COSY NMR spectrum of 2-chloro-3-(4-bromo-phenylamino)-1,4-naphthoquinone **1c**.



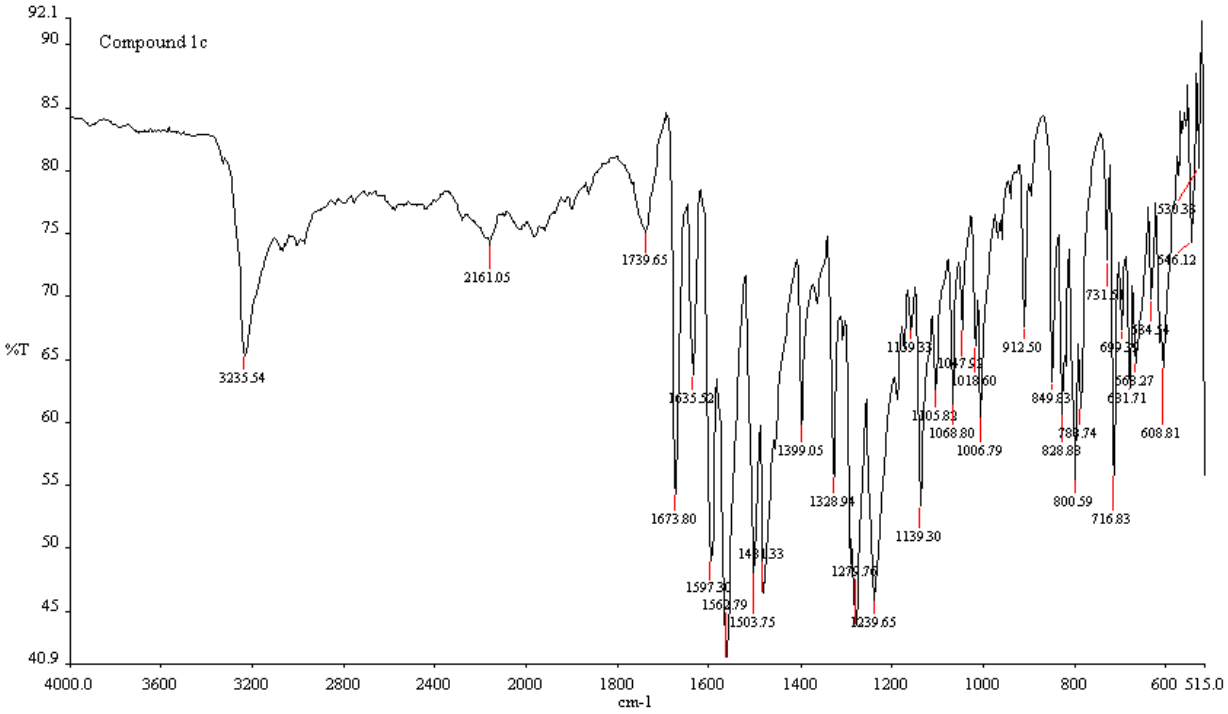
Full HSQC NMR spectrum of 2-chloro-3-(4-bromo-phenylamino)-1,4-naphthoquinone **1c**.



Expansion of HSQC NMR spectrum of 2-chloro-3-(4-bromo-phenylamino)-1,4-naphthoquinone **1c**.

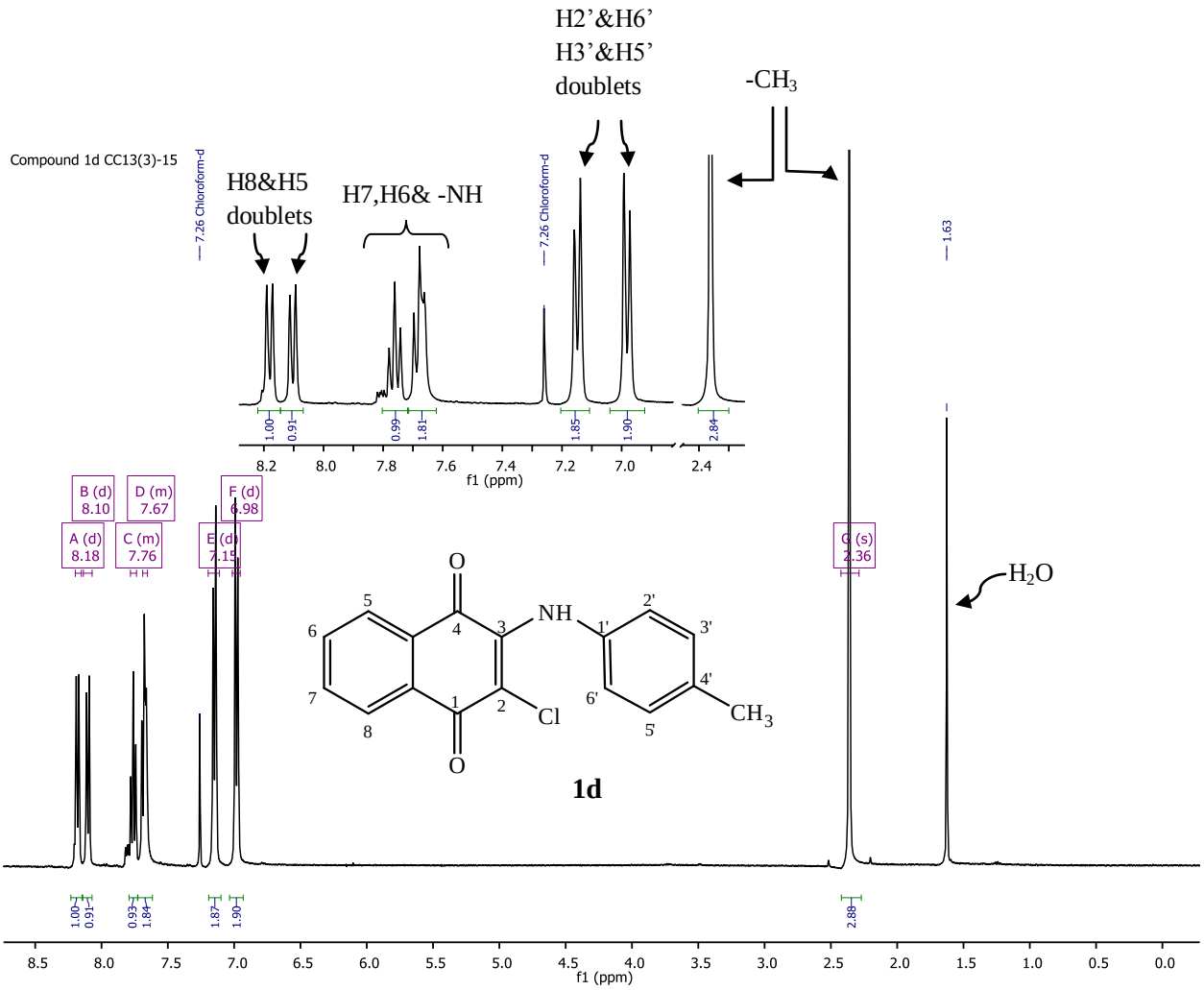


HPLC-ESI-MS spectrum of 2-chloro-3-(4-bromo-phenylamino)-1,4-naphthoquinone **1c**.

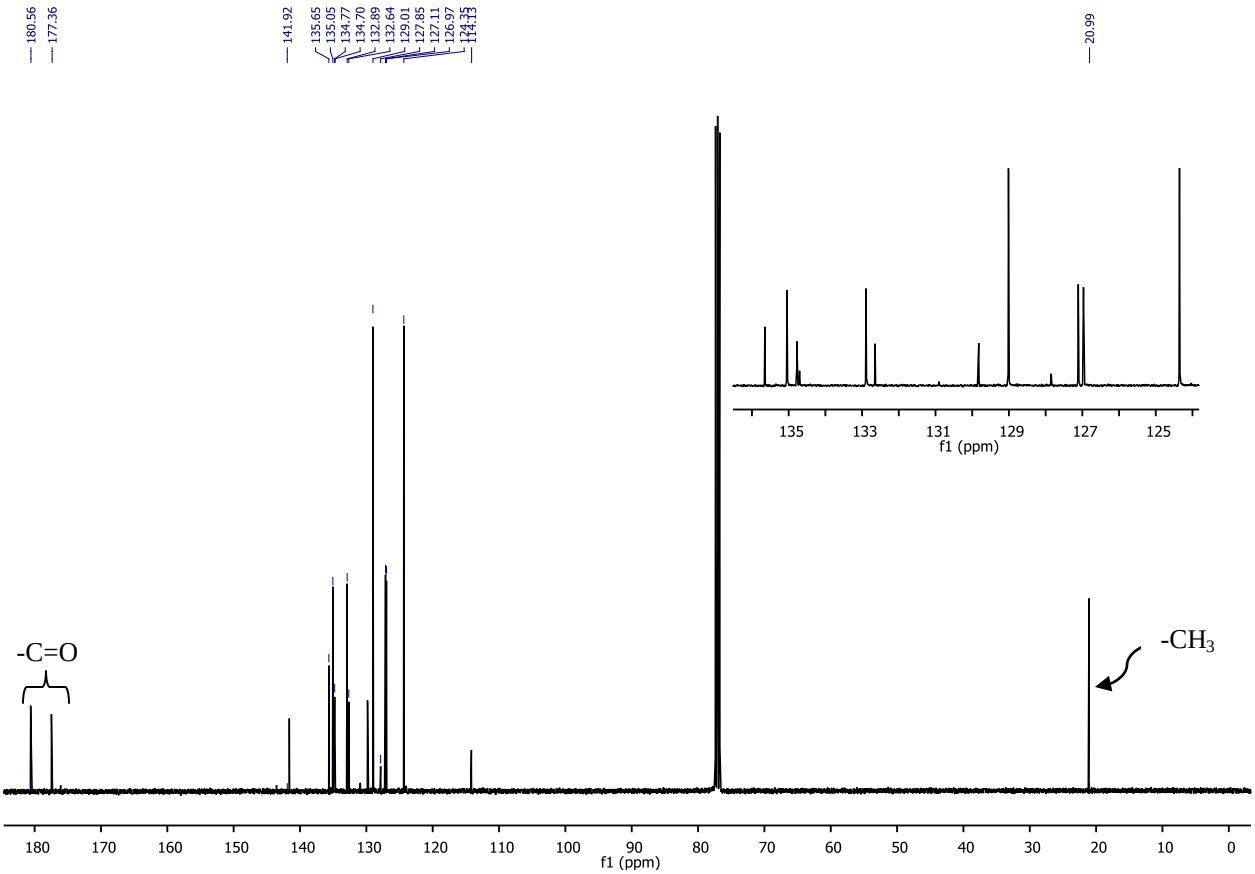


IR (KBr, cm^{-1}) spectrum of 2-chloro-3-(4-bromo-phenylamino)-1,4-naphthoquinone **1c**.

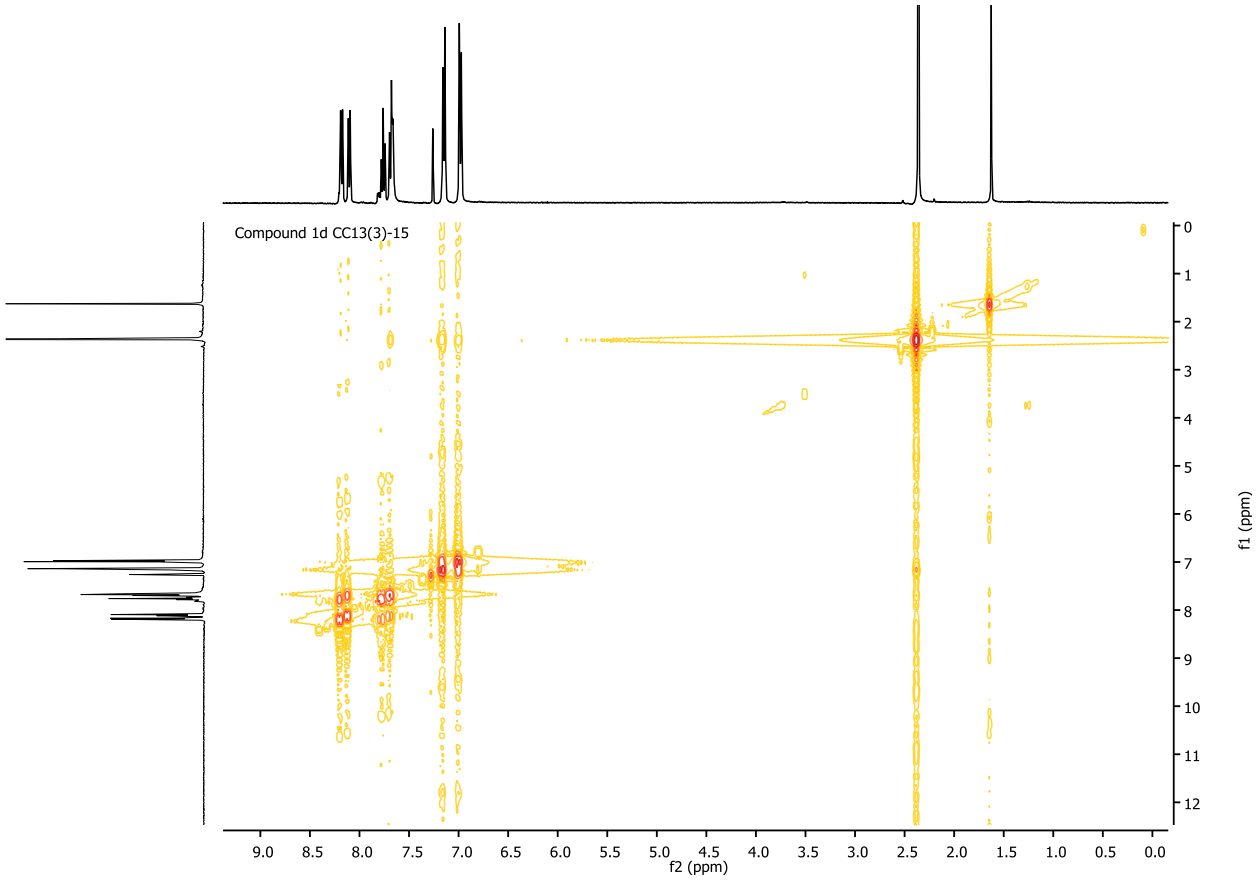
COMPOUND 1d



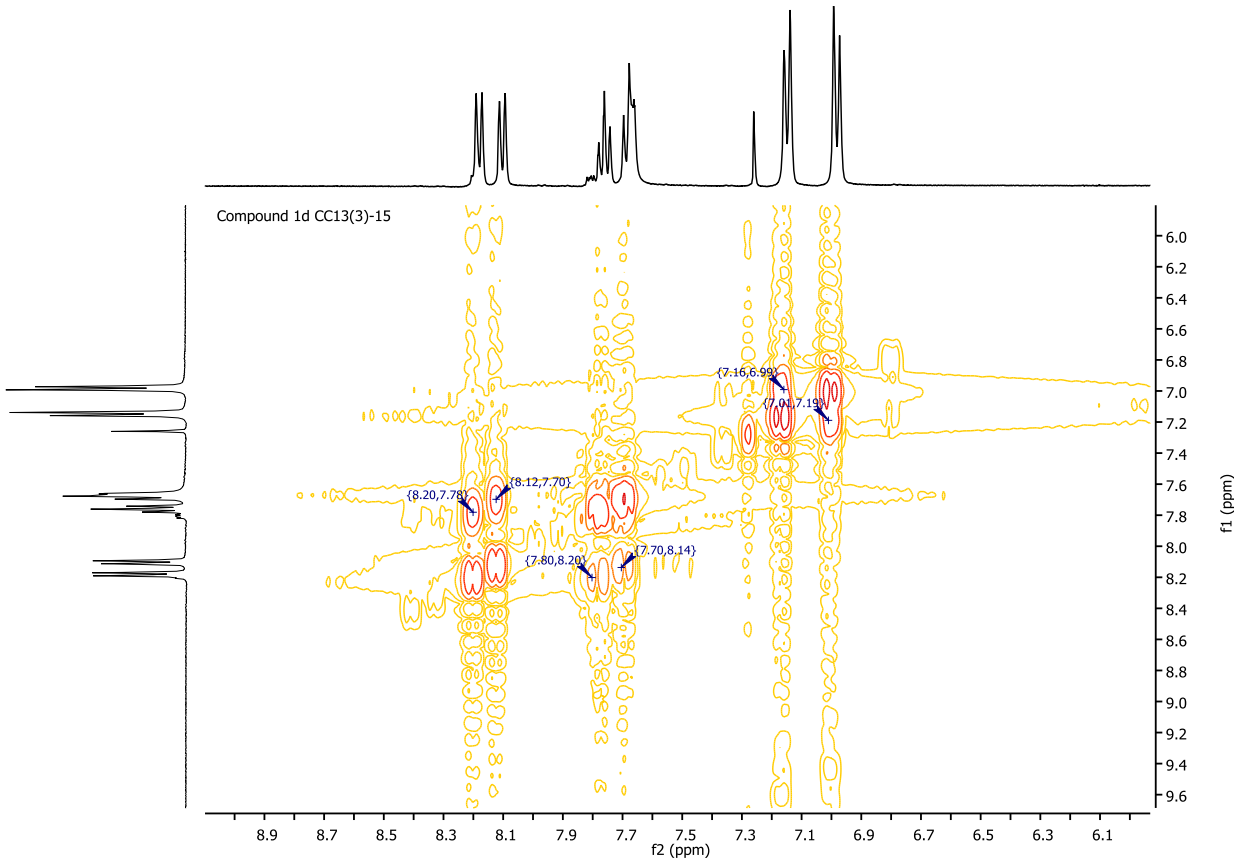
^{1}H NMR (400MHz, $CDCl_3$) spectrum of 2-chloro-3-(p-tolylamino)-1,4-naphthoquinone **1d**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 4-methylaniline. Expansions of signals in the aromatic region as well as the methyl signal are shown in the off-set plot.



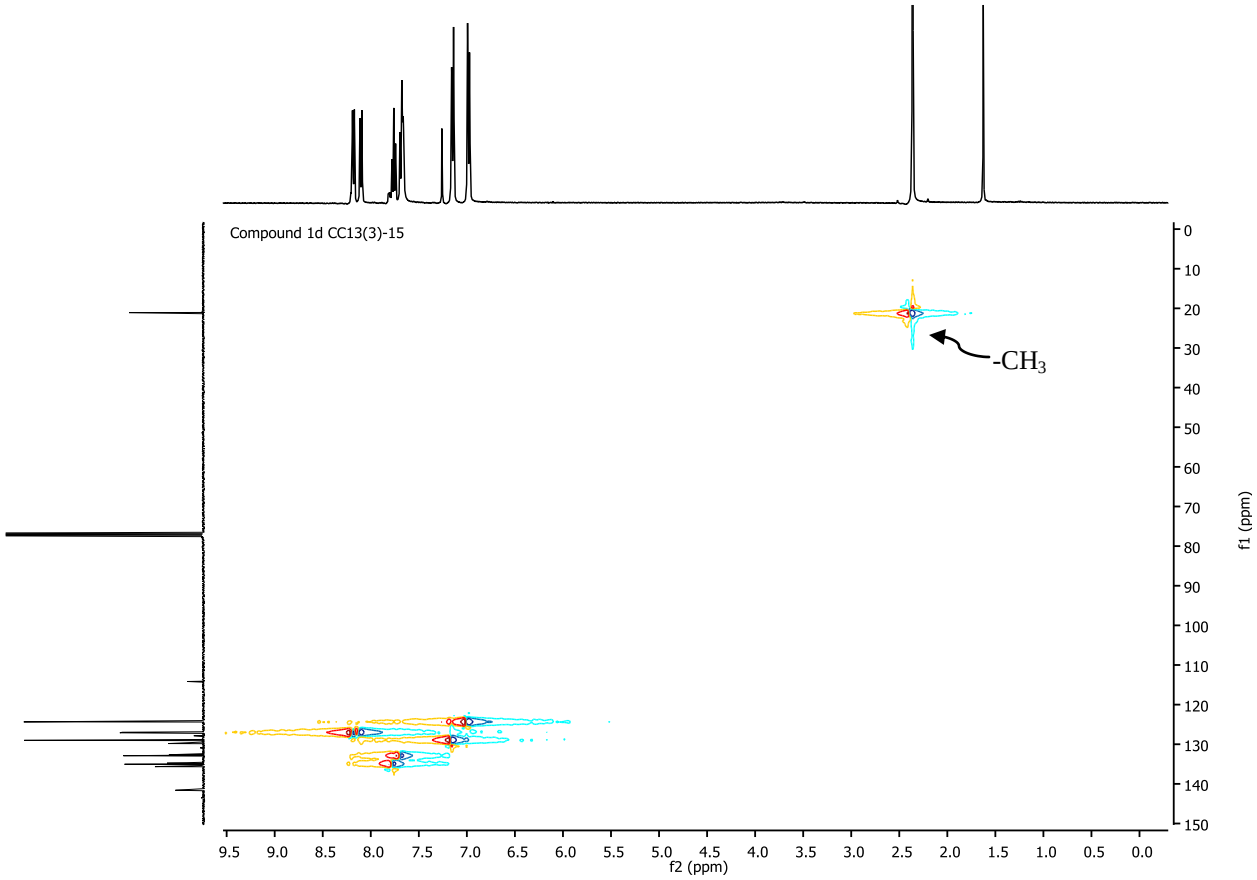
^{13}C NMR (100MHz, $CDCl_3$) spectrum of 2-chloro-3-(p-tolylamino)-1,4-naphthoquinone **1d**. Expansion of the signals between 124 ppm and 136 ppm are shown in the off-set plot.



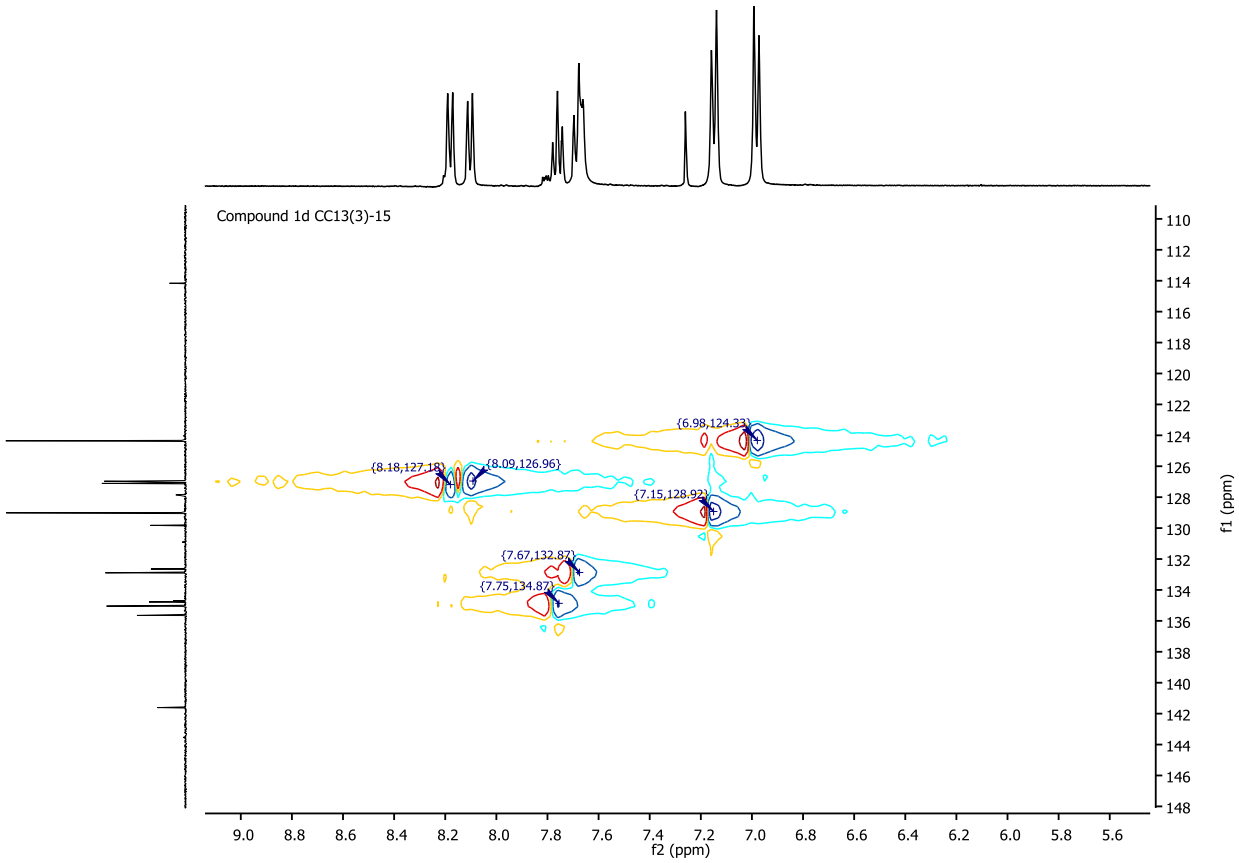
Full COSY NMR spectrum of 2-chloro-3-(p-tolylamino)-1,4-naphthoquinone **1d**.



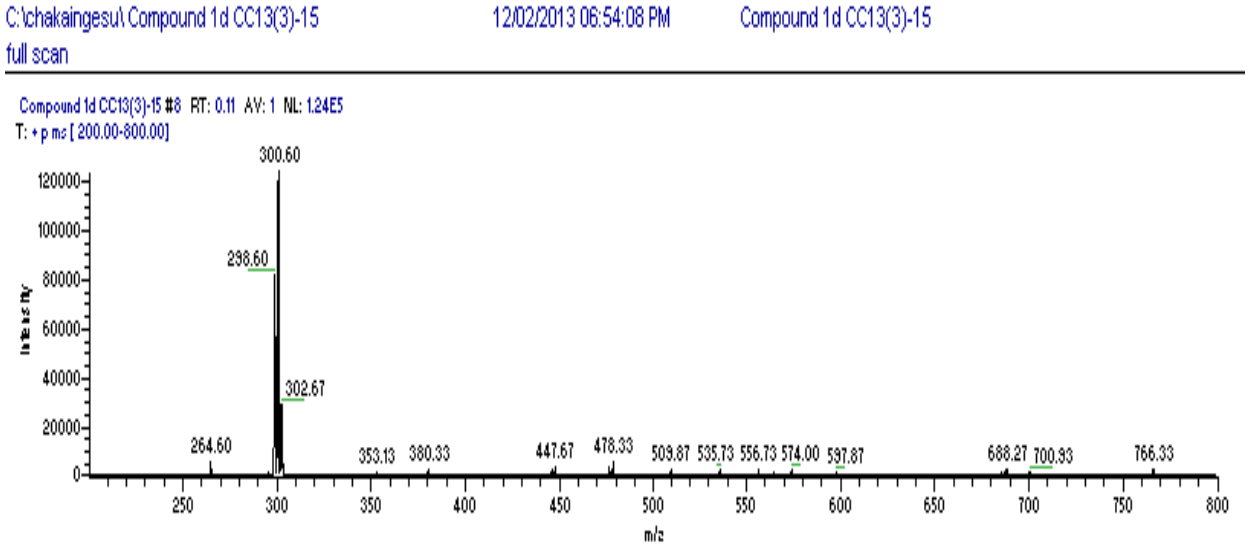
Expansion of the COSY NMR spectrum of 2-chloro-3-(p-tolylamino)-1,4-naphthoquinone **1d**.



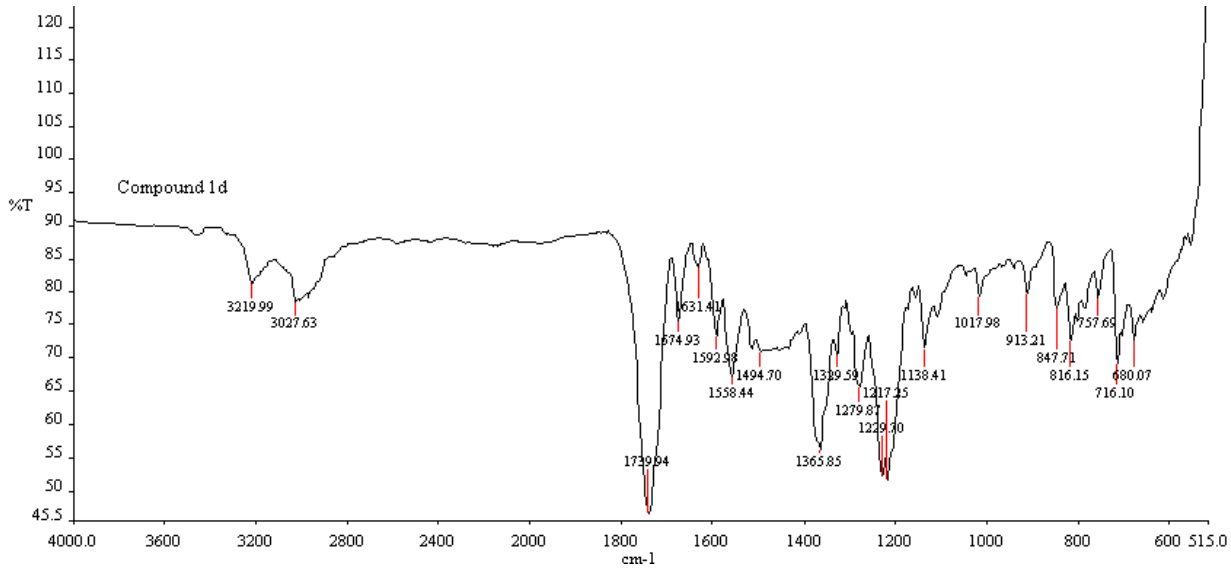
Full HSQC NMR spectrum of 2-chloro-3-(p-tolylamino)-1,4-naphthoquinone **1d**.



Expansion of the HSQC NMR spectrum of 2-chloro-3-(p-tolylamino)-1,4-naphthoquinone **1d**. The methyl correlation cannot be seen here.

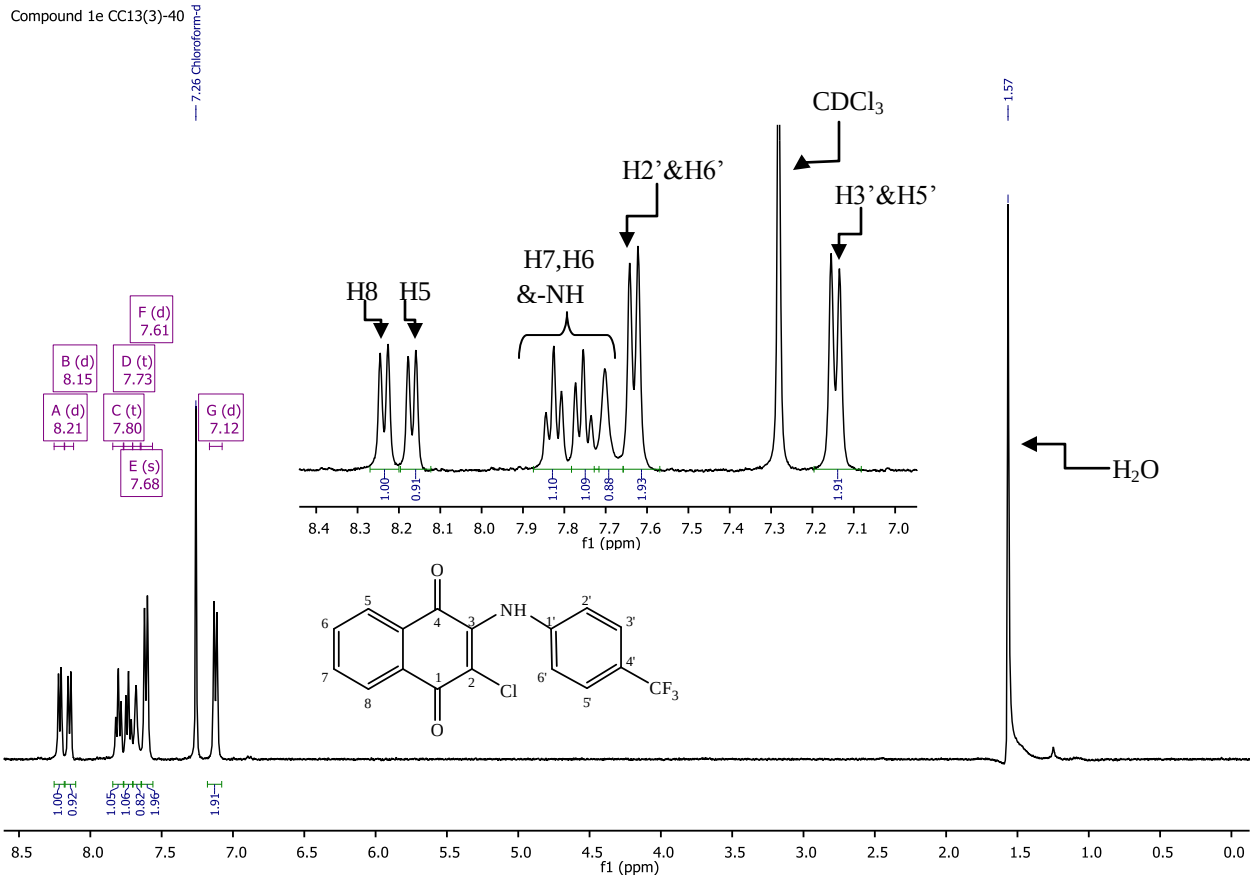


HPLC-ESI-MS spectrum of 2-chloro-3-(p-tolylamino)-1,4-naphthoquinone **1d**.

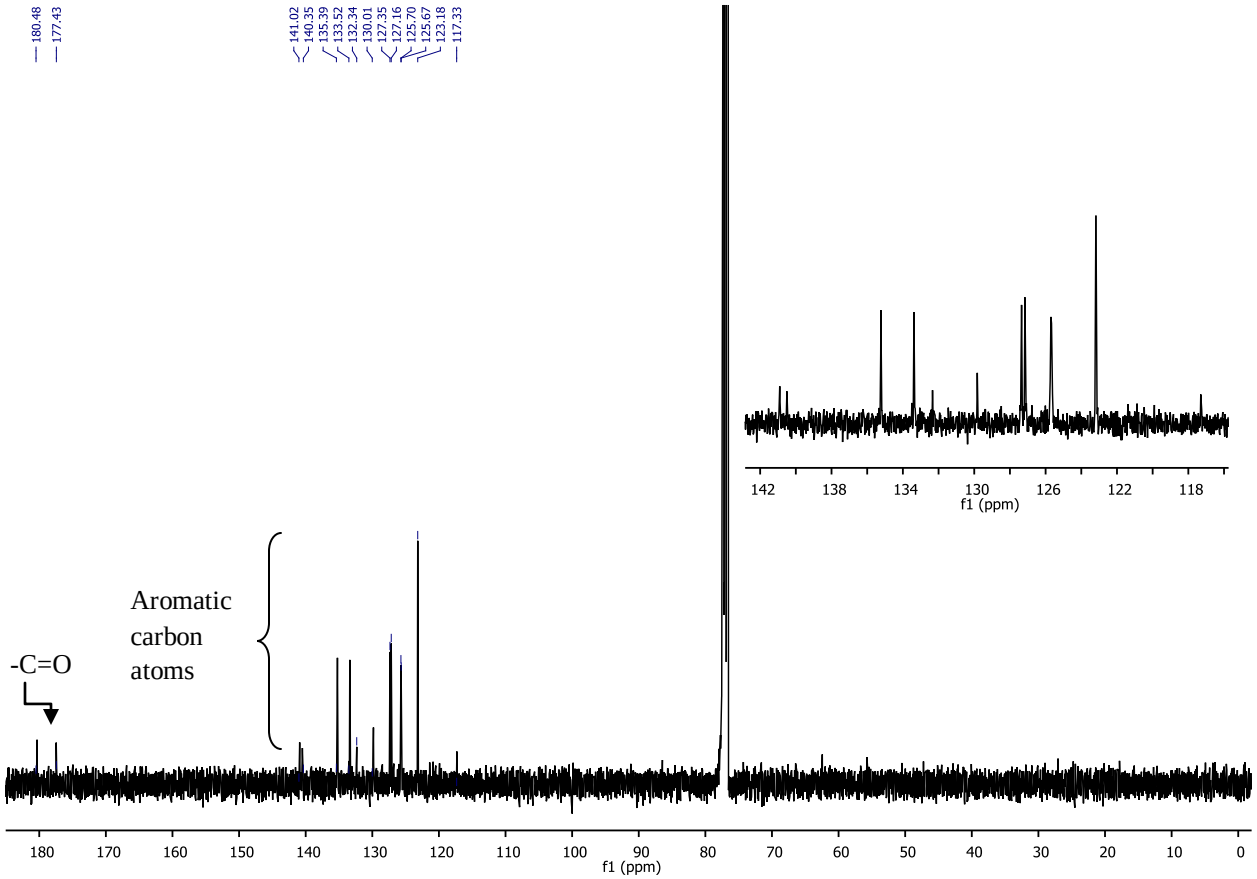


IR (KBr, cm^{-1}) spectrum of 2-chloro-3-(p-tolylamino)-1,4-naphthoquinone **1d**.

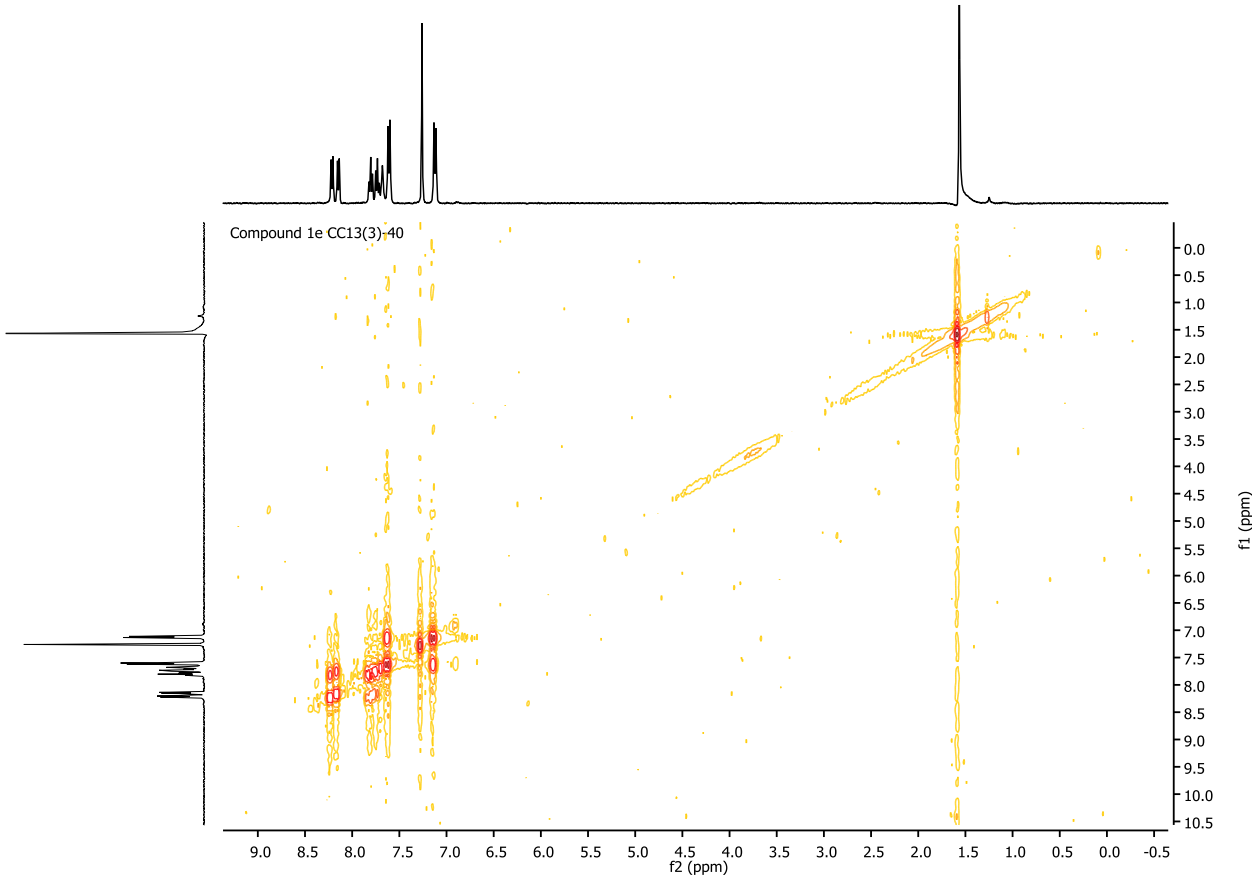
COMPOUND 1e



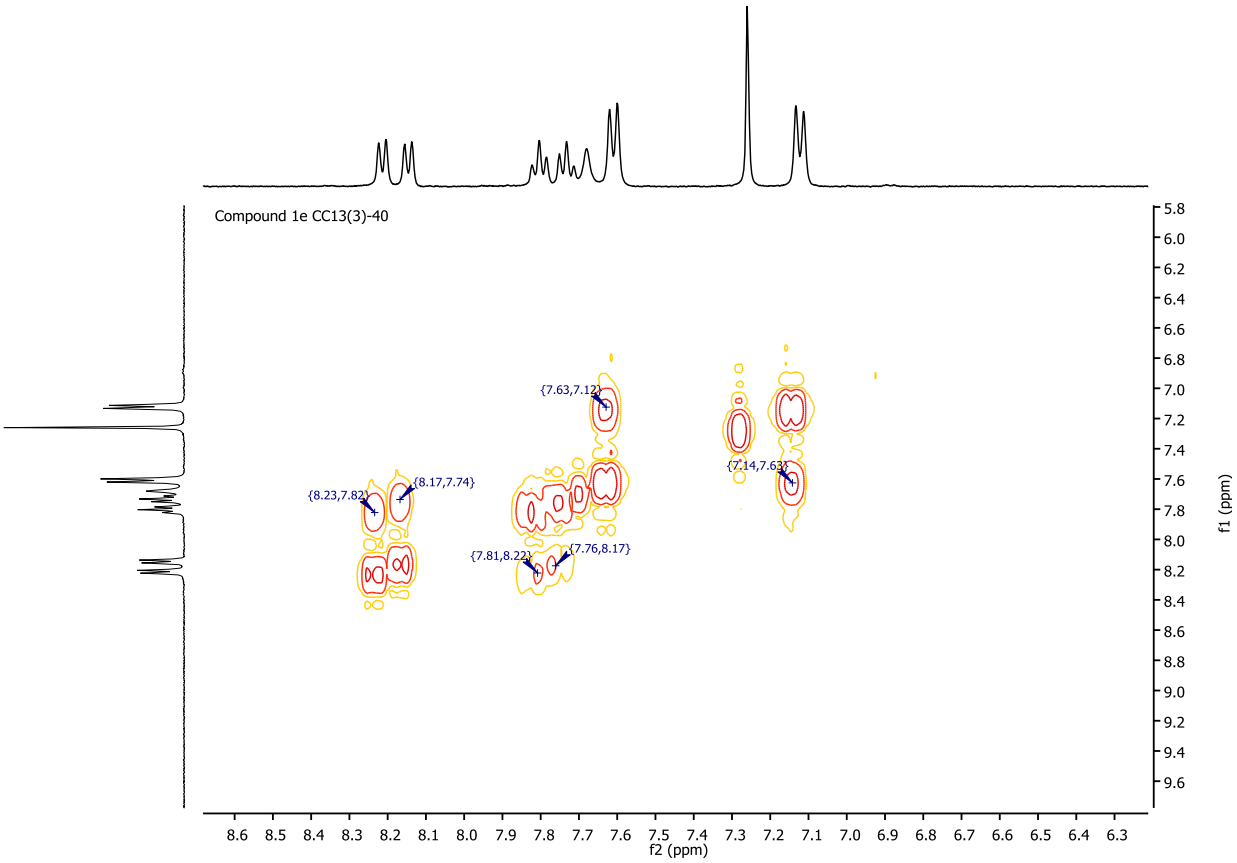
^{1}HNMR (400MHz,$CDCl_3$) spectrum of 2-chloro-3-(4-(trifluoro-methyl)phenylamino)-1,4-naphthoquinone **1e**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 4-(trifluoromethyl)aniline.



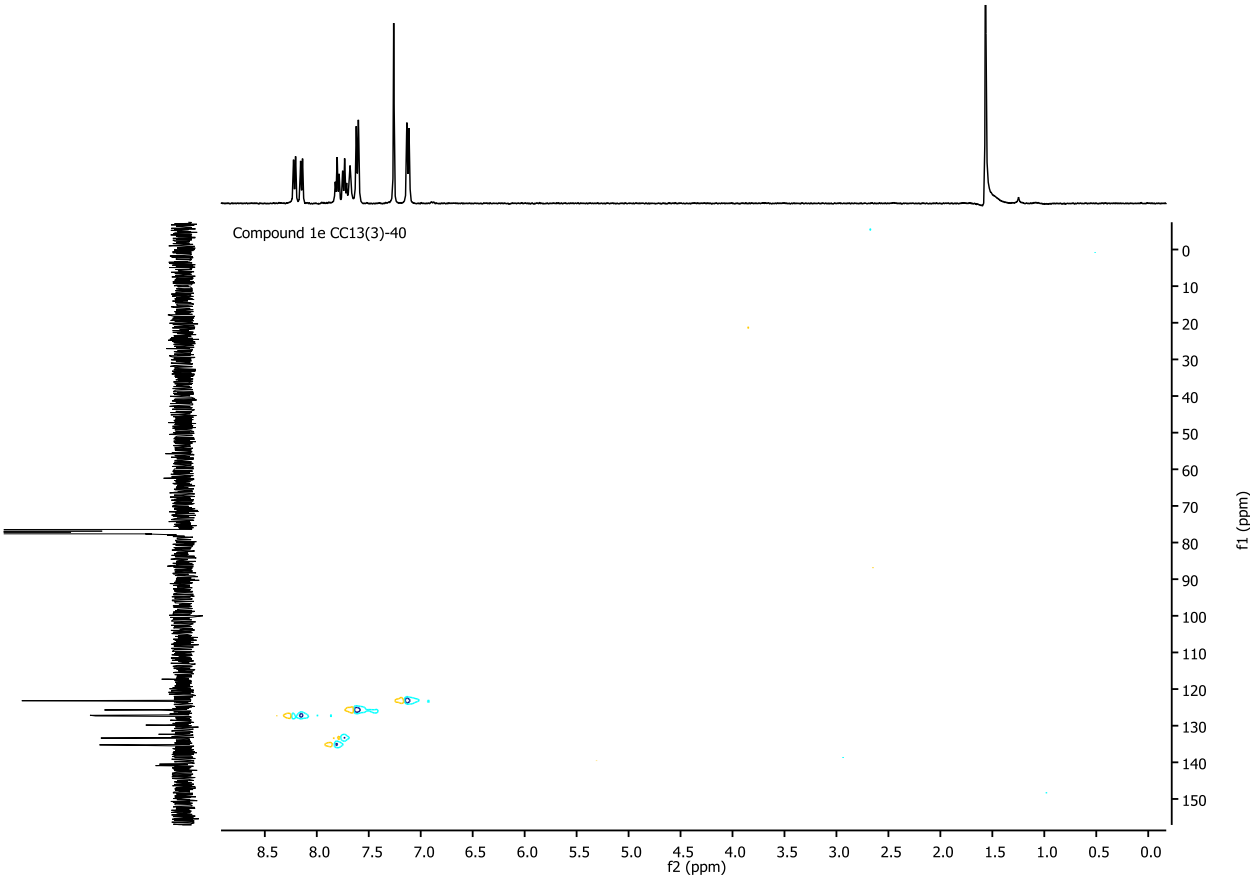
^{13}C NMR (100MHz, $CDCl_3$) spectrum of 2-chloro-3-(4-(trifluoro-methyl)phenylamino)-1,4-naphthoquinone **1e**. Expansion of the signals between 118 ppm and 142 ppm are shown in the off-set plot.



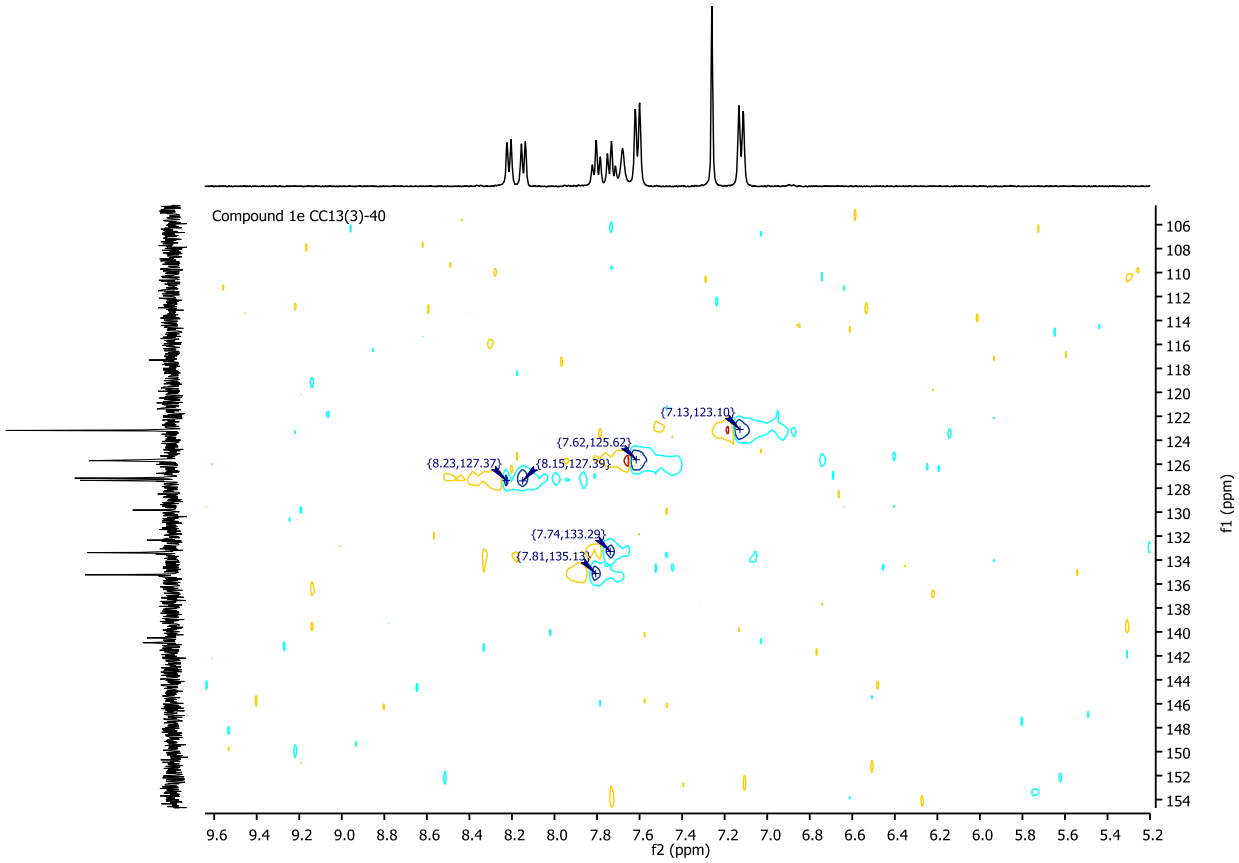
Full COSY NMR spectrum of 2-chloro-3-(4-(trifluoro-methyl)phenylamino)-1,4-naphthoquinone **1e**.



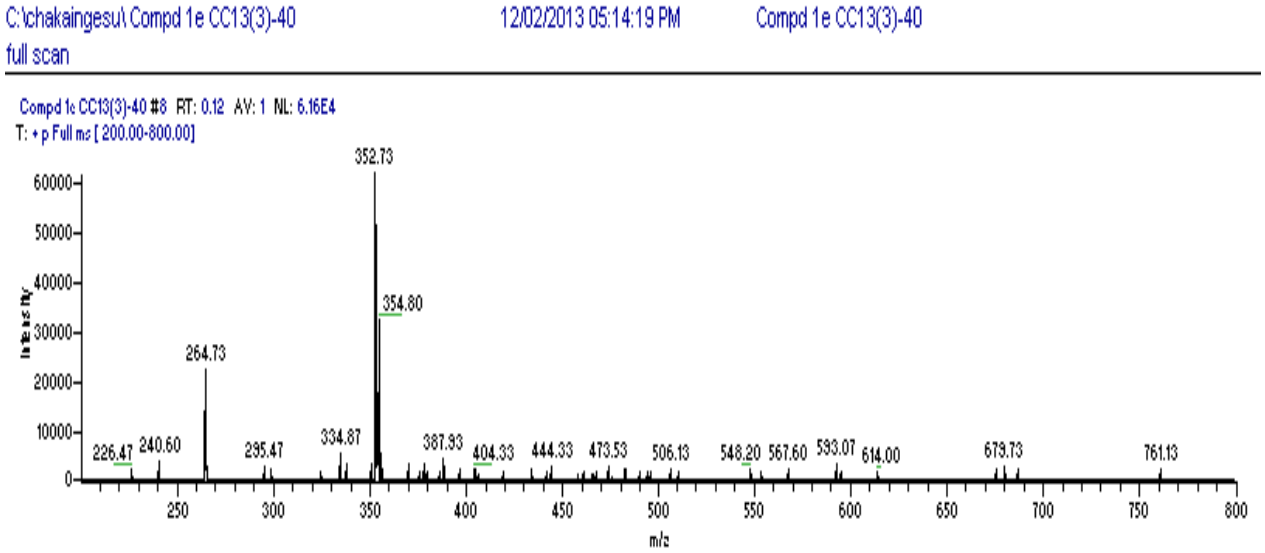
Expansion of the COSY NMR spectrum of 2-chloro-3-(4-(trifluoromethyl)-phenylamino)-1,4-naphthoquinone **1e**.



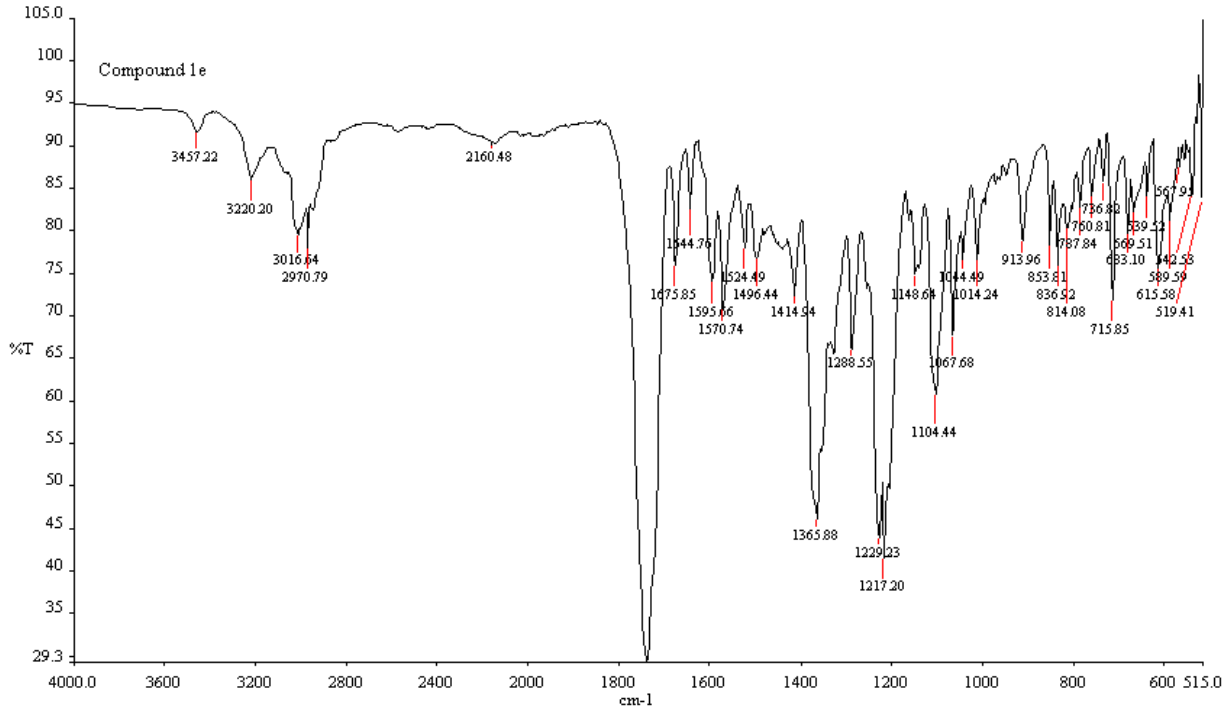
Full HSQC NMR spectrum of 2-chloro-3-(4-(trifluoromethyl)-phenylamino)-1,4-naphthoquinone **1e**.



Full HSQC NMR spectrum of 2-chloro-3-(4-(trifluoro-methyl)phenylamino)-1,4-naphthoquinone **1e**.

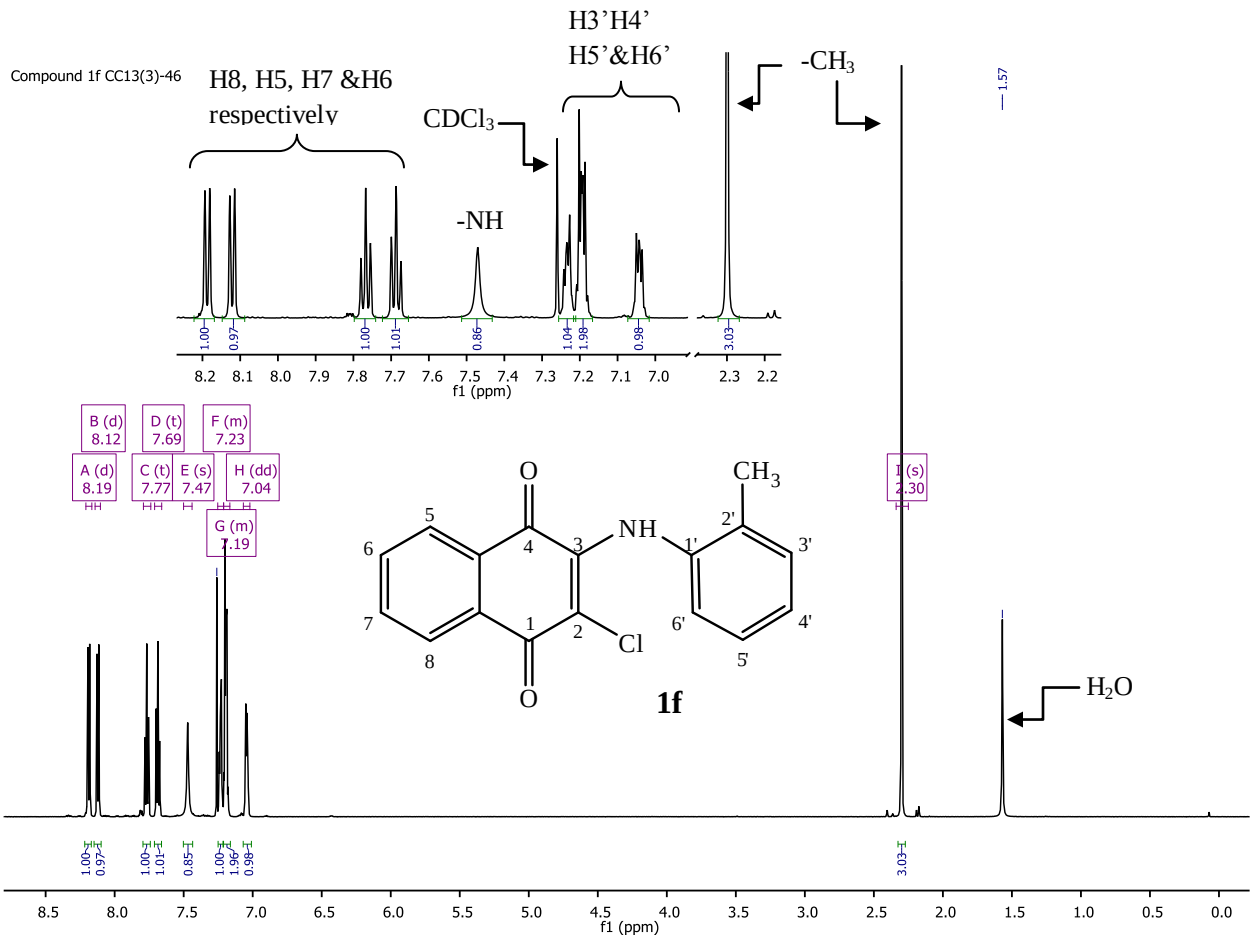


HPLC-ESI-MS spectrum of 2-chloro-3-(4-(trifluoromethyl)phenylamino)-1,4-naphthoquinone **1e**.

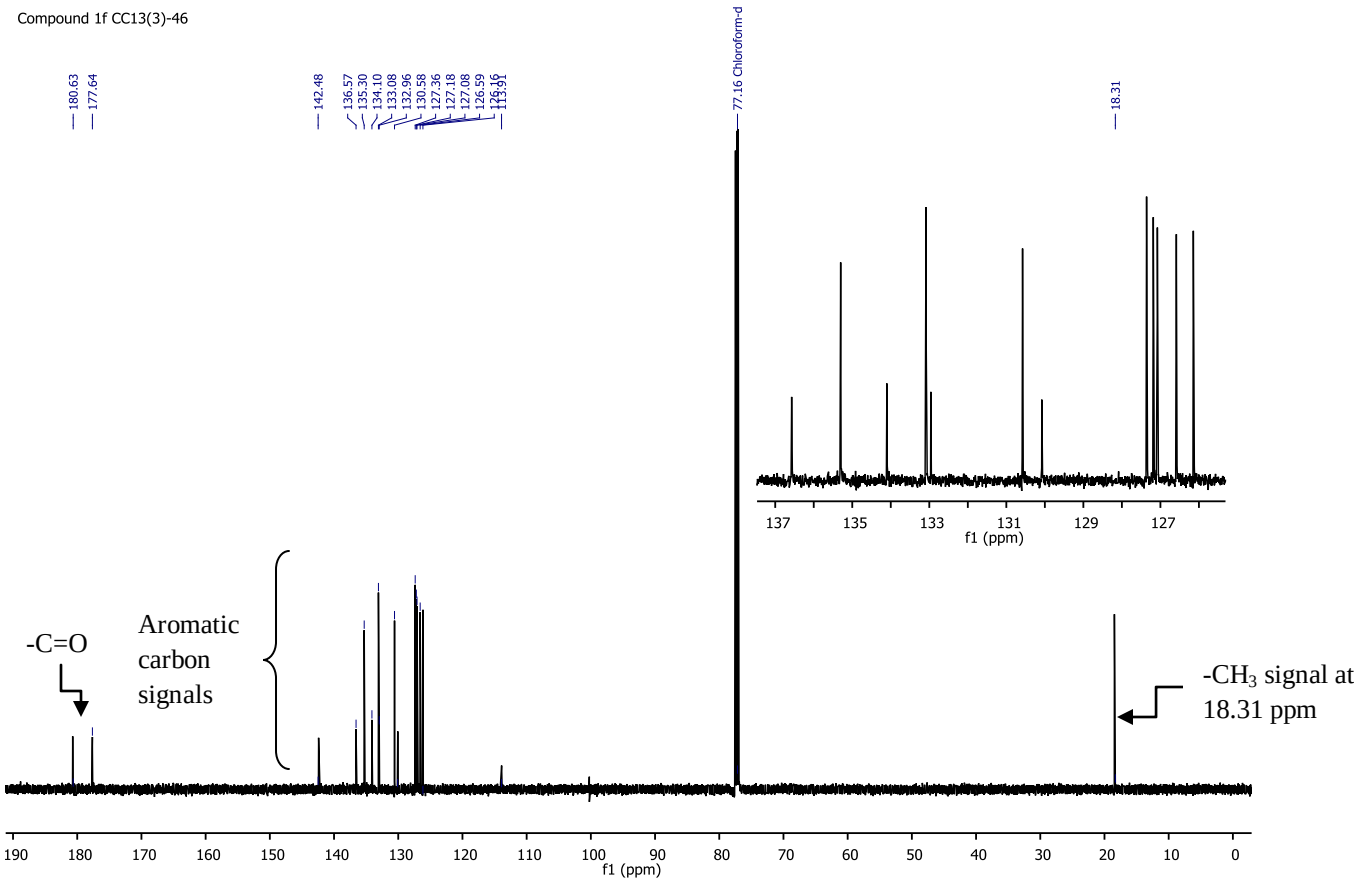


IR (KBr, cm^{-1}) spectrum of 2-chloro-3-(4-(trifluoromethyl)phenylamino)-1,4-naphtho-quinone **1e**.

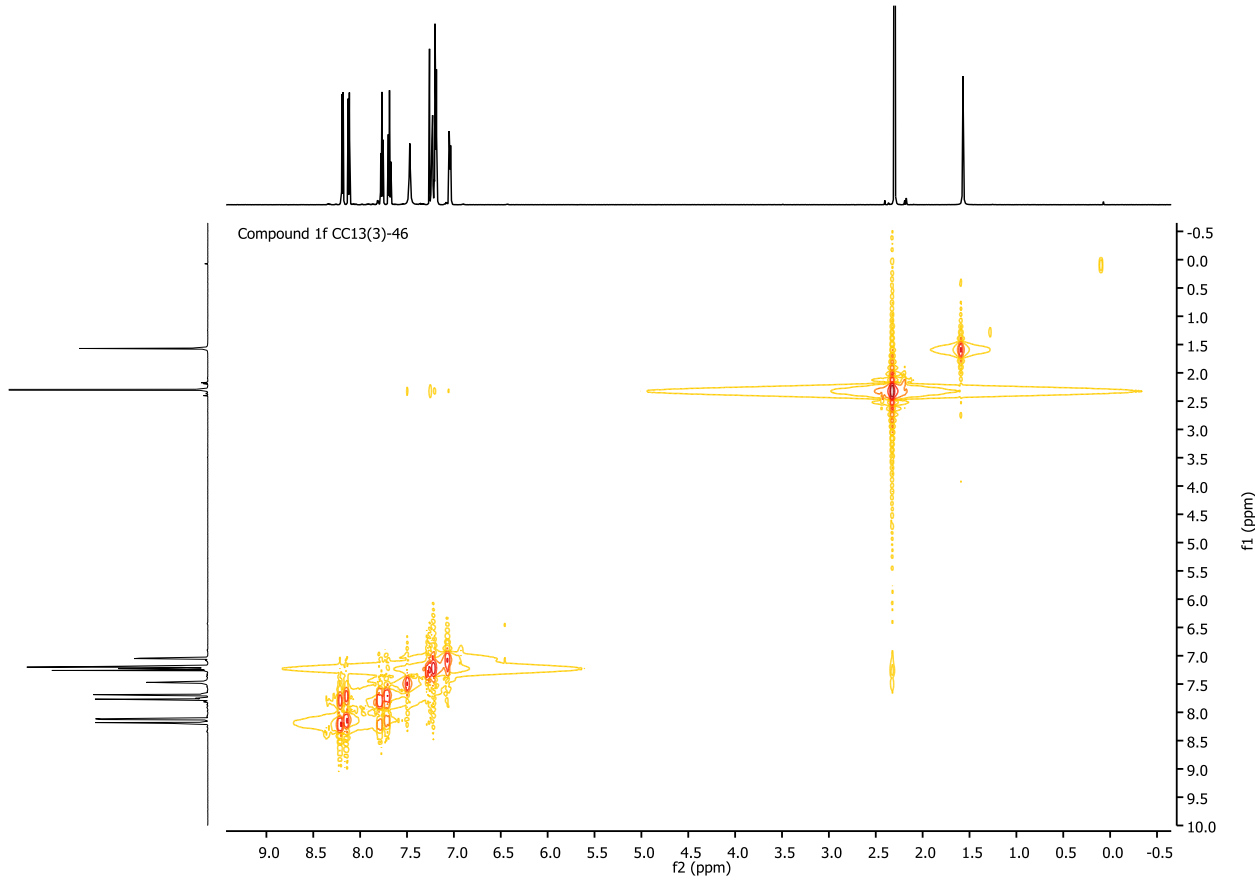
COMPOUND 1f



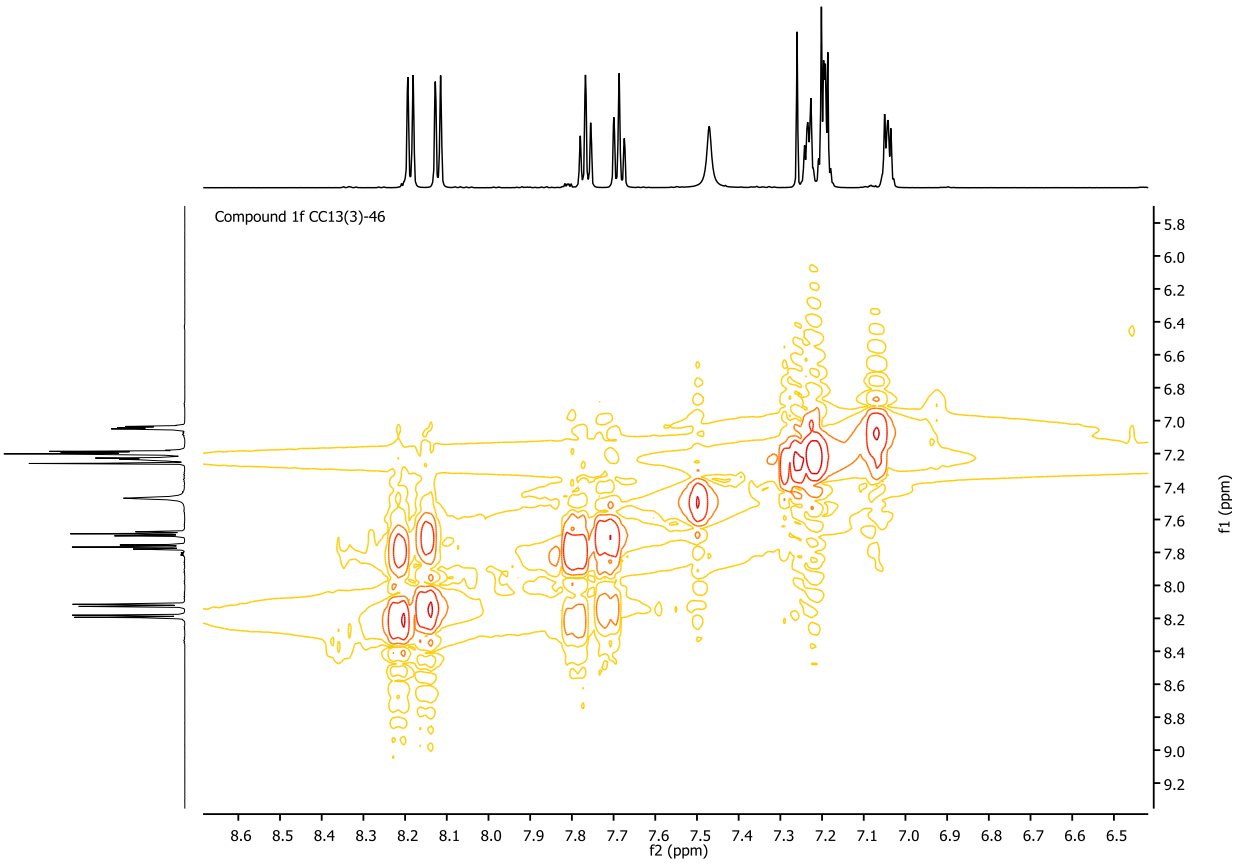
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2-chloro-3-(o-tolylamino)-1,4-naphthoquinone **1f**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 2-methylaniline. Expansions of signals in the aromatic region as well as the methyl signal are shown in the off-set plot.



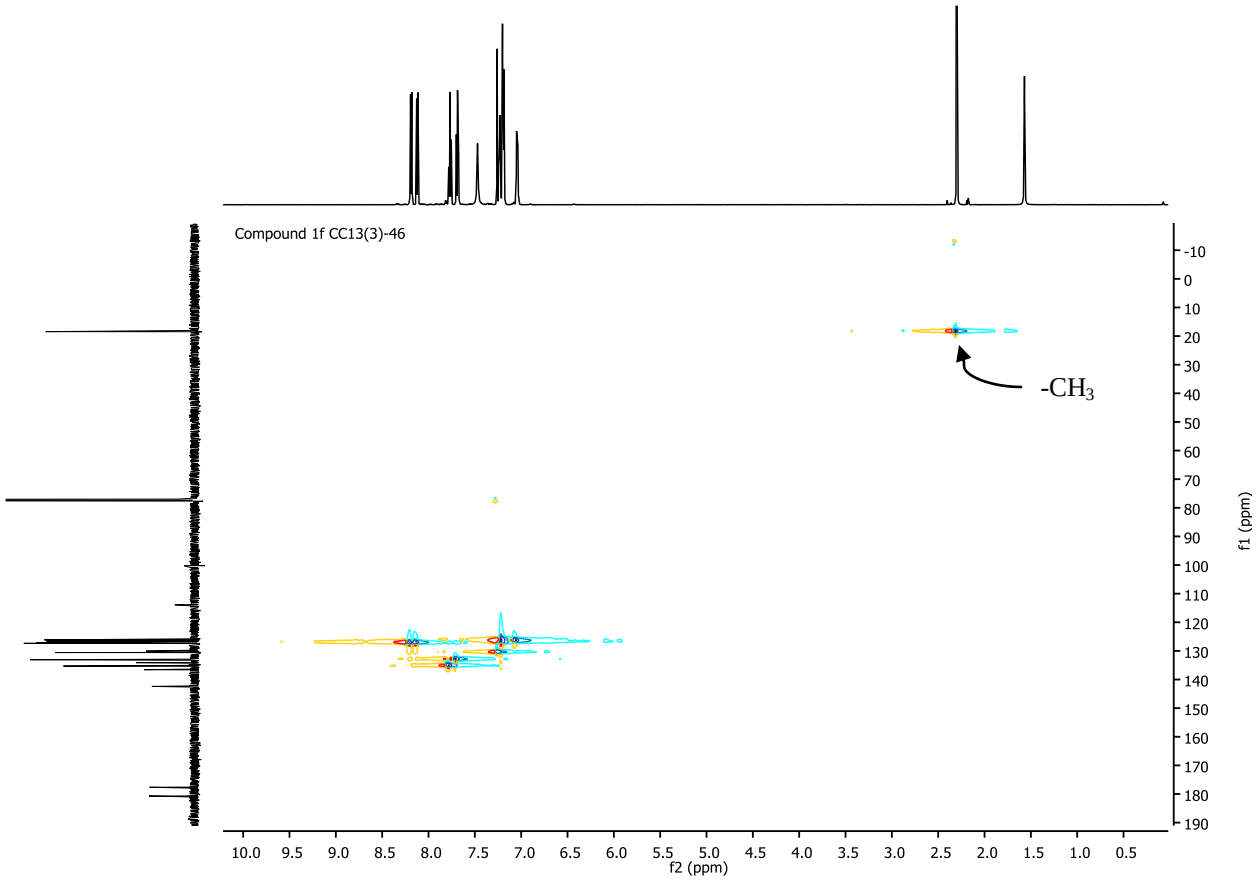
^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2-chloro-3-(o-tolylamino)-1,4-naphthoquinone **1f**. Expansion of the signals between 126 ppm and 137 ppm are shown in the off-set plot.



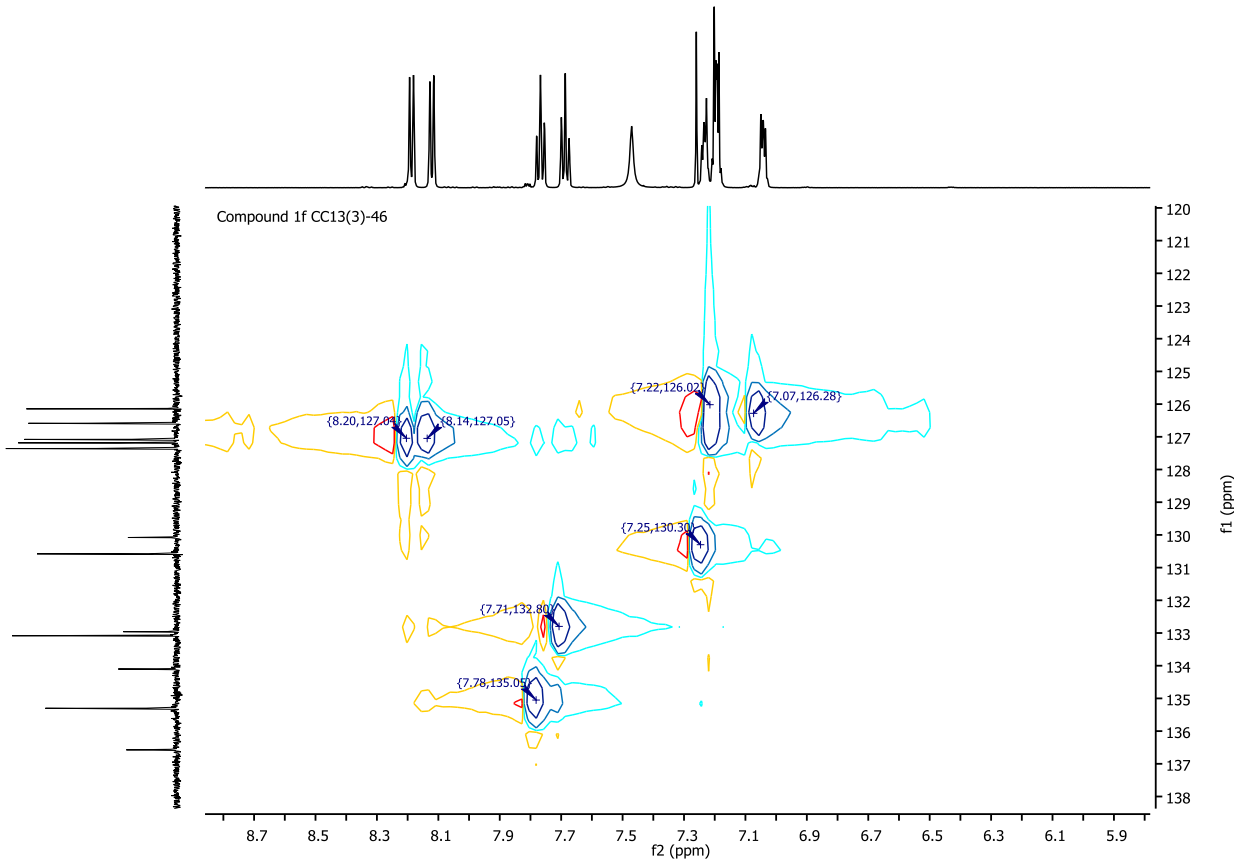
Full COSY NMR spectrum of 2-chloro-3-(o-tolylamino)-1,4-naphthoquinone **1f**.



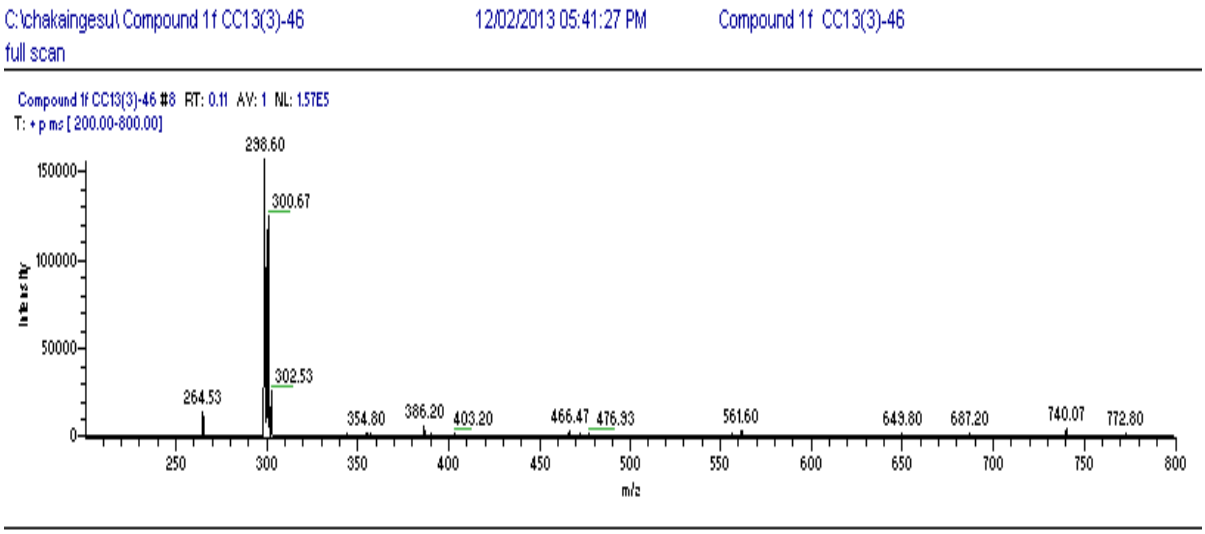
Expansion of the COSY NMR spectrum of 2-chloro-3-(o-tolylamino)-1,4-naphthoquinone **1f**.



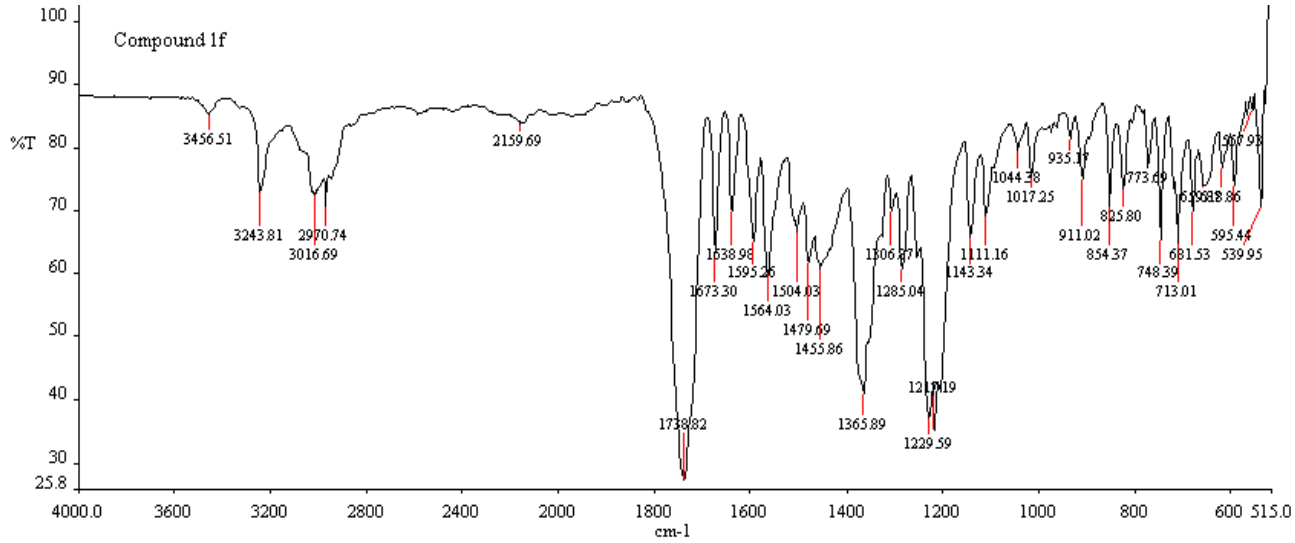
Full HSQC NMR spectrum of 2-chloro-3-(o-tolylamino)-1,4-naphthoquinone **1f**.



Expansion of the HSQC NMR spectrum of 2-chloro-3-(o-tolylamino)-1,4-naphthoquinone **1f**.

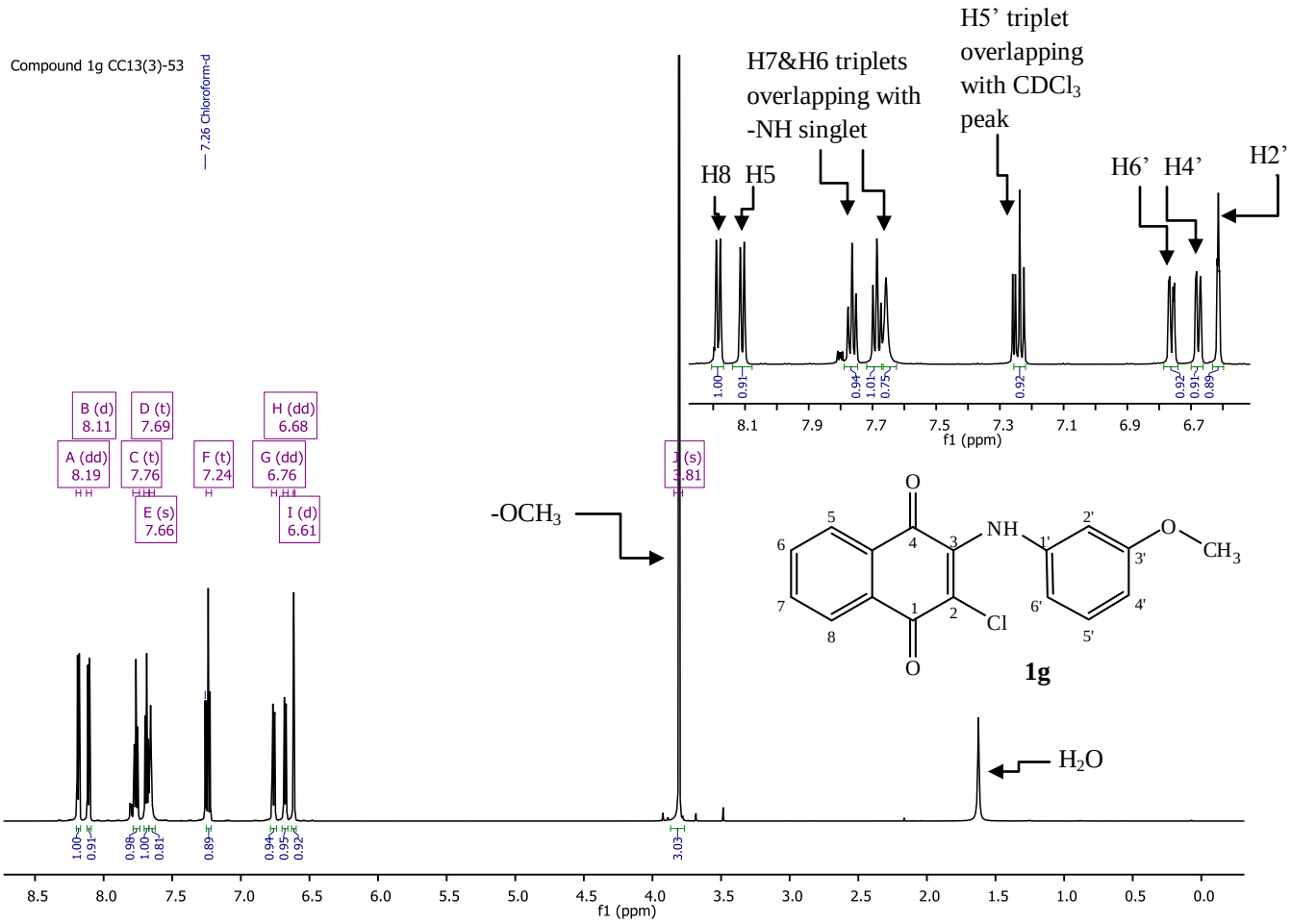


HPLC-ESI-MS spectrum of 2-chloro-3-(o-tolylamino)-1,4-naphthoquinone **1f**.

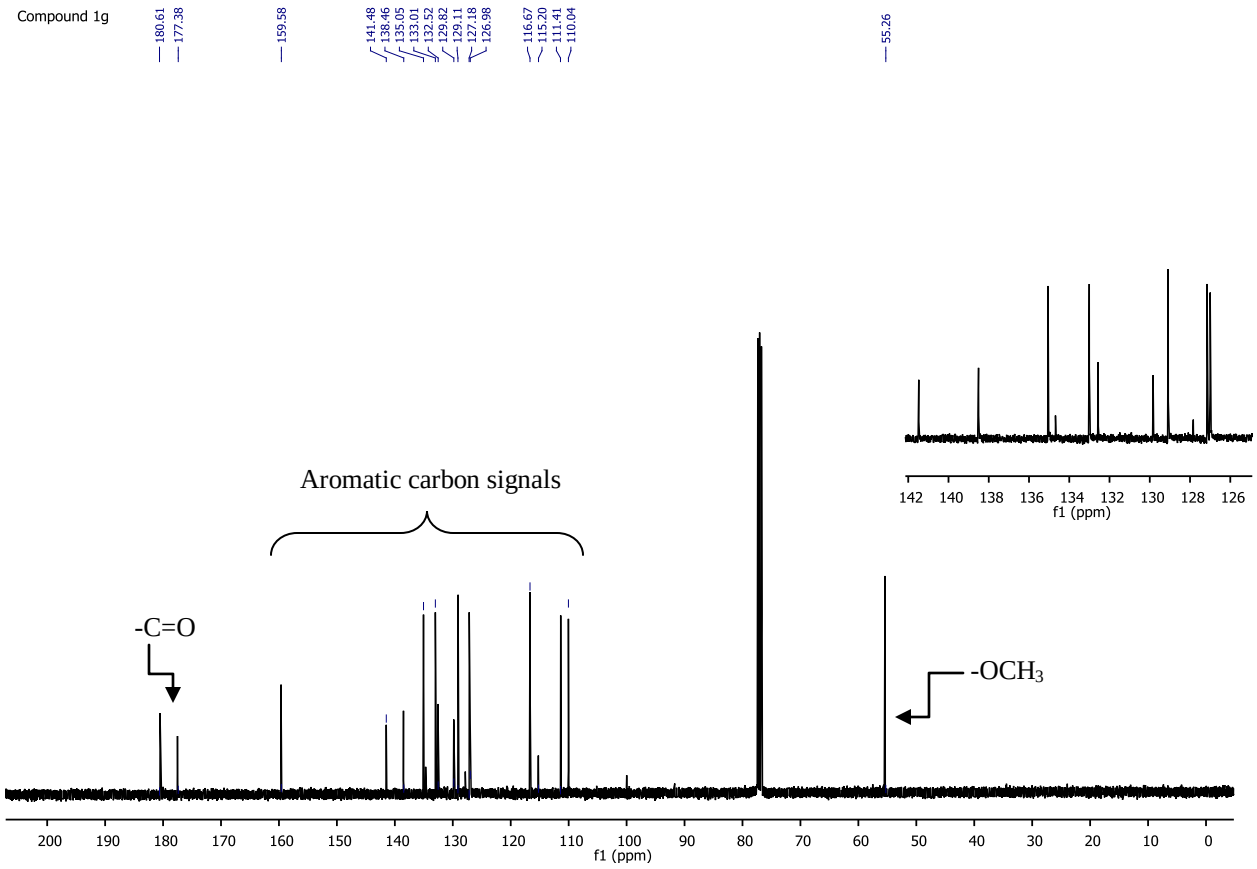


IR (KBr, cm^{-1}) spectrum of 2-chloro-3-(o-tolylamino)-1,4-naphthoquinone **1f**.

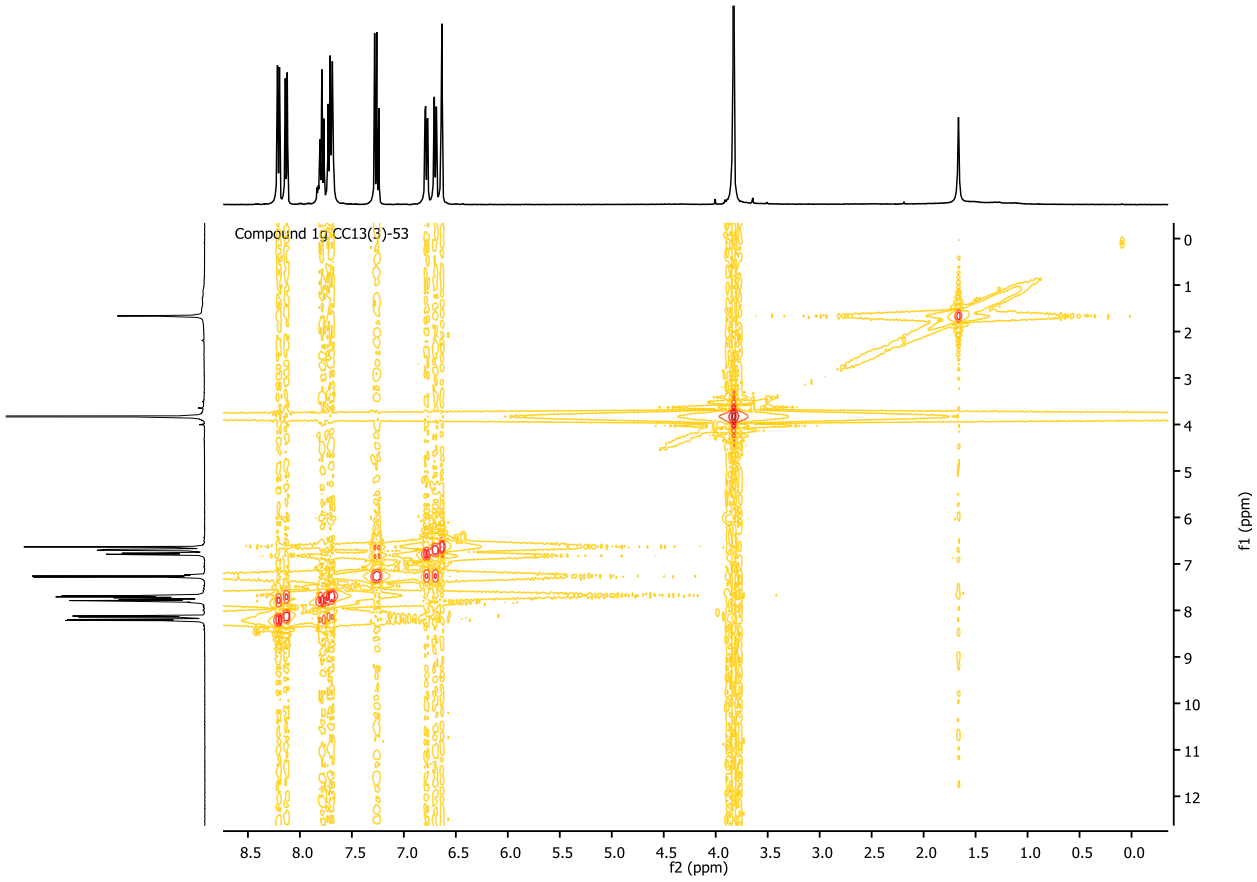
COMPOUND 1g



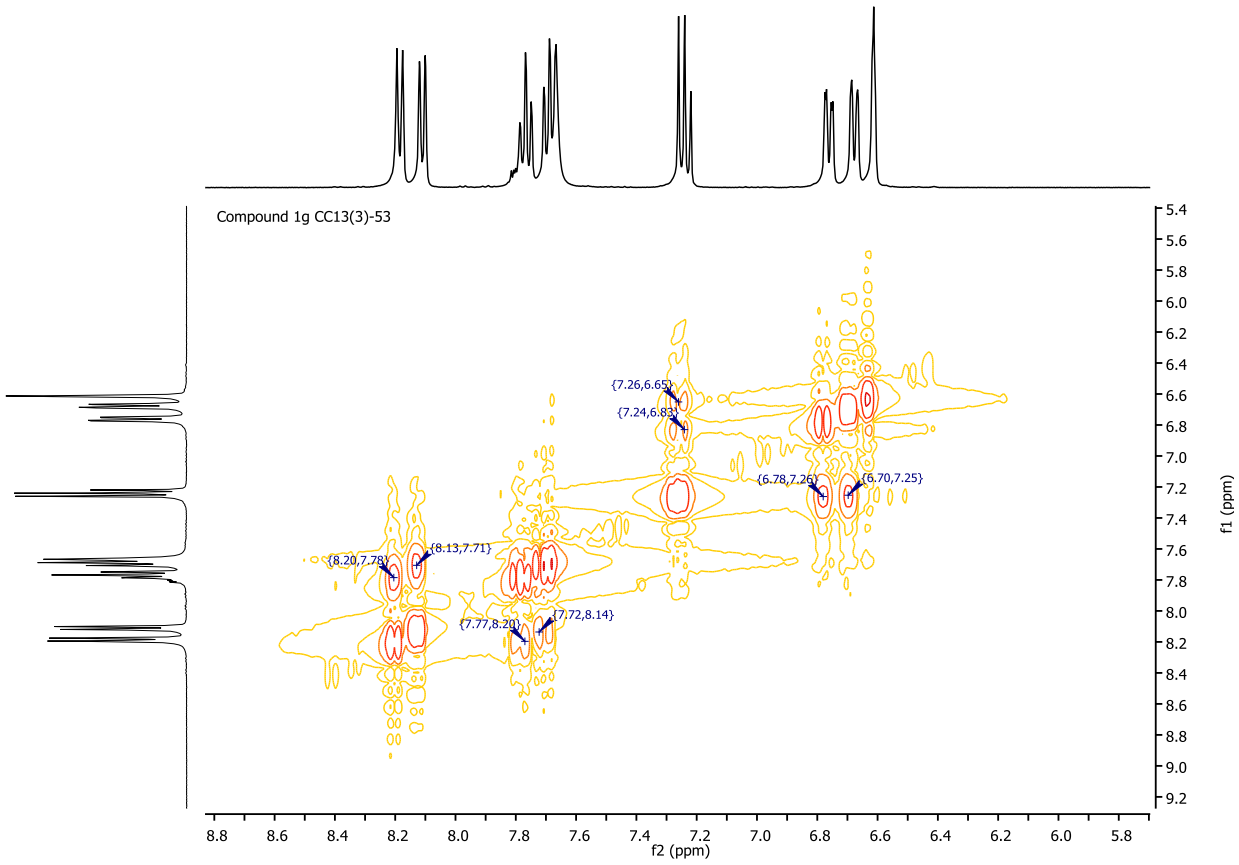
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2-chloro-3-(3-methoxyphenylamino)-1,4-naphthoquinone **1g**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 3-methoxyaniline. Expansions of signals in the aromatic region are shown in the off-set plot.



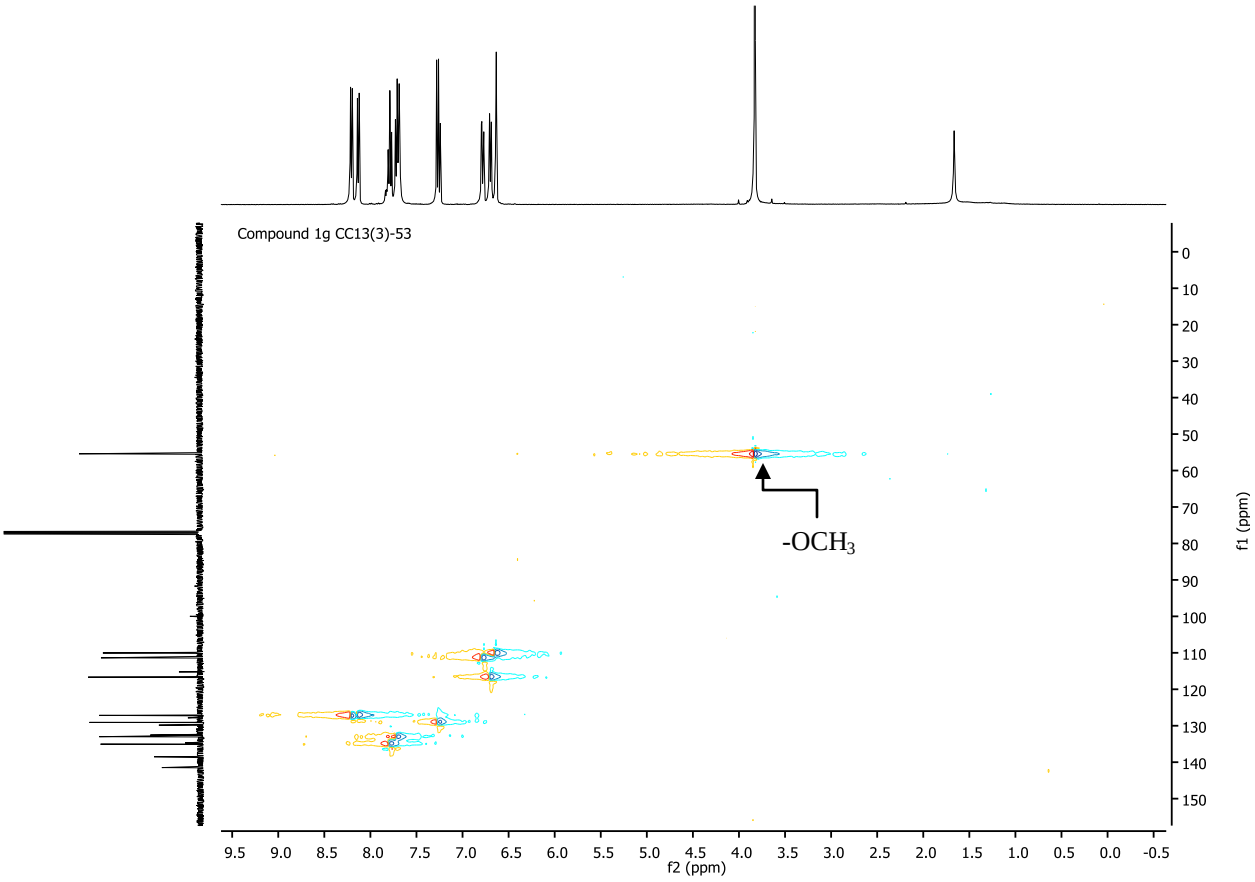
^{13}C NMR (400MHz, $CDCl_3$) spectrum of 2-chloro-3-(3-methoxyphenylamino)-1,4-naphthoquinone **1g**. Expansion of the signals between 126 ppm and 142 ppm are shown in the off-set plot.



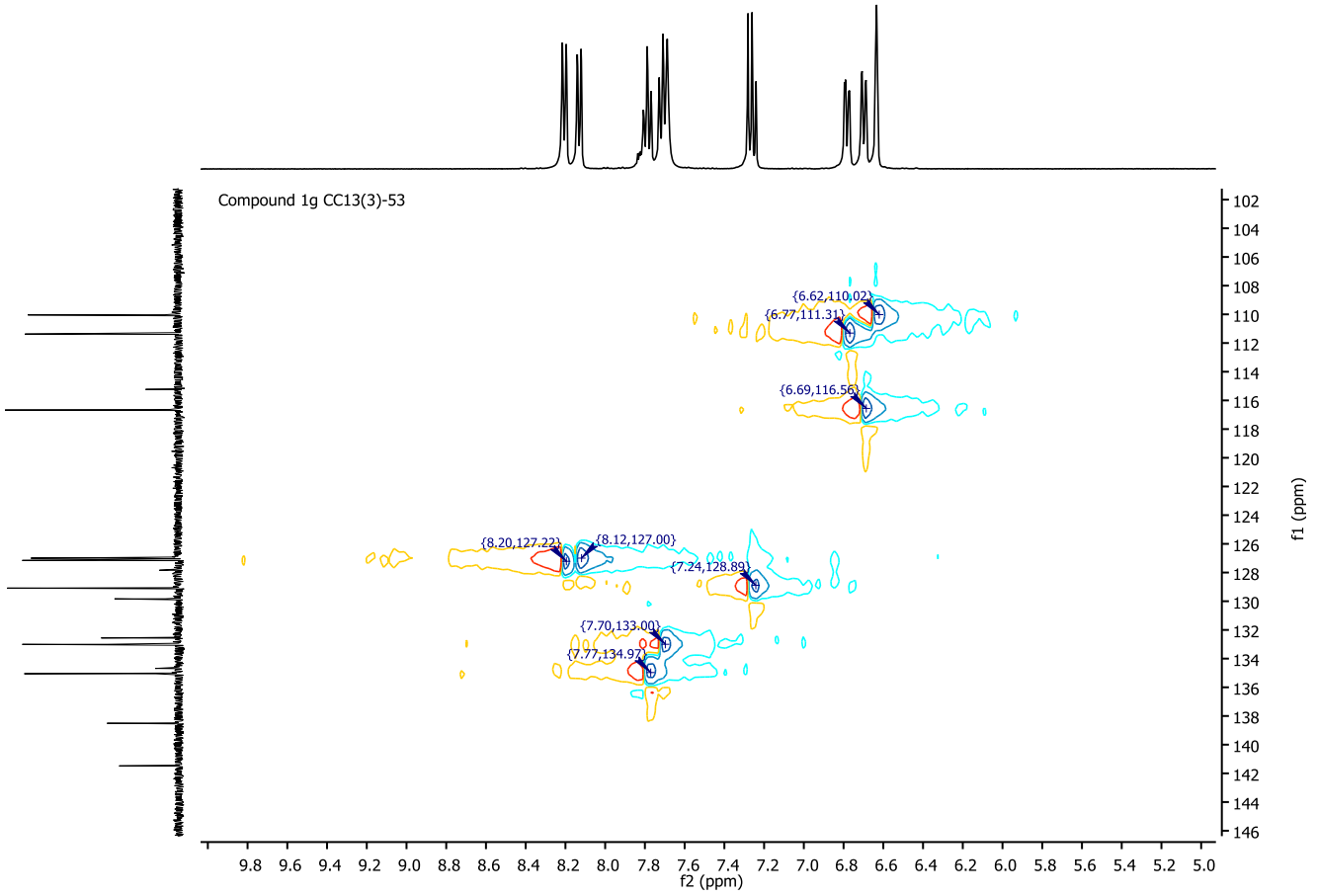
Full COSY NMR spectrum of 2-chloro-3-(3-methoxyphenylamino)-1,4-naphthoquinone **1g**.



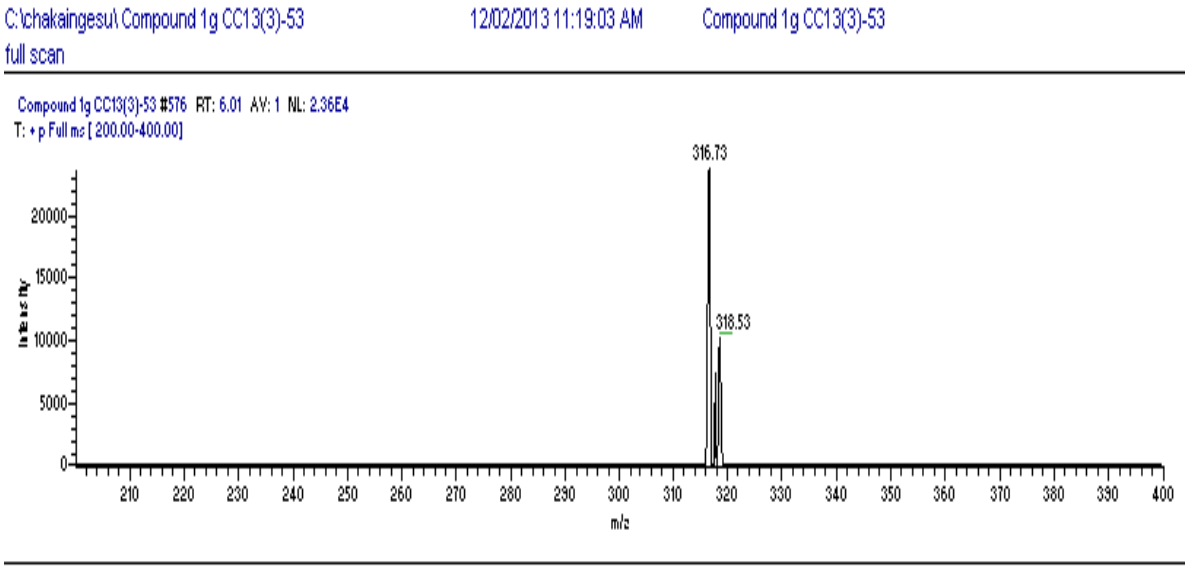
Expansion of the COSY NMR spectrum of 2-chloro-3-(3-methoxy-phenylamino)-1,4-naphthoquinone **1g**.



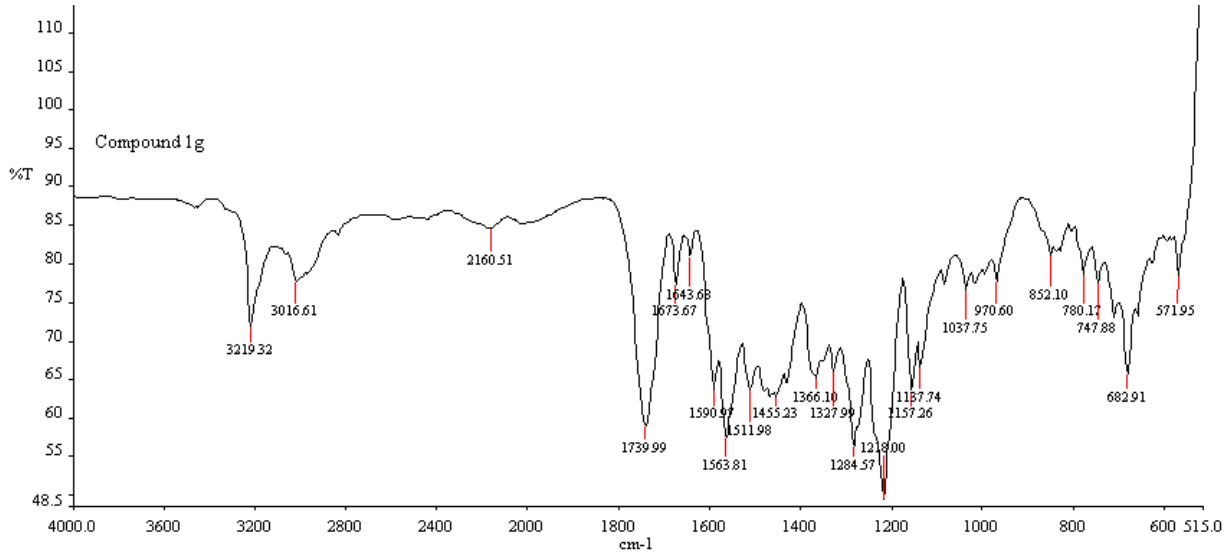
Full HSQC NMR spectrum of 2-chloro-3-(3-methoxyphenylamino)-1,4-naphthoquinone **1g**.



Expansion of the HSQC NMR spectrum of 2-chloro-3-(3-methoxy-phenylamino)-1,4-naphthoquinone **1g**. The methoxy group correlation has been left out.

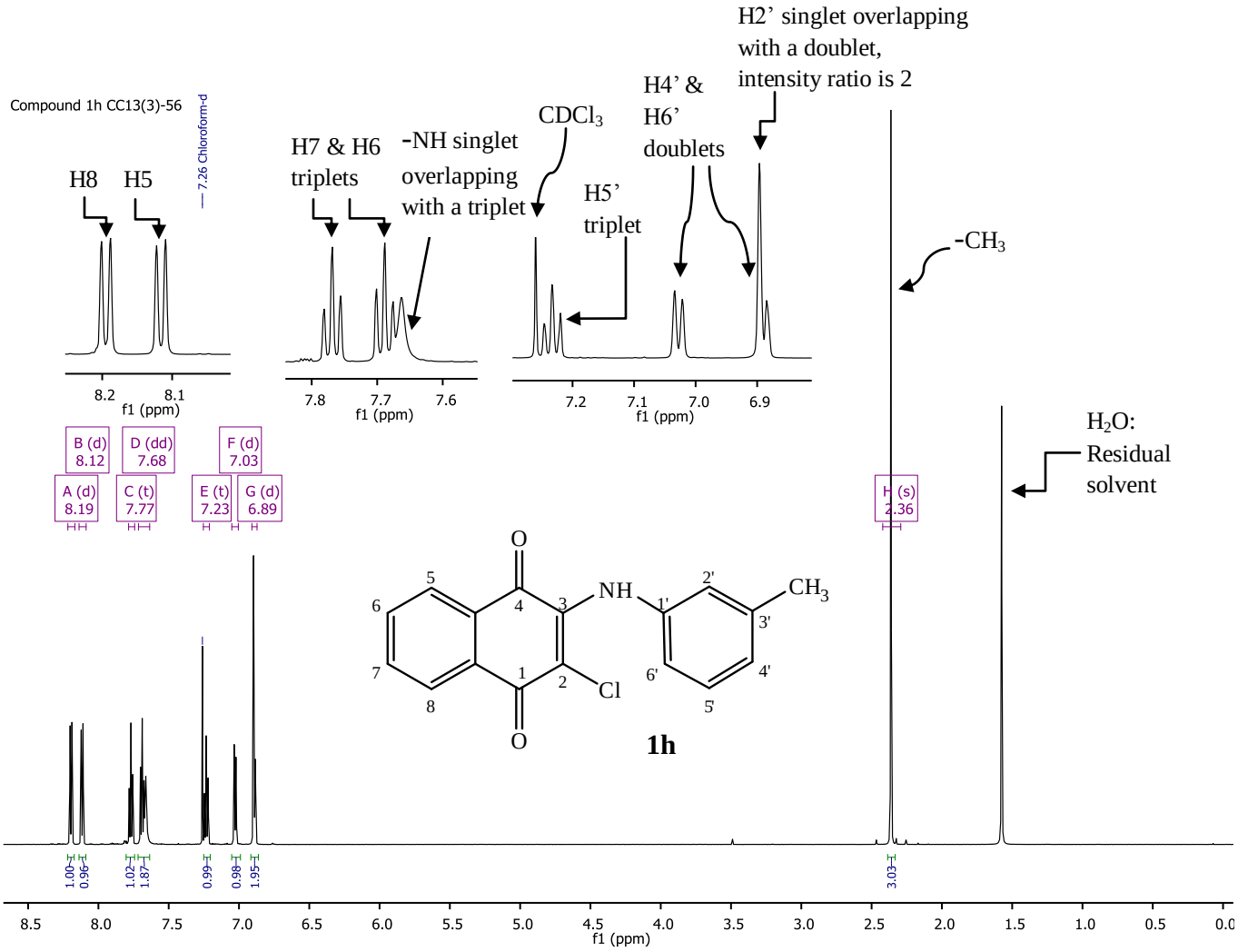


HPLC-ESI-MS spectrum of 2-chloro-3-(3-methoxy-phenylamino)-1,4-naphthoquinone **1g**.

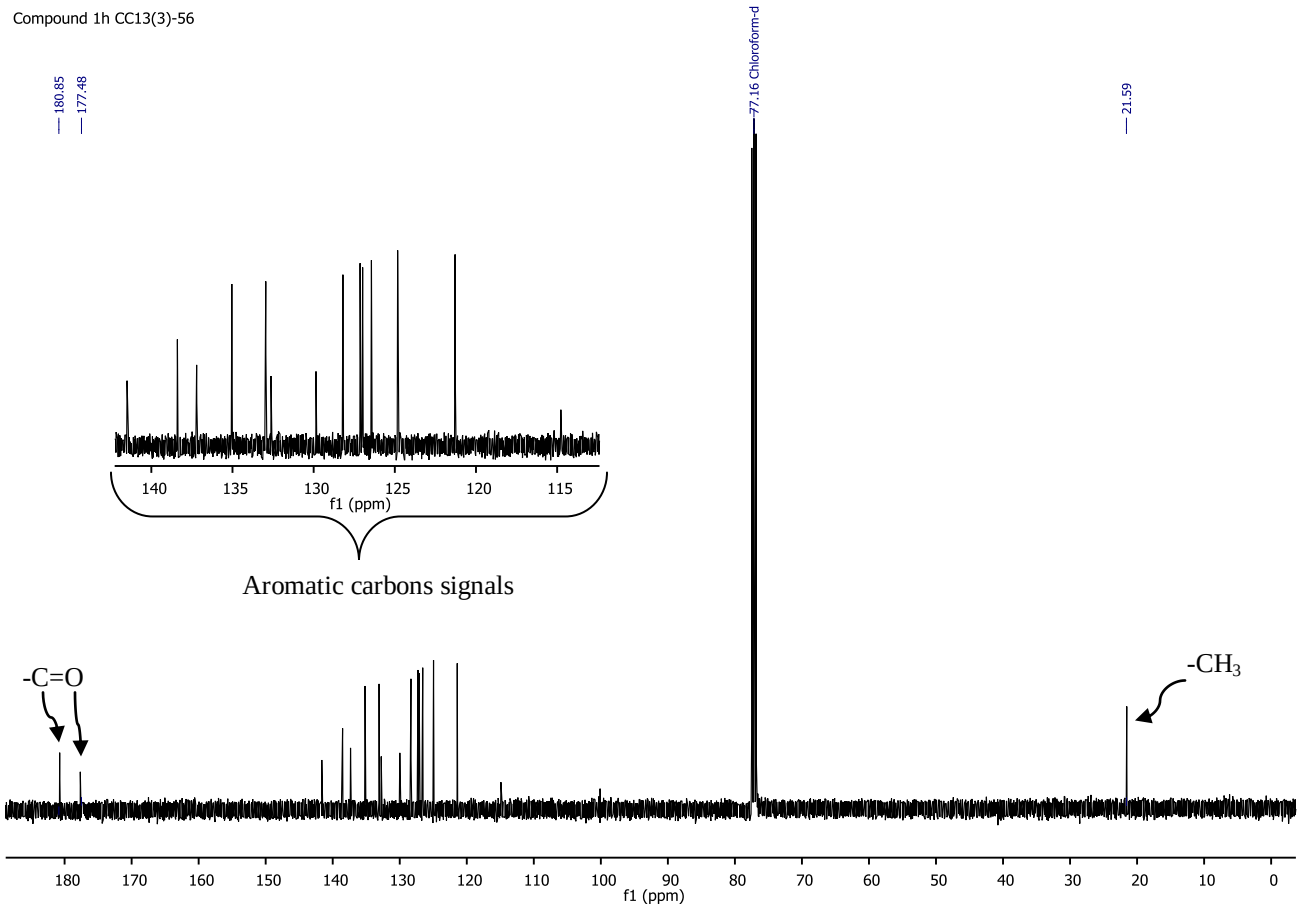


IR (KBr, cm^{-1}) spectrum of 2-chloro-3-(3-methoxy-phenylamino)-1,4-naphthoquinone **1g**.

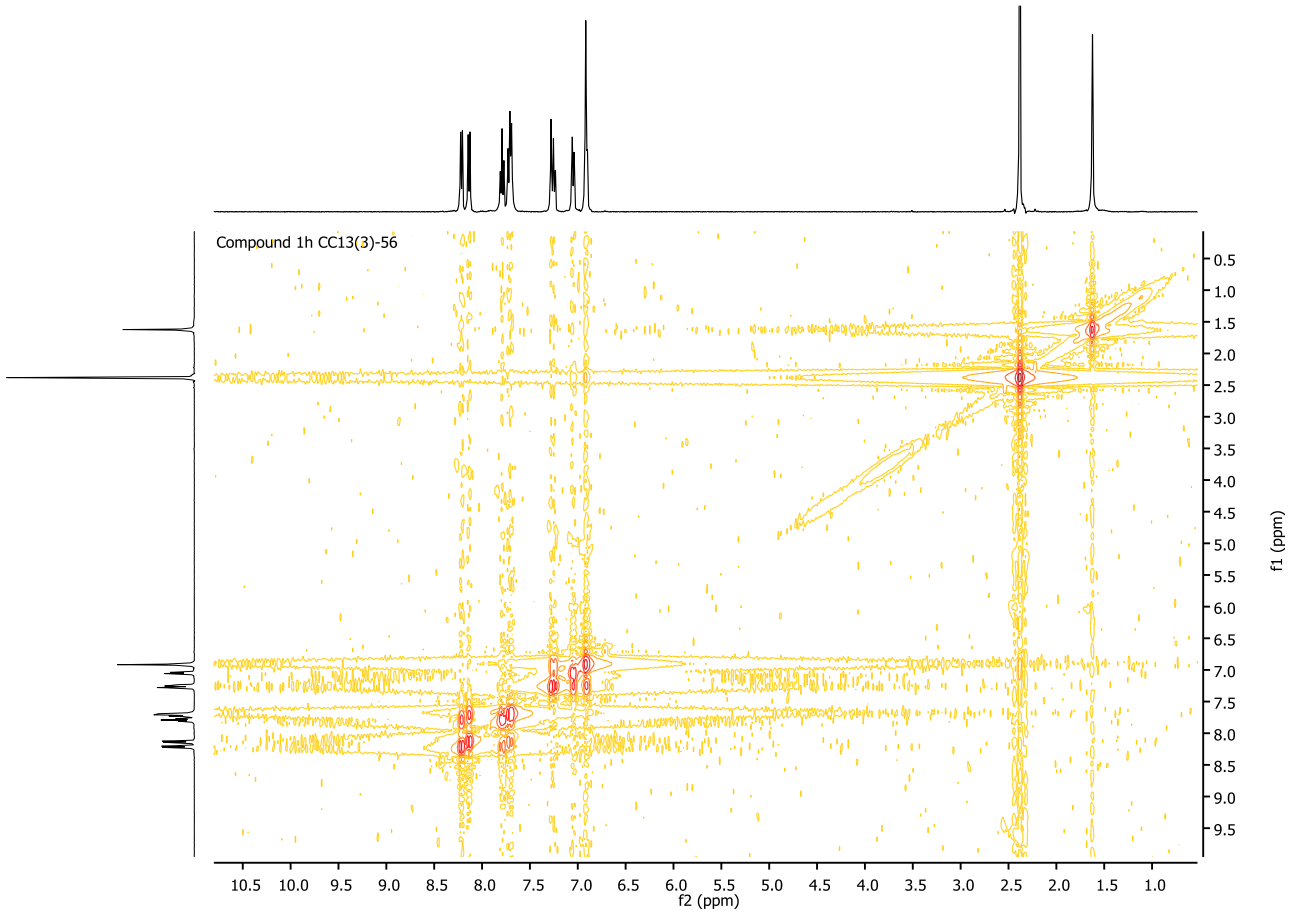
COMPOUND 1h



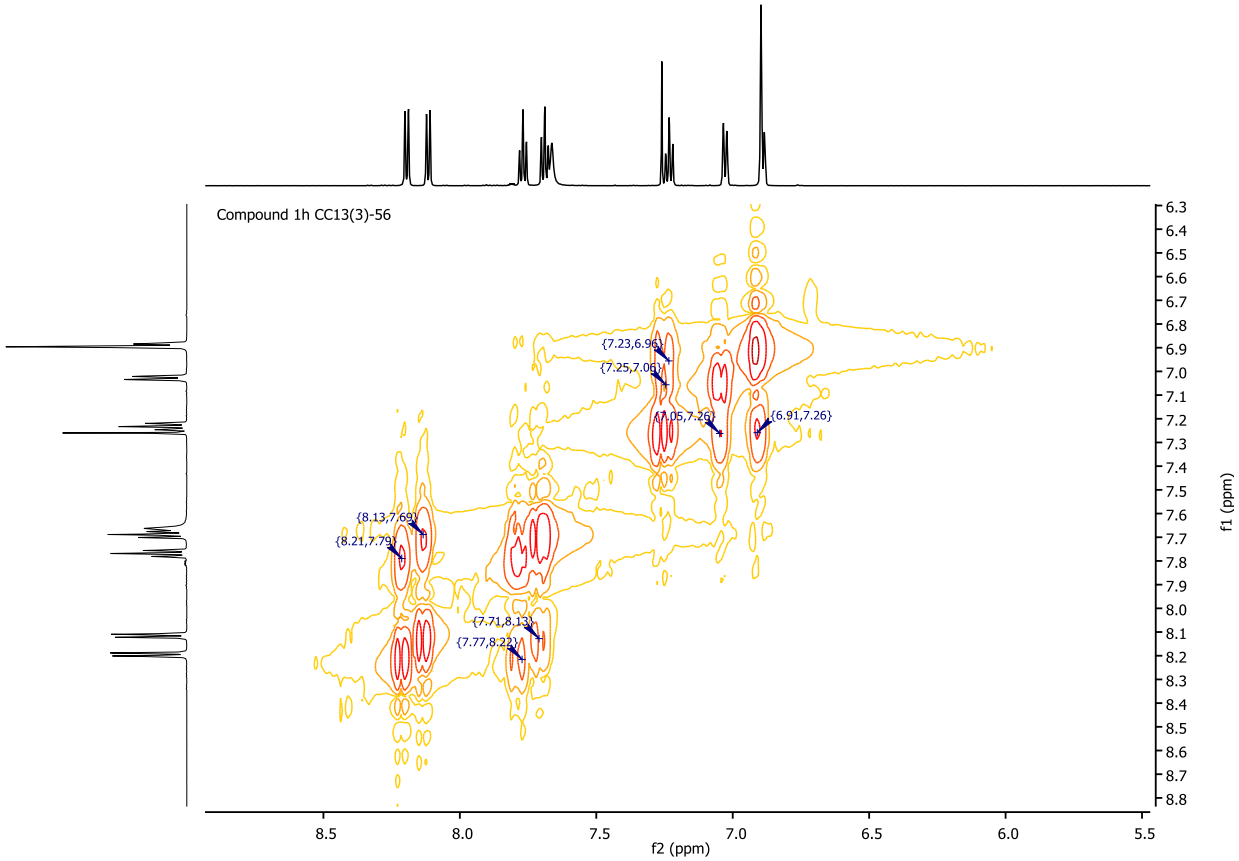
^{1}H NMR (600MHz, $CDCl_3$) spectrum of the 2-chloro-3-(m-tolylamino)-1,4-naphthoquinone **1h**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 3-methylaniline. Expansions of signals in the aromatic region are shown in the off-set plot.



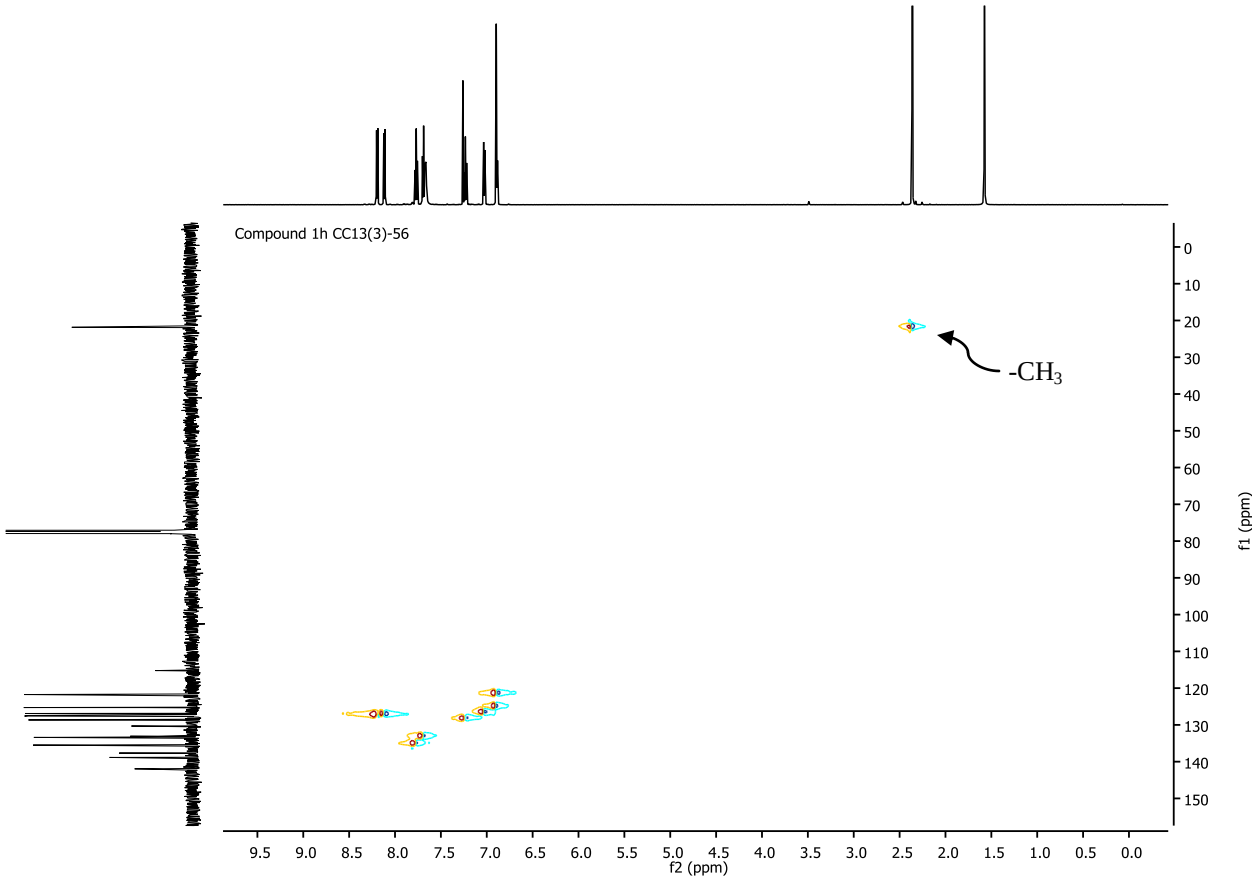
^{13}C NMR (400MHz, $CDCl_3$) spectrum of 2-chloro-3-(m-tolylamino)-1,4-naphthoquinone **1h** to show aromatic region. Expansion of the signals between 115 ppm and 142 ppm are shown in the off-set plot.



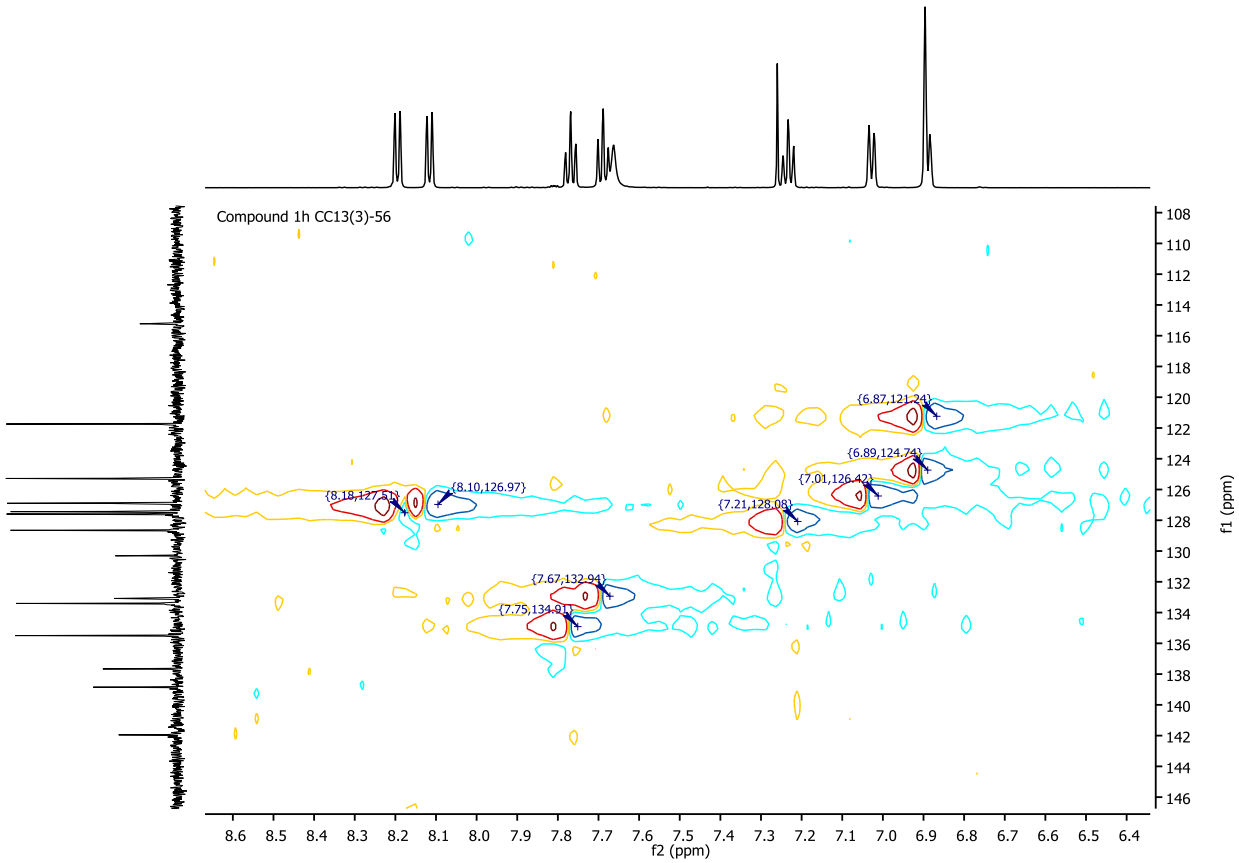
Full COSY NMR spectrum of 2-chloro-3-(m-tolylamino)-1,4-naphthoquinone **1h**.



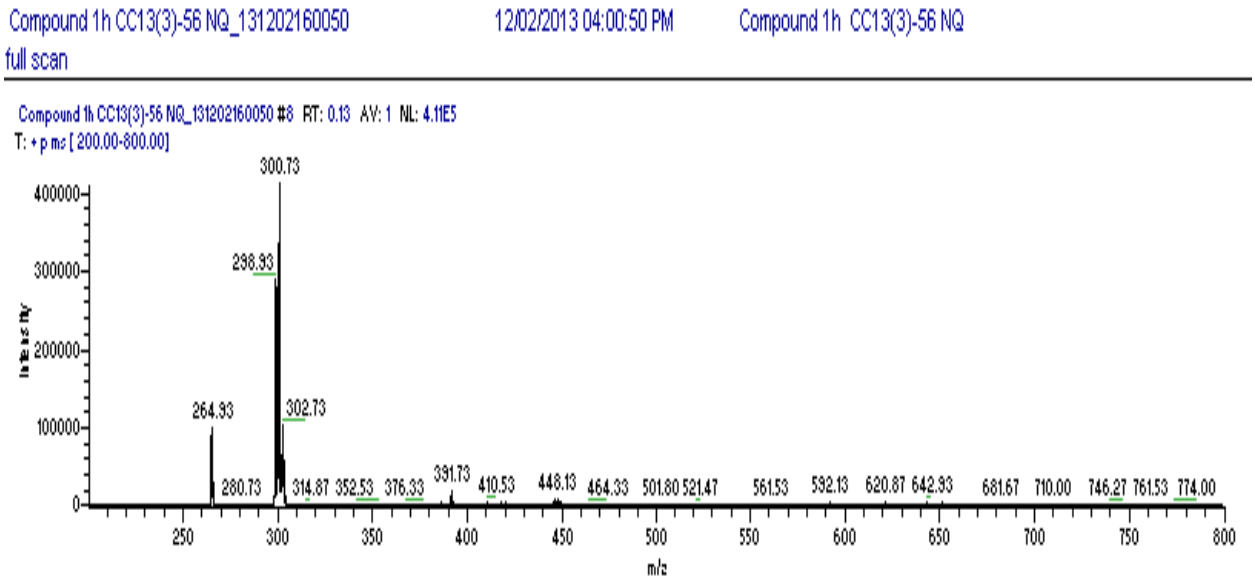
Expansion of the COSY NMR spectrum of 2-chloro-3-(m-tolylamino)-1,4-naphtho-quinone **1h** showing the aromatic region only.



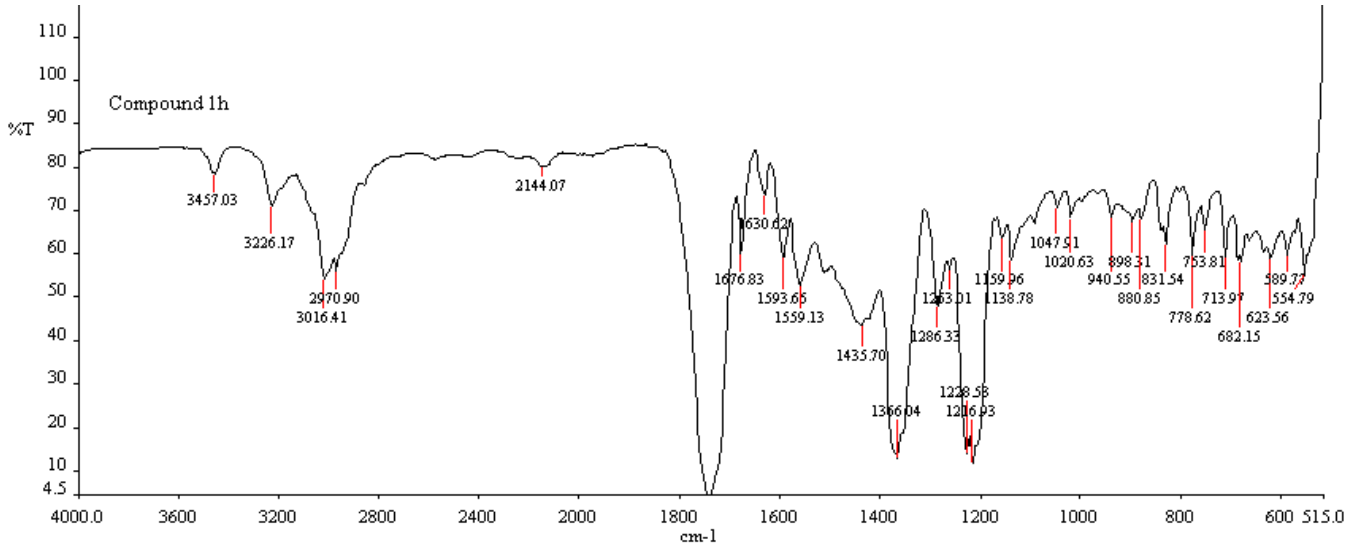
Full HSQC NMR spectrum of 2-chloro-3-(m-tolylamino)-1,4-naphthoquinone **1h**.



Expansion of the HSQC NMR spectrum of 2-chloro-3-(m-tolylamino)-1,4-naphthoquinone **1h** showing the aromatic region only. The methyl correlation has been left out.

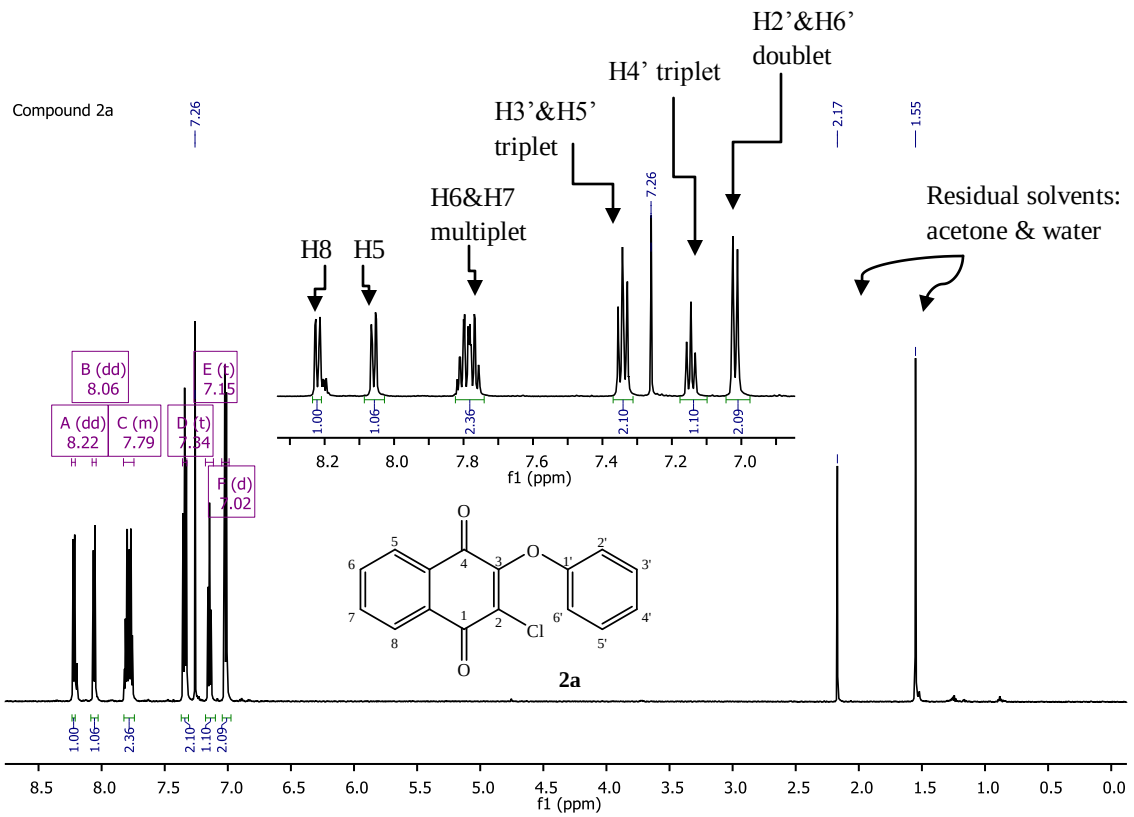


HPLC-ESI-MS spectrum of 2-chloro-3-(m-tolylamino)-1,4-naphthoquinone **1h**.

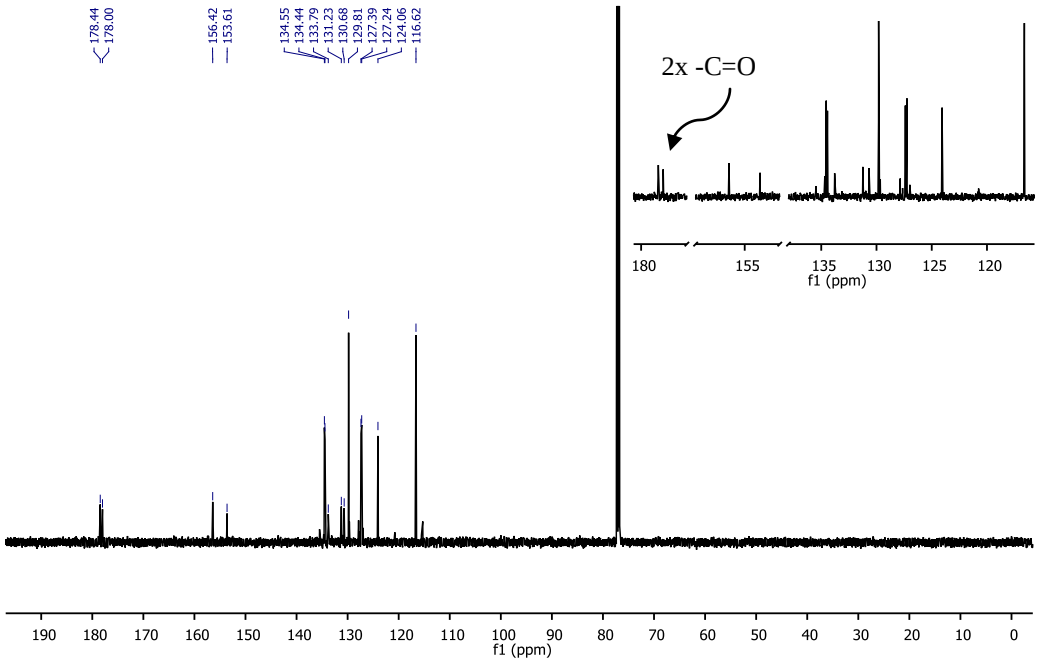


IR (KBr, cm^{-1}) spectrum of 2-chloro-3-(m-tolylamino)-1,4-naphthoquinone **1h**.

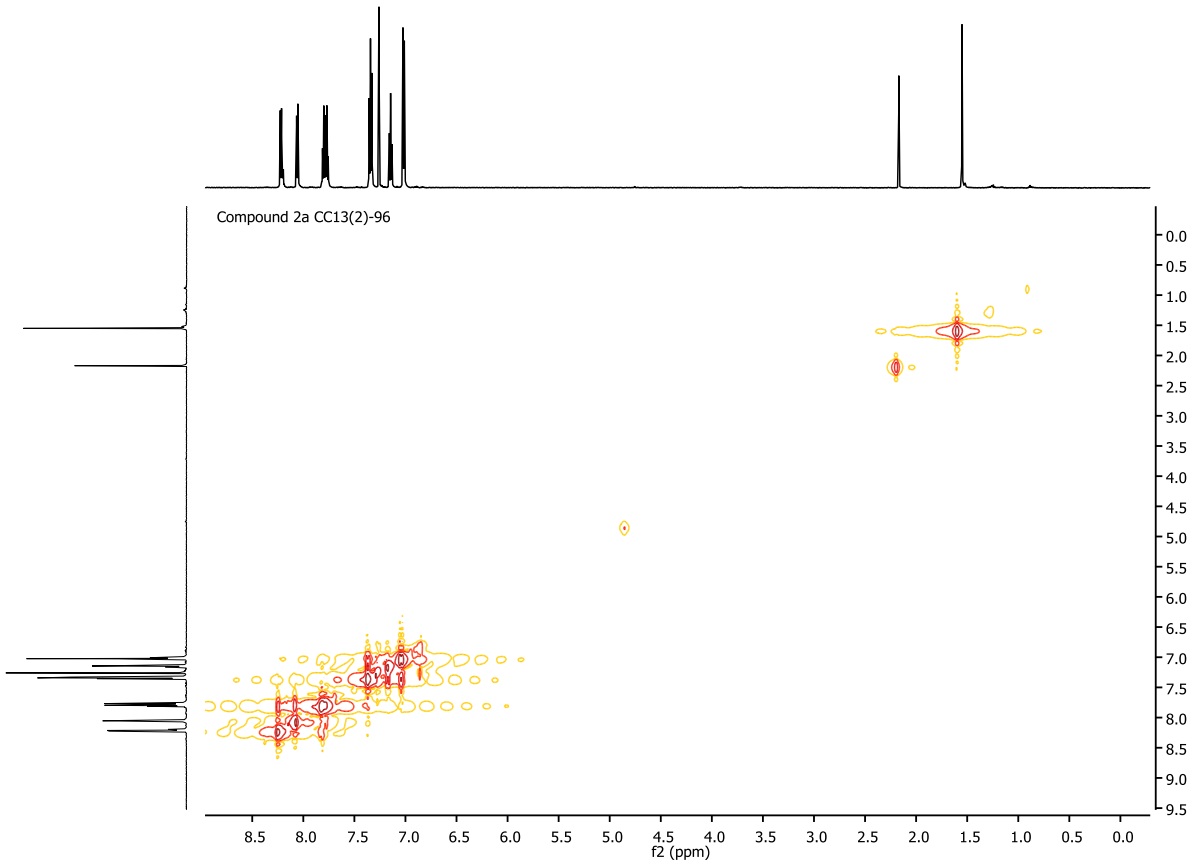
COMPOUND 2a



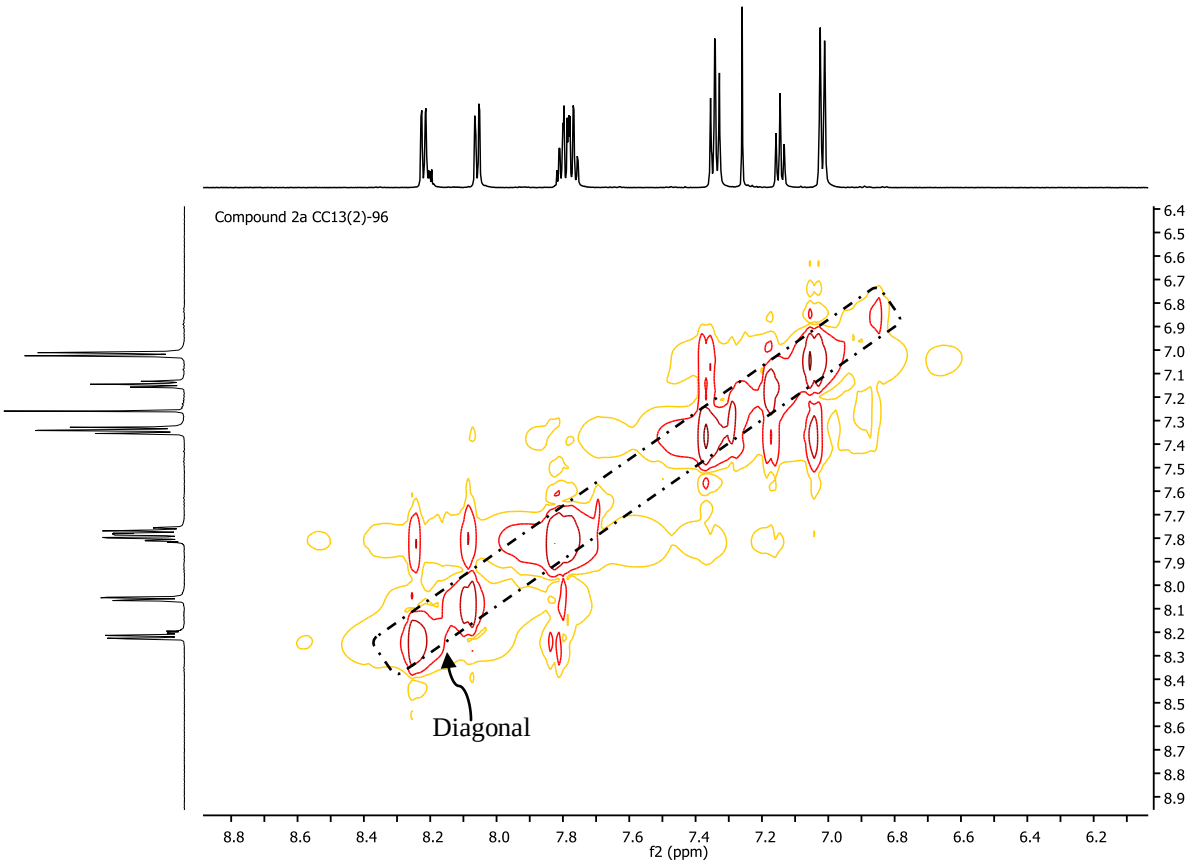
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2-chloro-3-phenoxy-1,4-naphthoquinone **2a**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with phenol. Expansions of signals in the aromatic region are shown in the off-set plot.



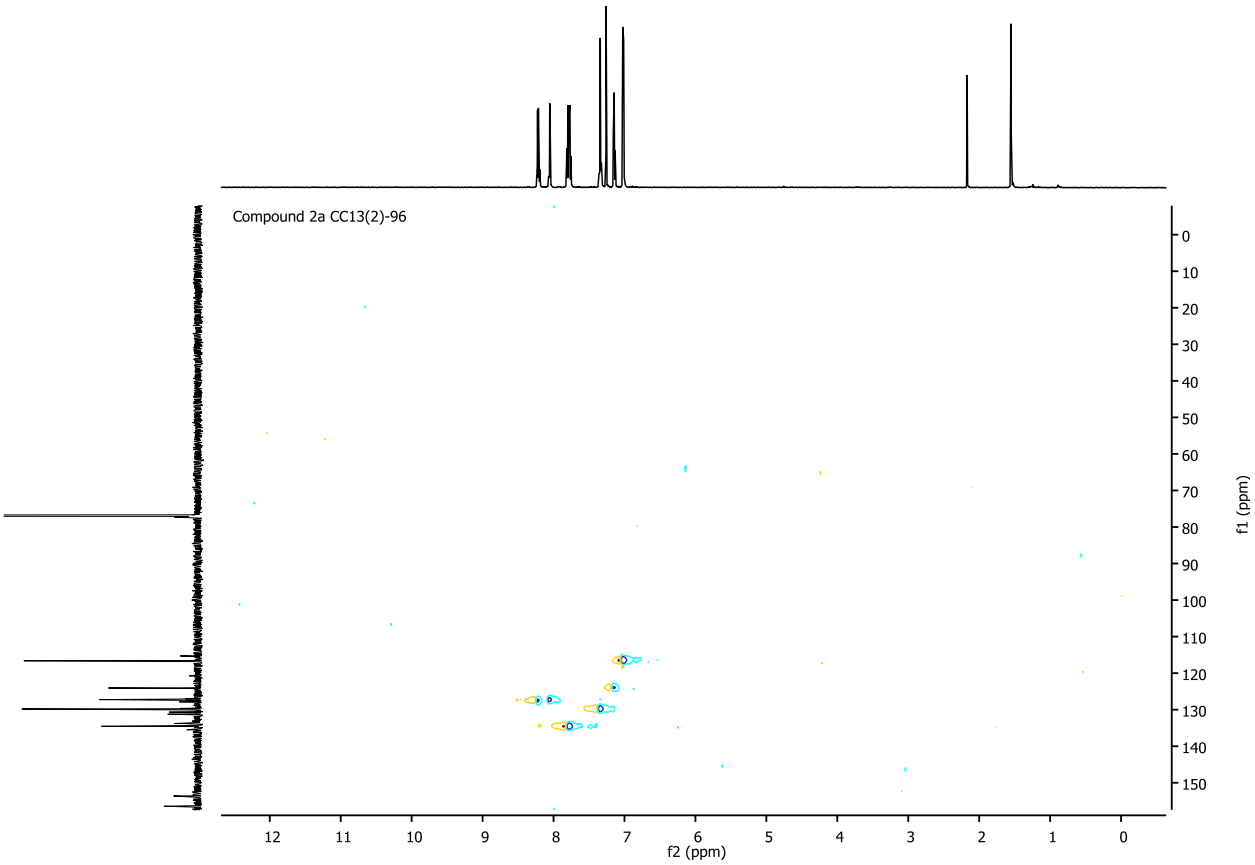
^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2-chloro-3-phenoxy-1,4naphthoquinone **2a**. Expansion of the signals between 115 ppm and 180 ppm are shown in the off-set plot.



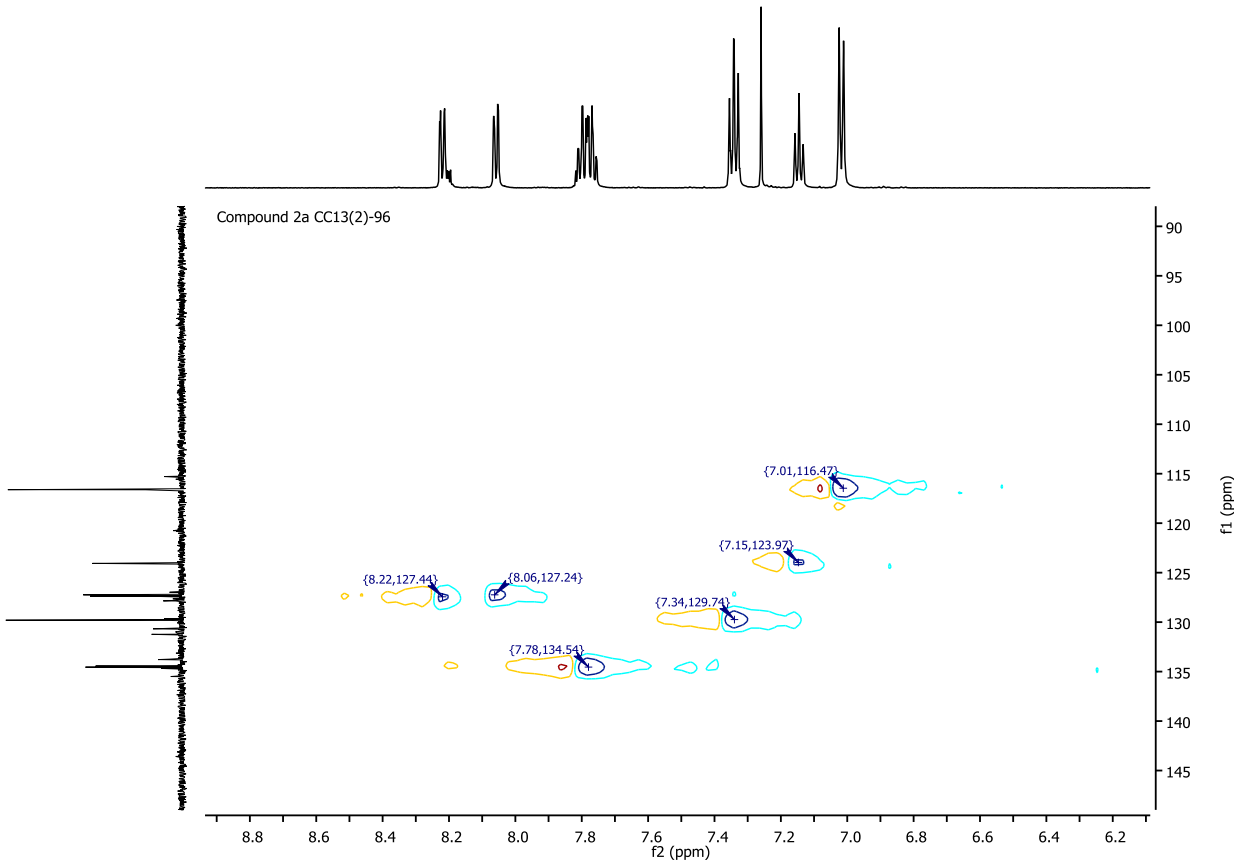
Full COSY spectrum of 2-chloro-3-phenoxy-1,4-naphthoquinone **2a**.



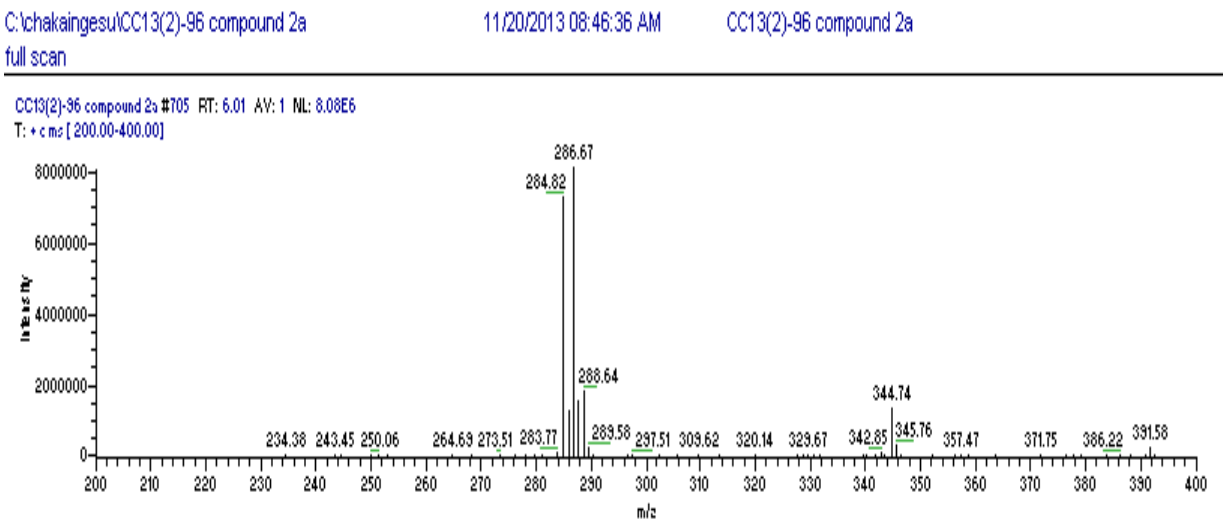
Expansion of the COSY spectrum of 2-chloro-3-phenoxy-1,4-naphthoquinone **2a**.



Full HSQC spectrum of 2-chloro-3-phenoxy-1,4-naphthoquinone **2a**.

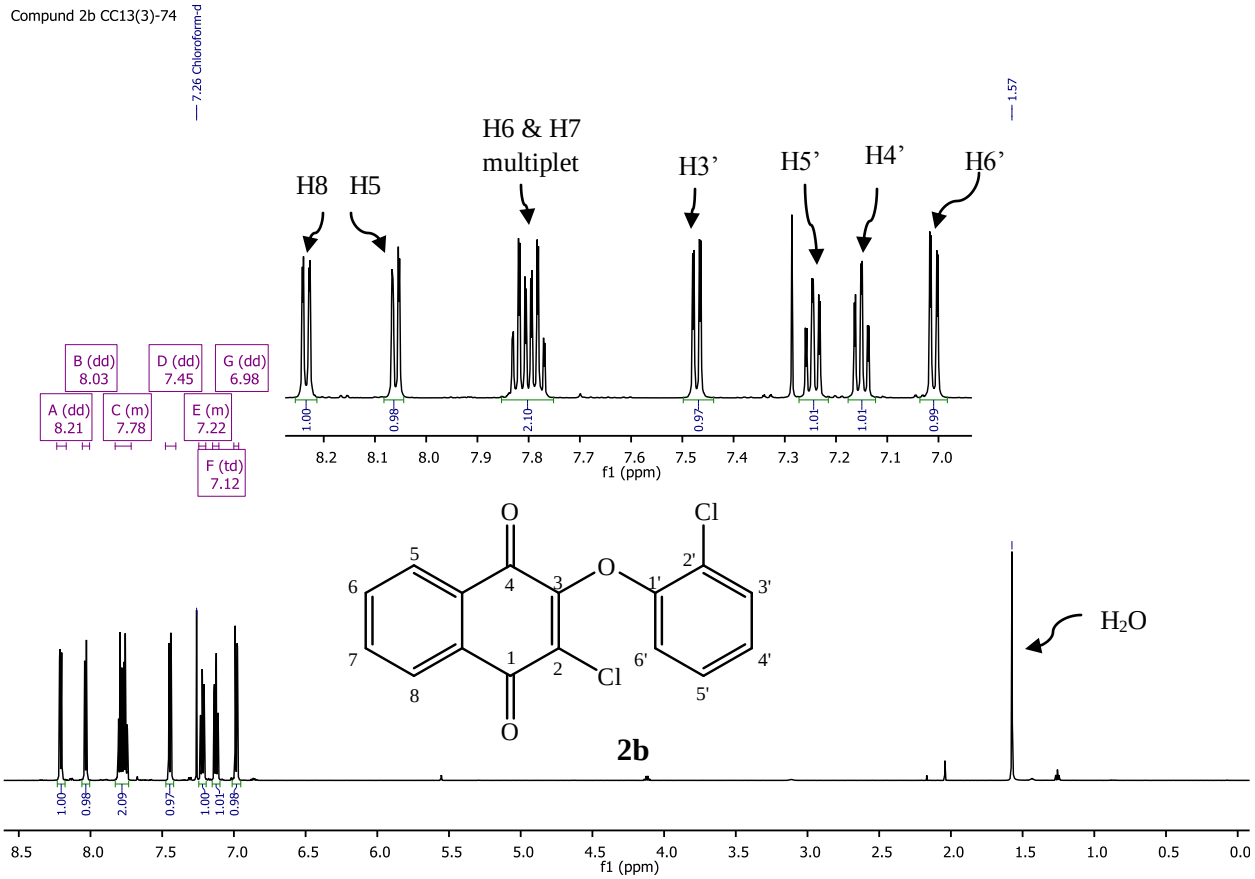


Expansion of the HSQC spectrum of 2-chloro-3-phenoxy-1,4-naphthoquinone **2a**.

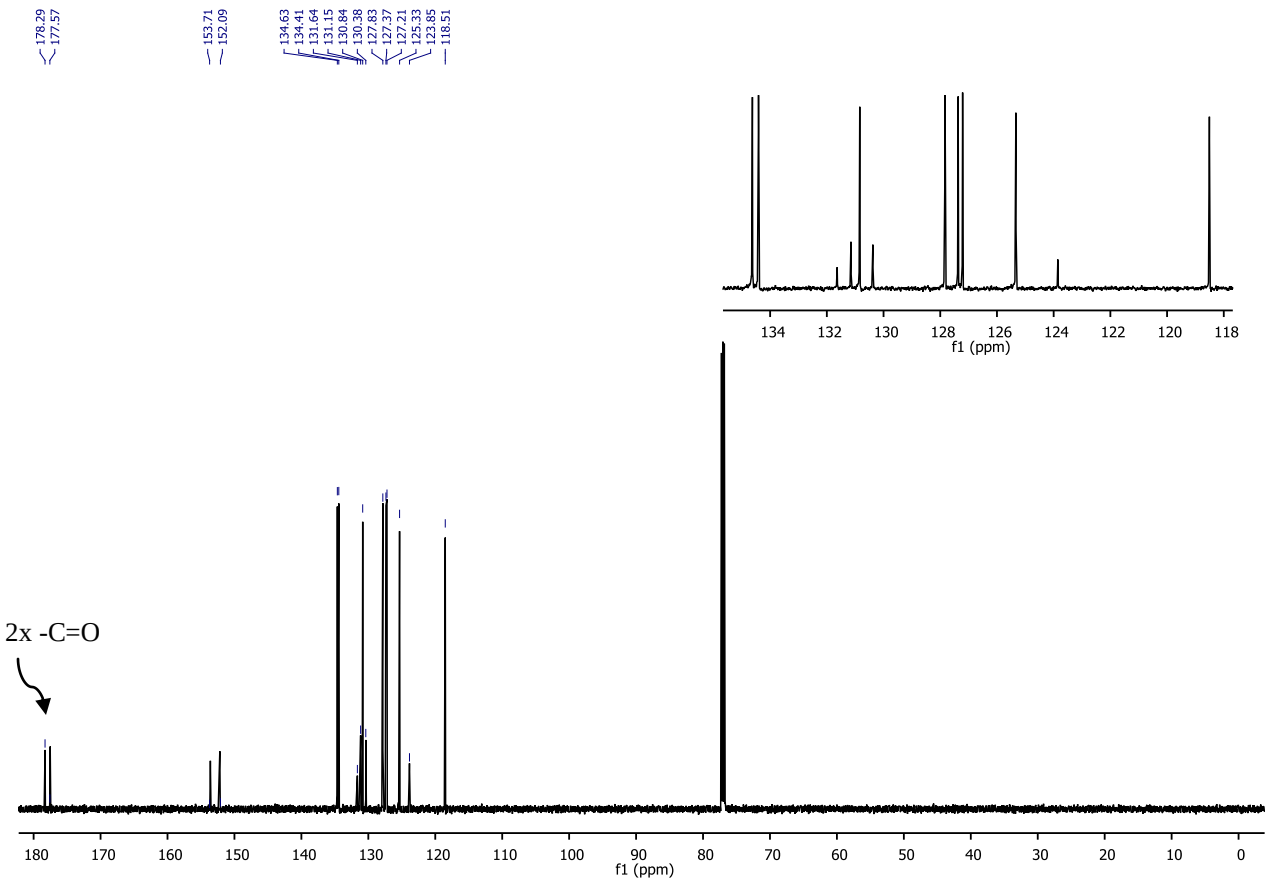


HPLC-ESI-MS spectrum of 2-chloro-3-phenoxy-1,4-naphthoquinone **2a**.

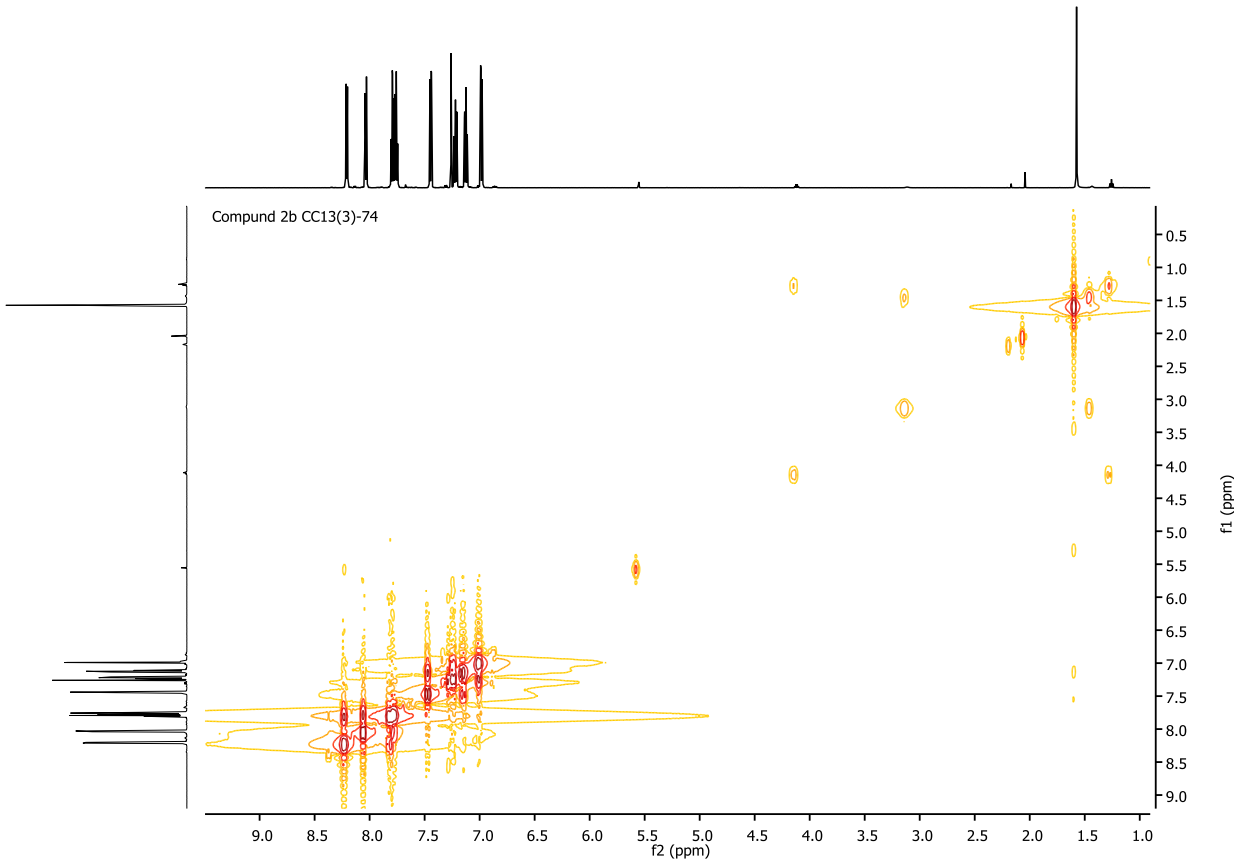
COMPOUND 2b



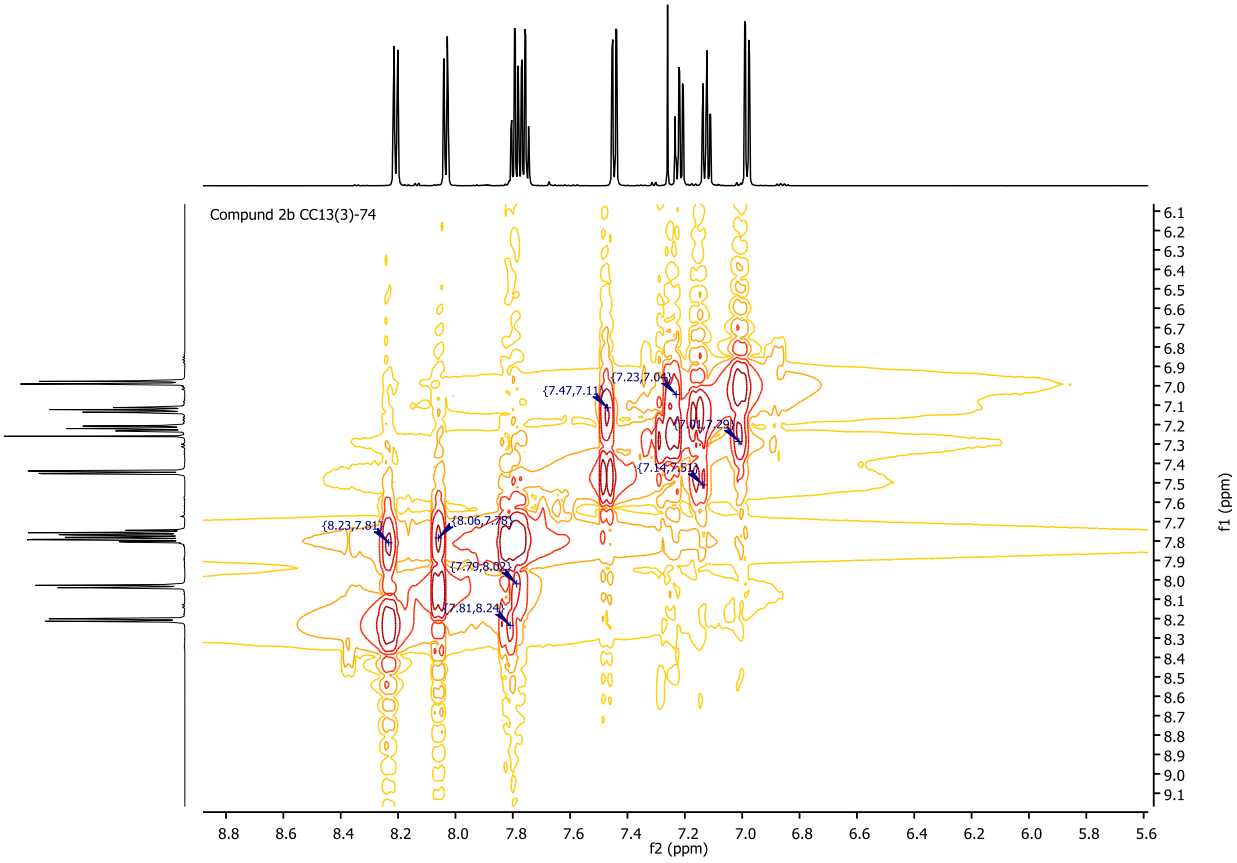
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 2-chlorophenol. Expansions of signals in the aromatic region are shown in the off-set plot.



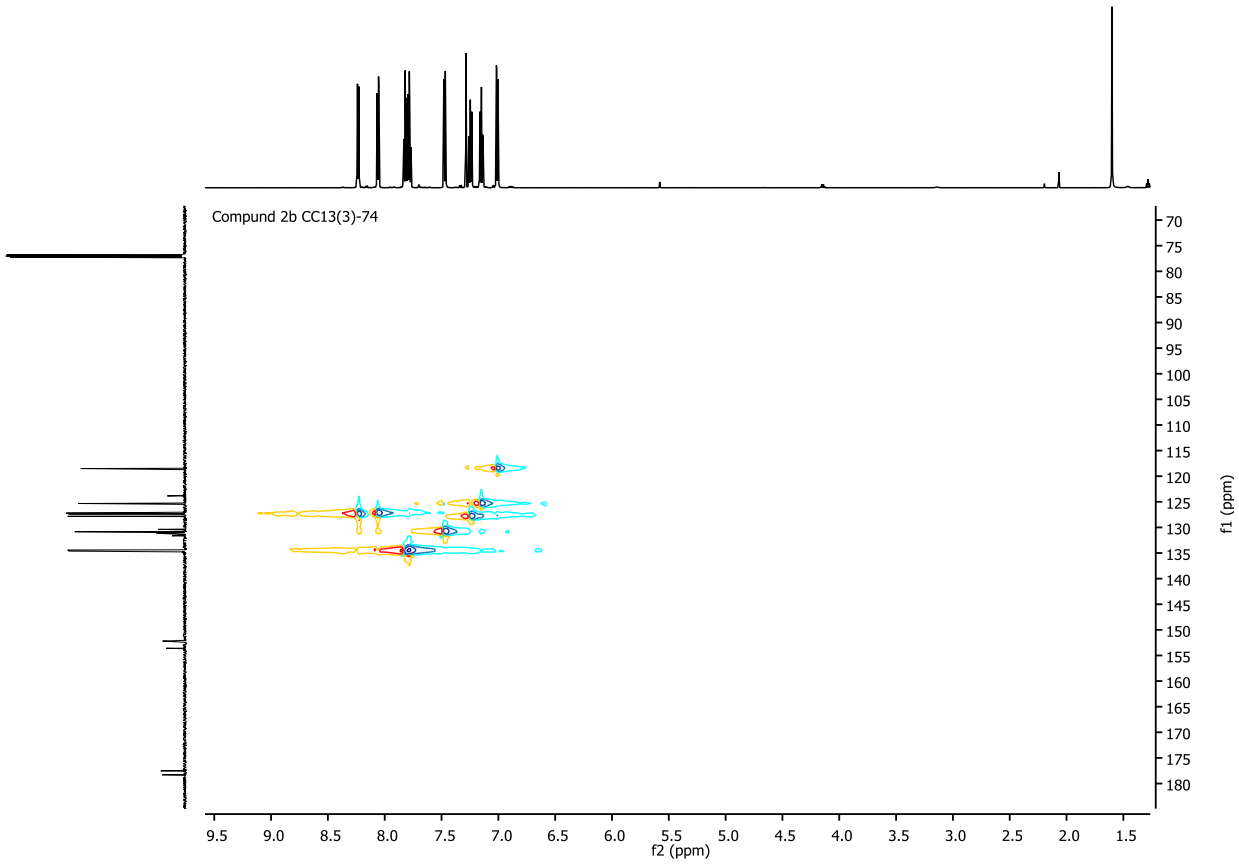
^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b**. Expansion of the signals between 118 ppm and 135 ppm are shown in the off-set plot.



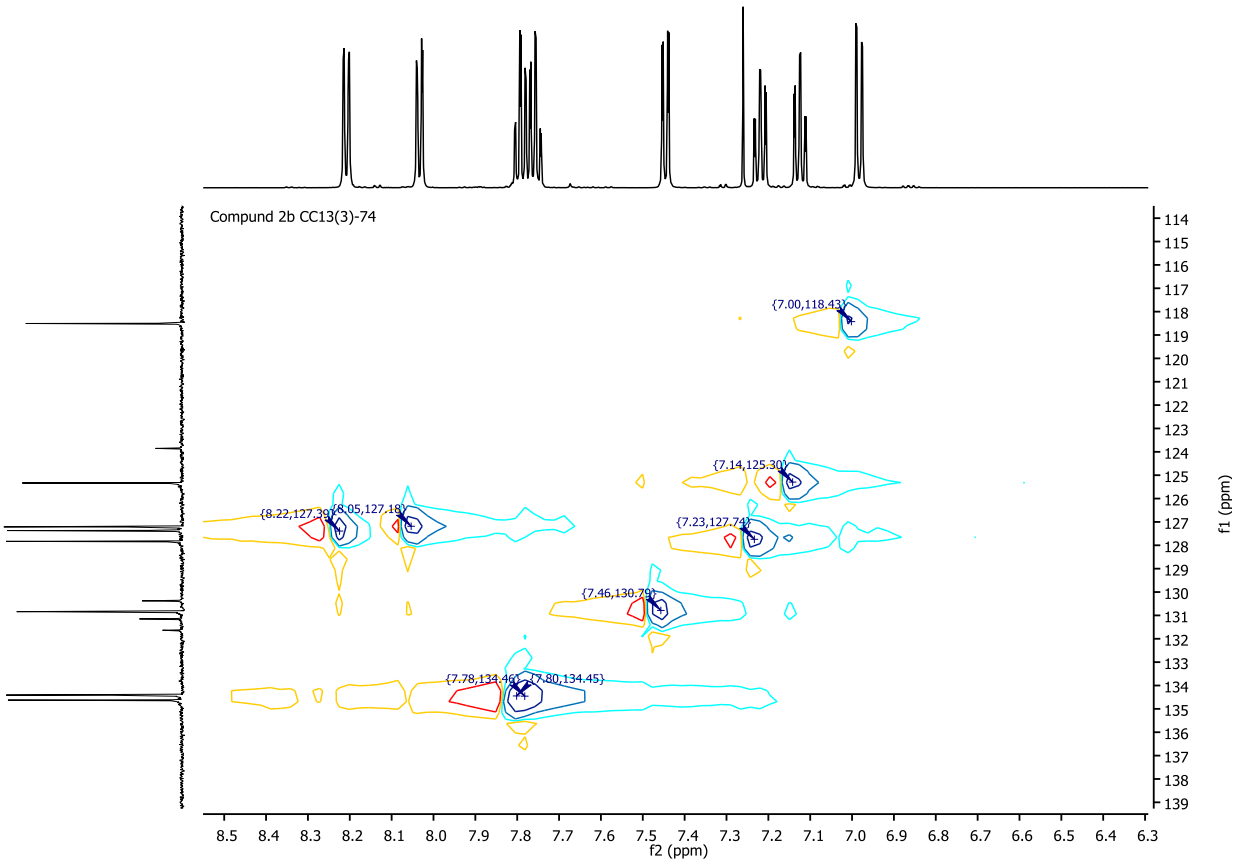
Full COSY NMR spectrum of 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b**.



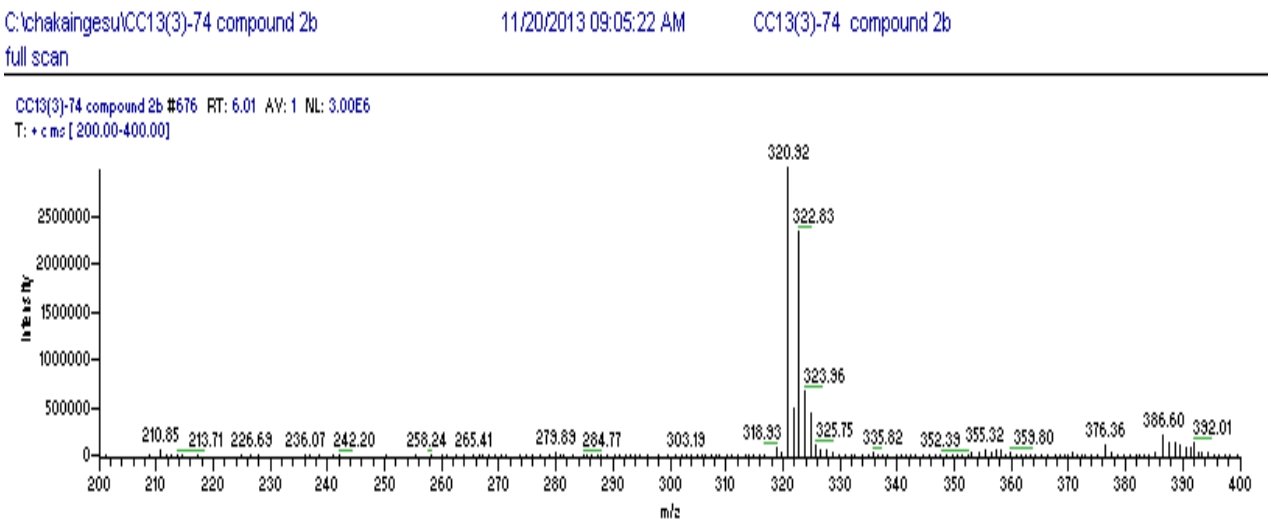
Expansion of the COSY NMR spectrum of 2-chloro-3-(2-chloro-phenoxy)-1,4-naphthoquinone **2b**.



Full HSQC NMR spectrum of 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b**.

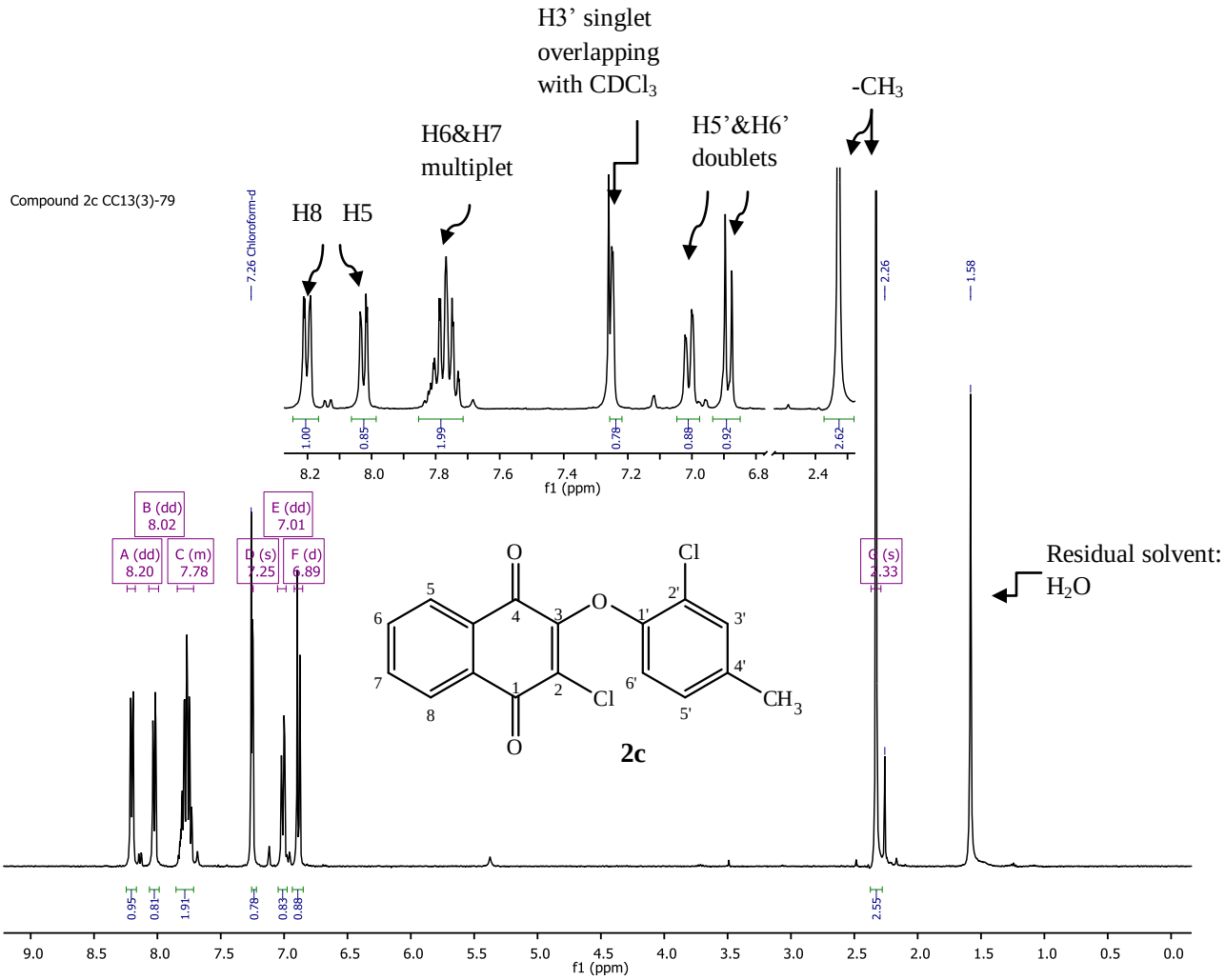


Expansion of the HSQC NMR spectrum of 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b**.

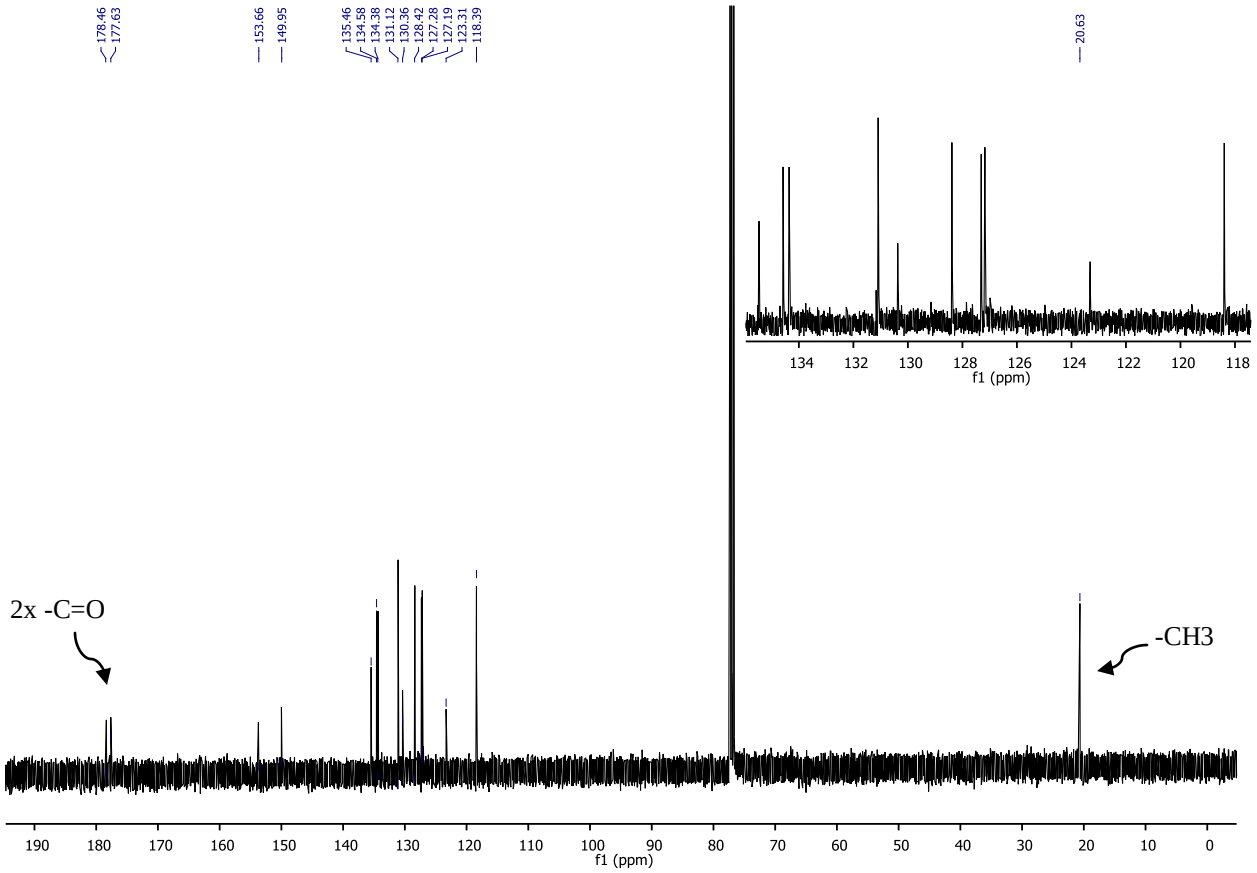


HPLC-ESI-MS spectrum of 2-chloro-3-(2-chlorophenoxy)-1,4-naphthoquinone **2b**.

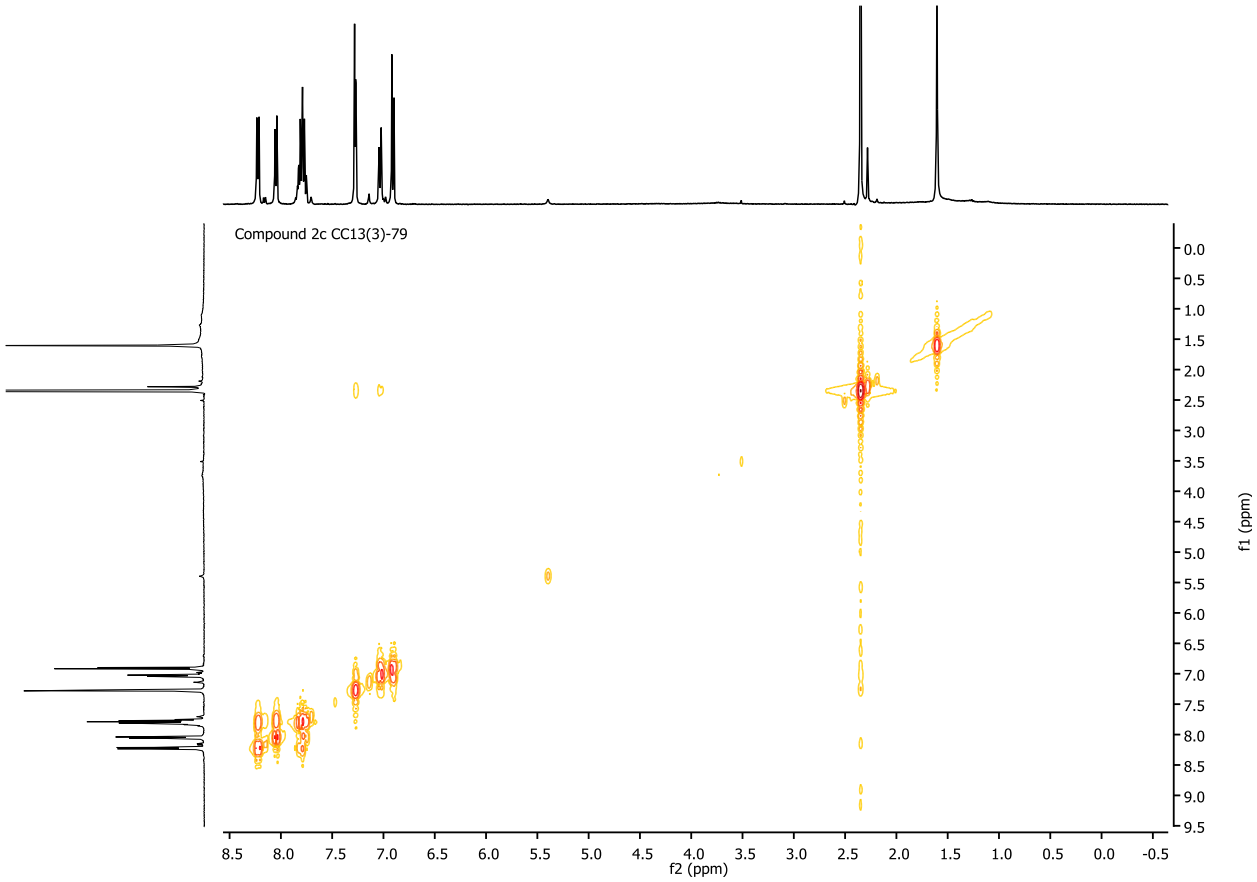
COMPOUND 2c



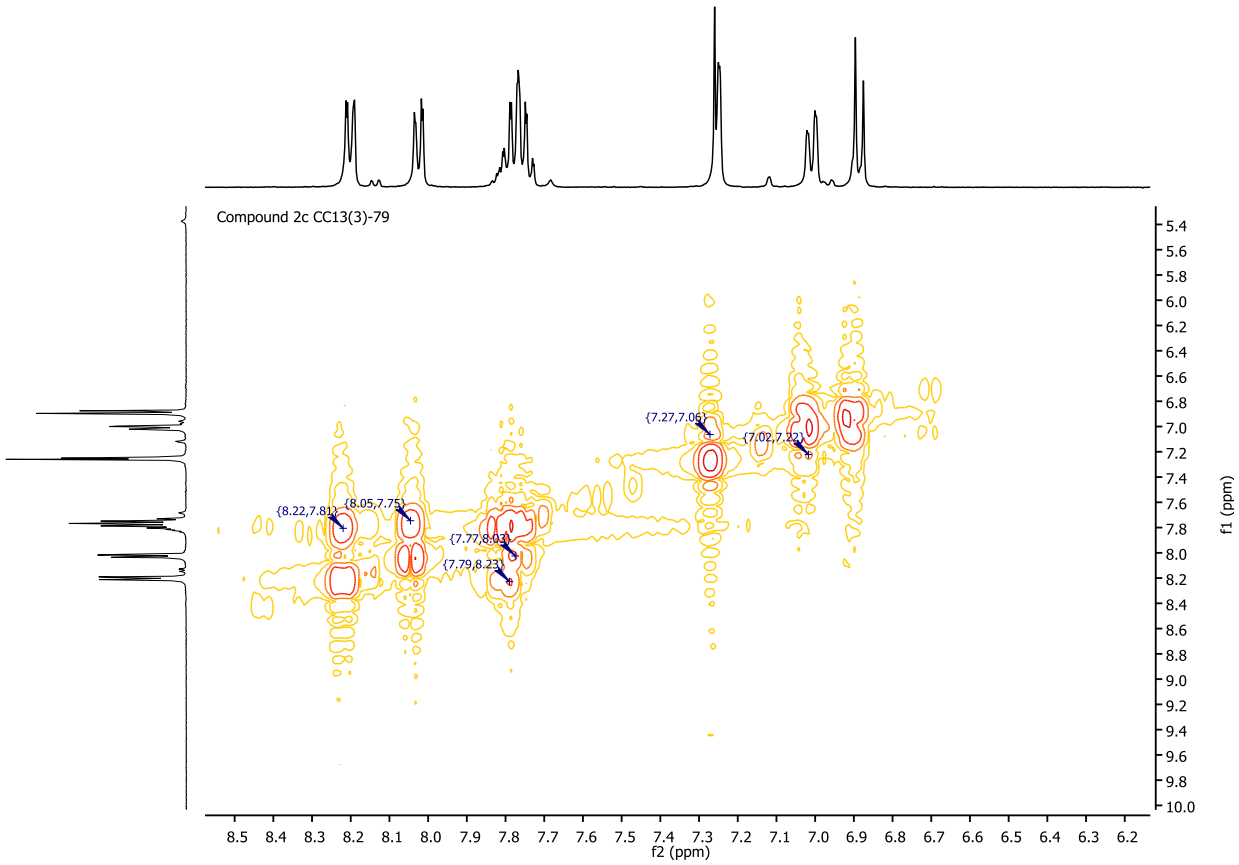
^{1}H NMR (400MHz, $CDCl_3$) spectrum of 2-chloro-3-(2-chloro-4-methylphenoxy)-1,4-naphthoquinone **2c**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 2-chloro-4-methylphenol. Expansions of signals in the aromatic region as well as the methyl signal are shown in the off-set plot.



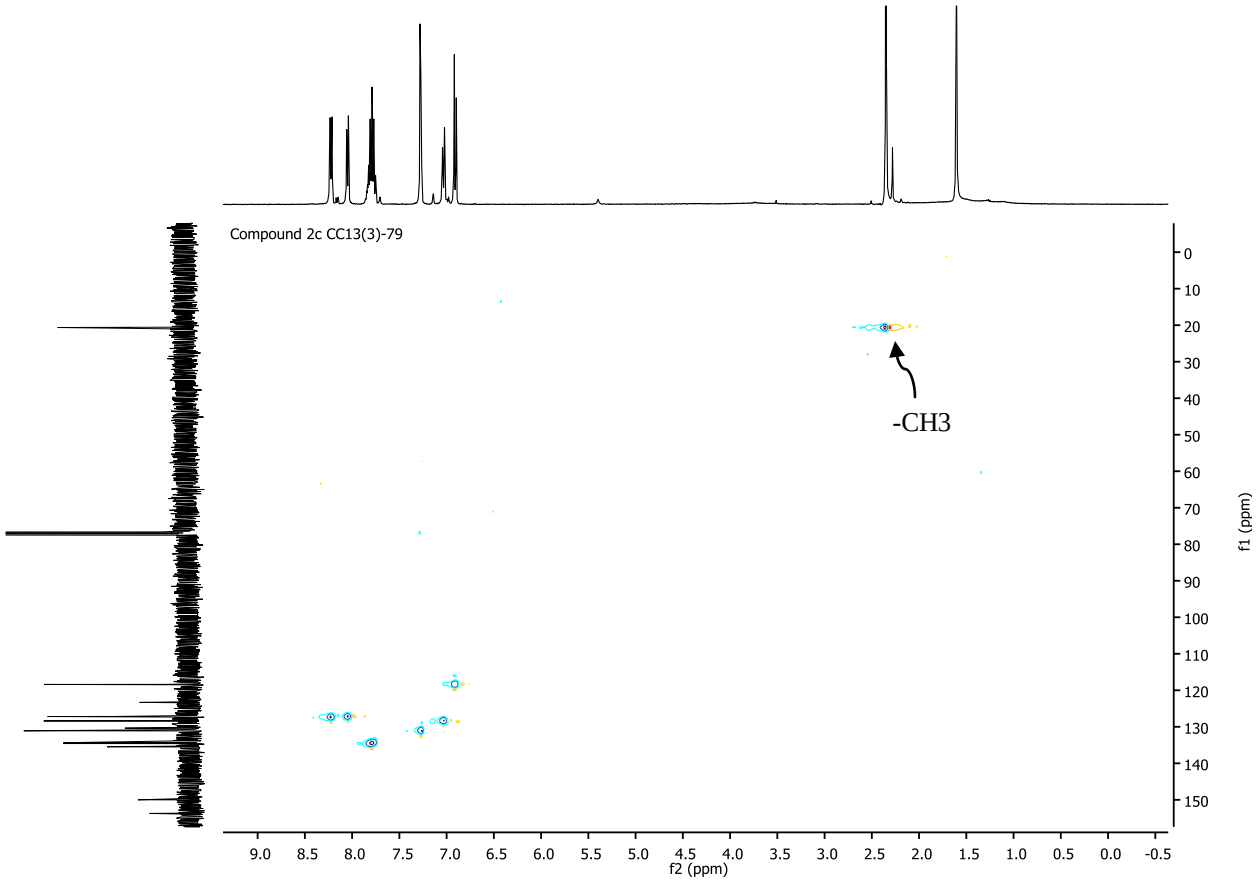
^{13}C NMR (100MHz, $CDCl_3$) spectrum of 2-chloro-3-(2-chloro-4-methylphenoxy)-1,4-naphthoquinone **2c**. Expansion of the signals between 118 ppm and 136 ppm are shown in the off-set plot.



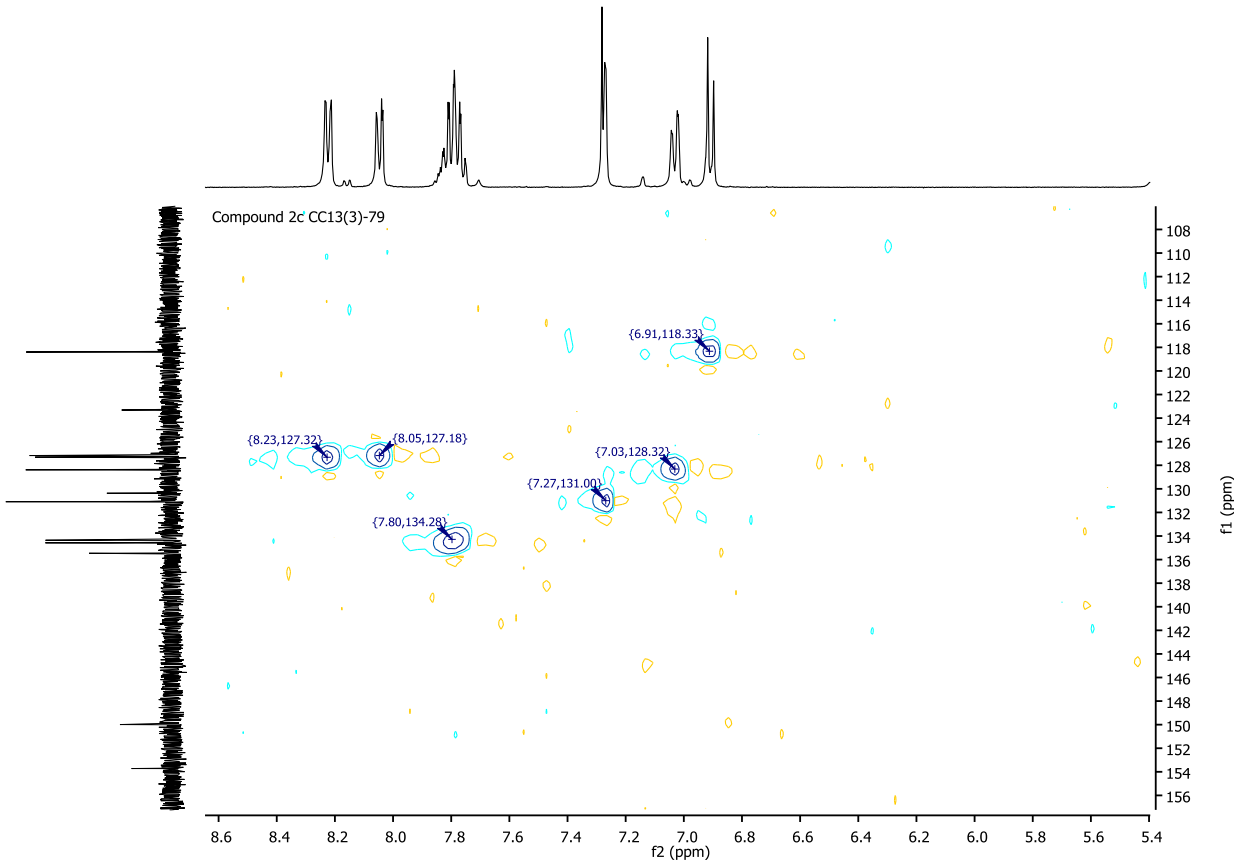
Full COSY NMR spectrum of 2-chloro-3-(2-chloro-4-methylphenoxy)-1,4-naphthoquinone **2c**.



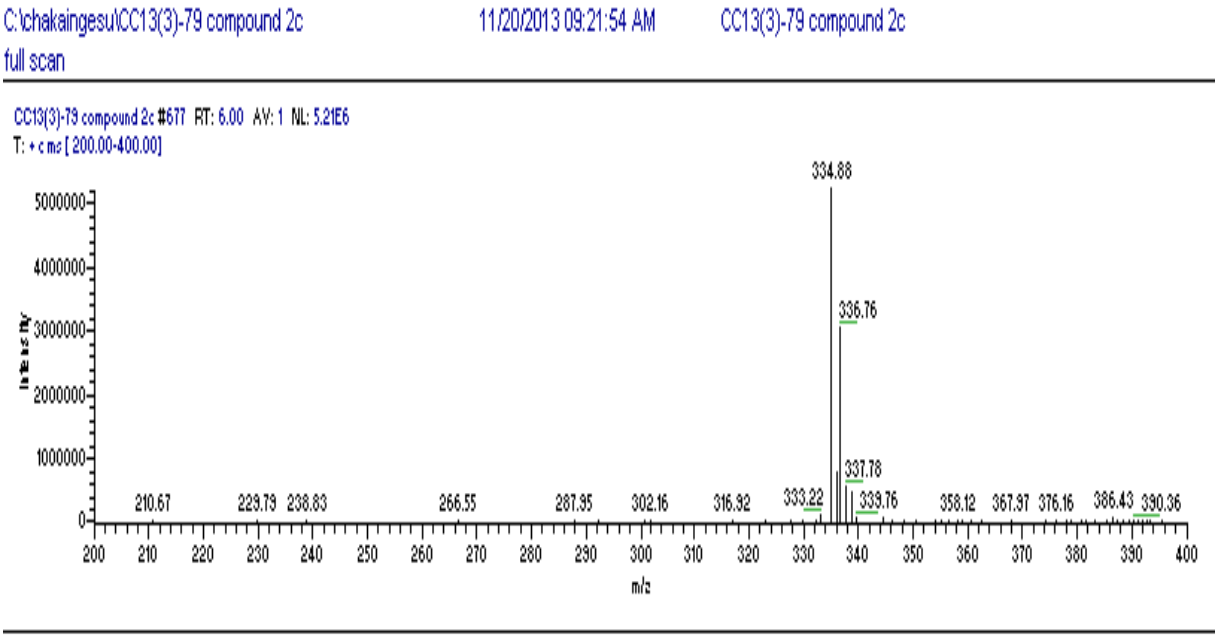
Expansion of the COSY NMR spectrum of 2-chloro-3-(2-chloro-4-methyl-phenoxy)-1,4-naphthoquinone **2c**.



Full HSQC NMR spectrum of 2-chloro-3-(2-chloro-4-methylphenoxy)-1,4-naphthoquinone **2c**.

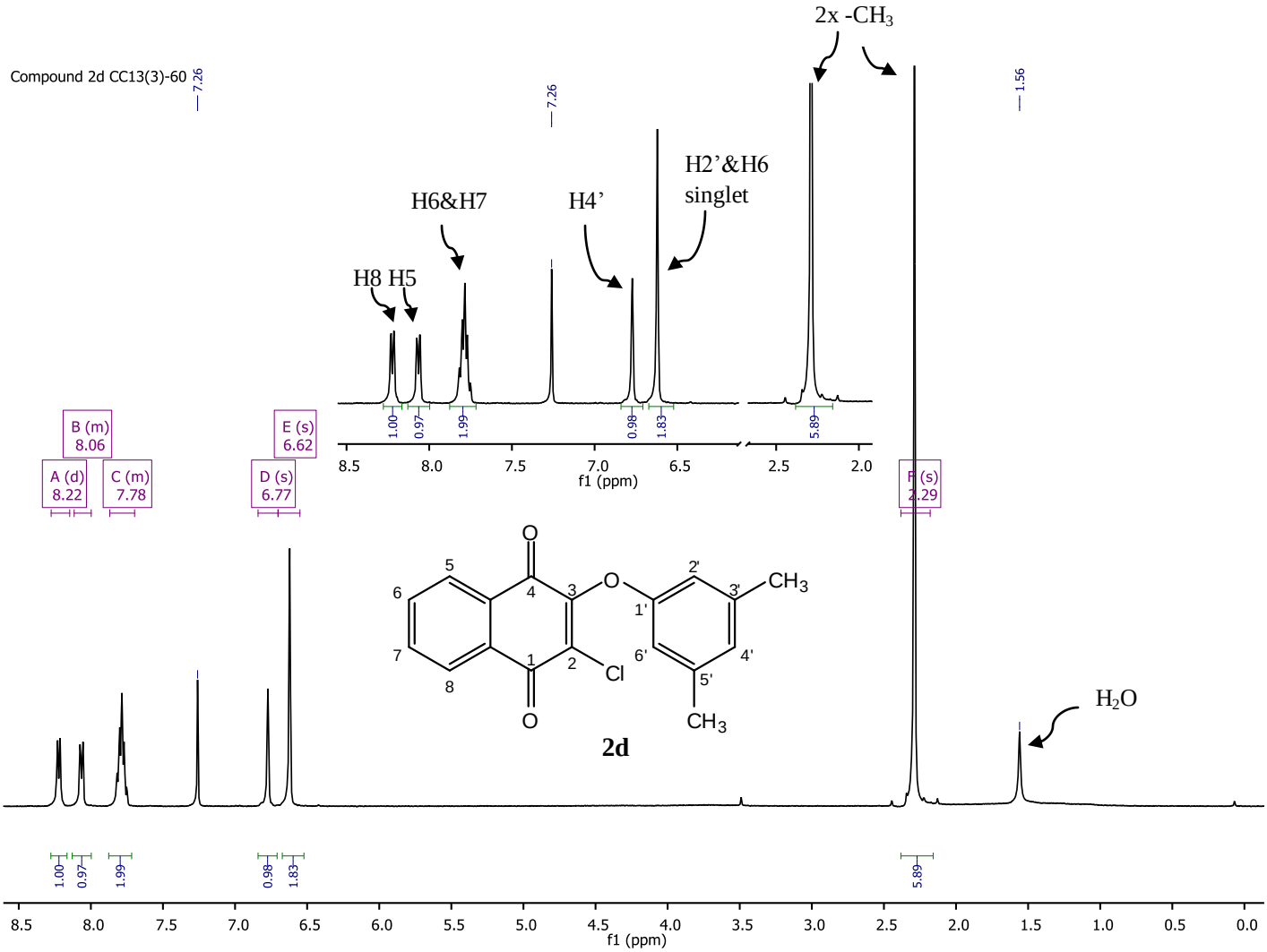


Expansion of the HSQC NMR spectrum of 2-chloro-3-(2-chloro-4-methyl-phenoxy)-1,4-naphthoquinone **2c**. The methyl cross-peak has been left out here.

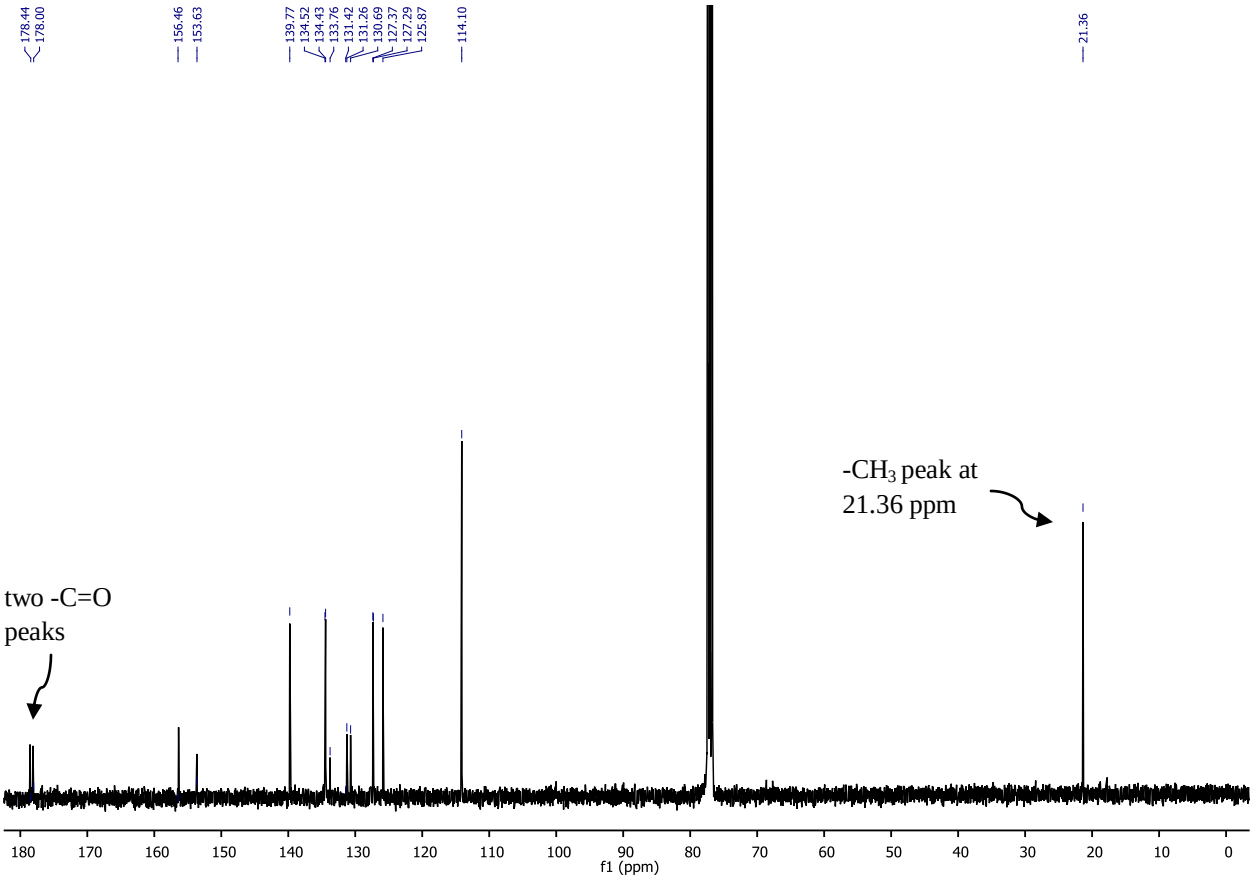


HPLC-ESI-MS spectrum of 2-chloro-3-(2-chloro-4-methyl-phenoxy)-1,4-naphthoquinone **2c**.

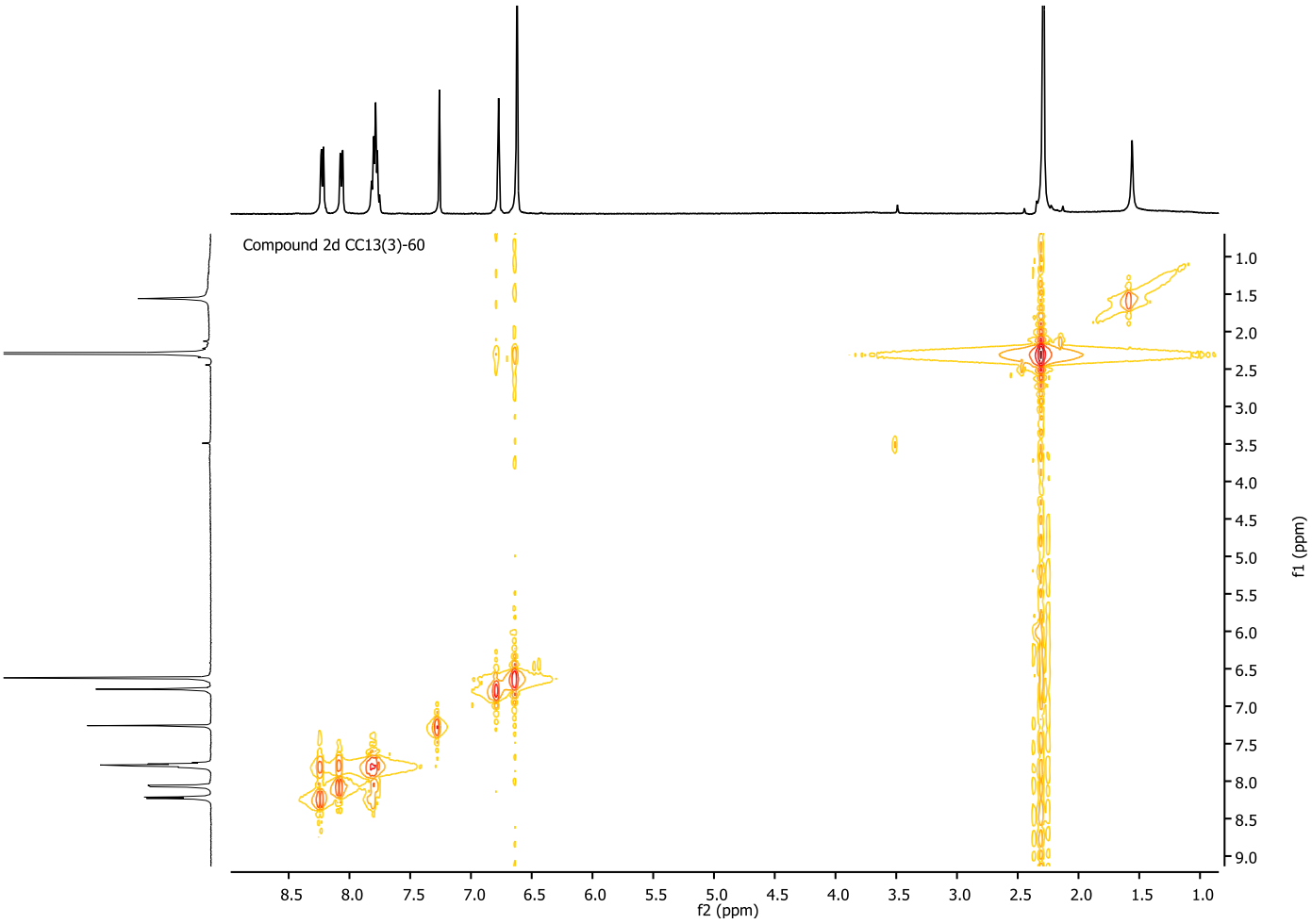
COMPOUND 2d



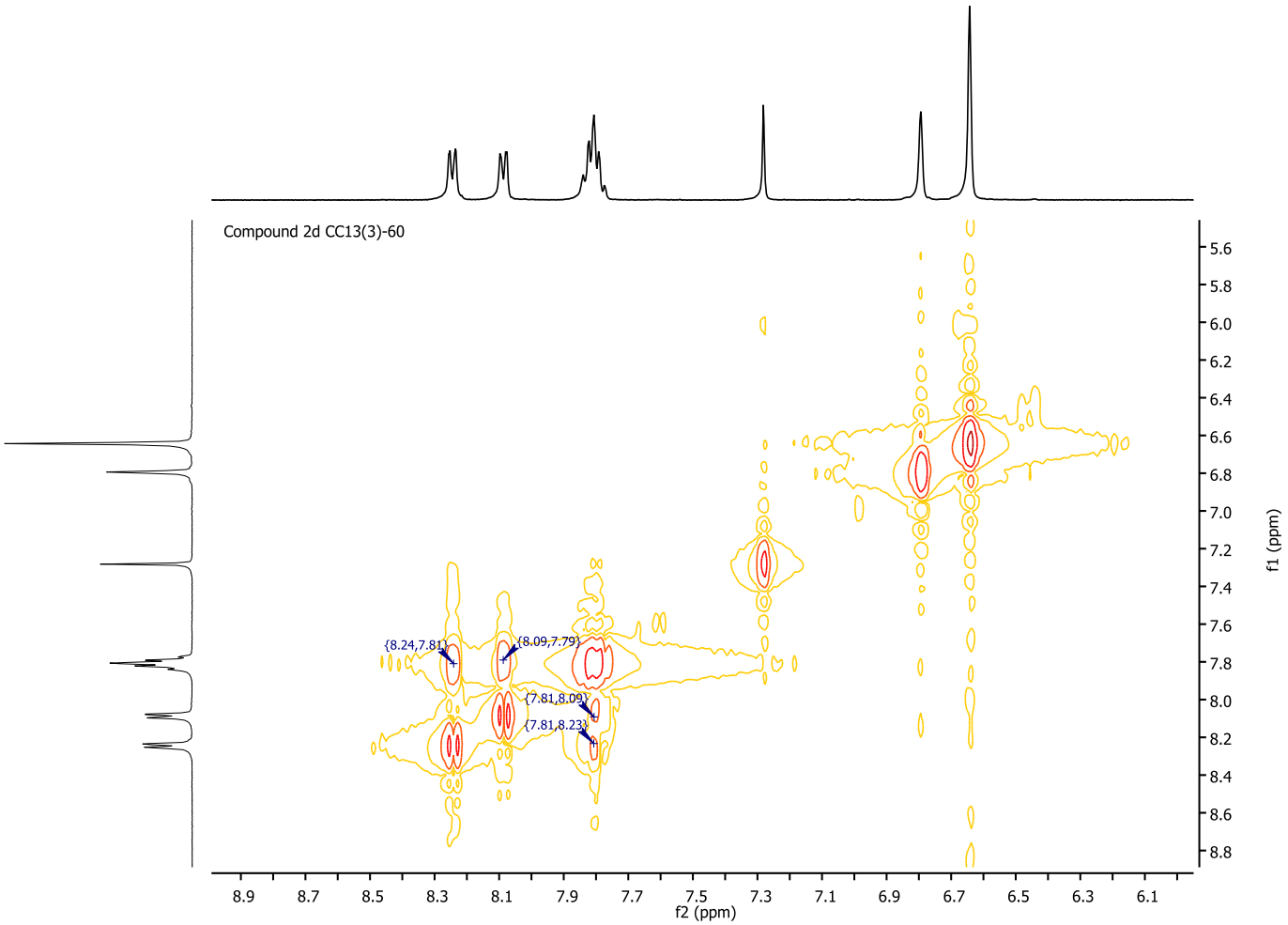
^{1}H NMR (400MHz, $CDCl_3$) spectrum of 2-chloro-3-(3,5-dimethylphenoxy)-1,4-naphthoquinone **2d**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 3,5-dimethylphenol. Expansions of signals in the aromatic region as well as the methyl signal are shown in the off-set plot.



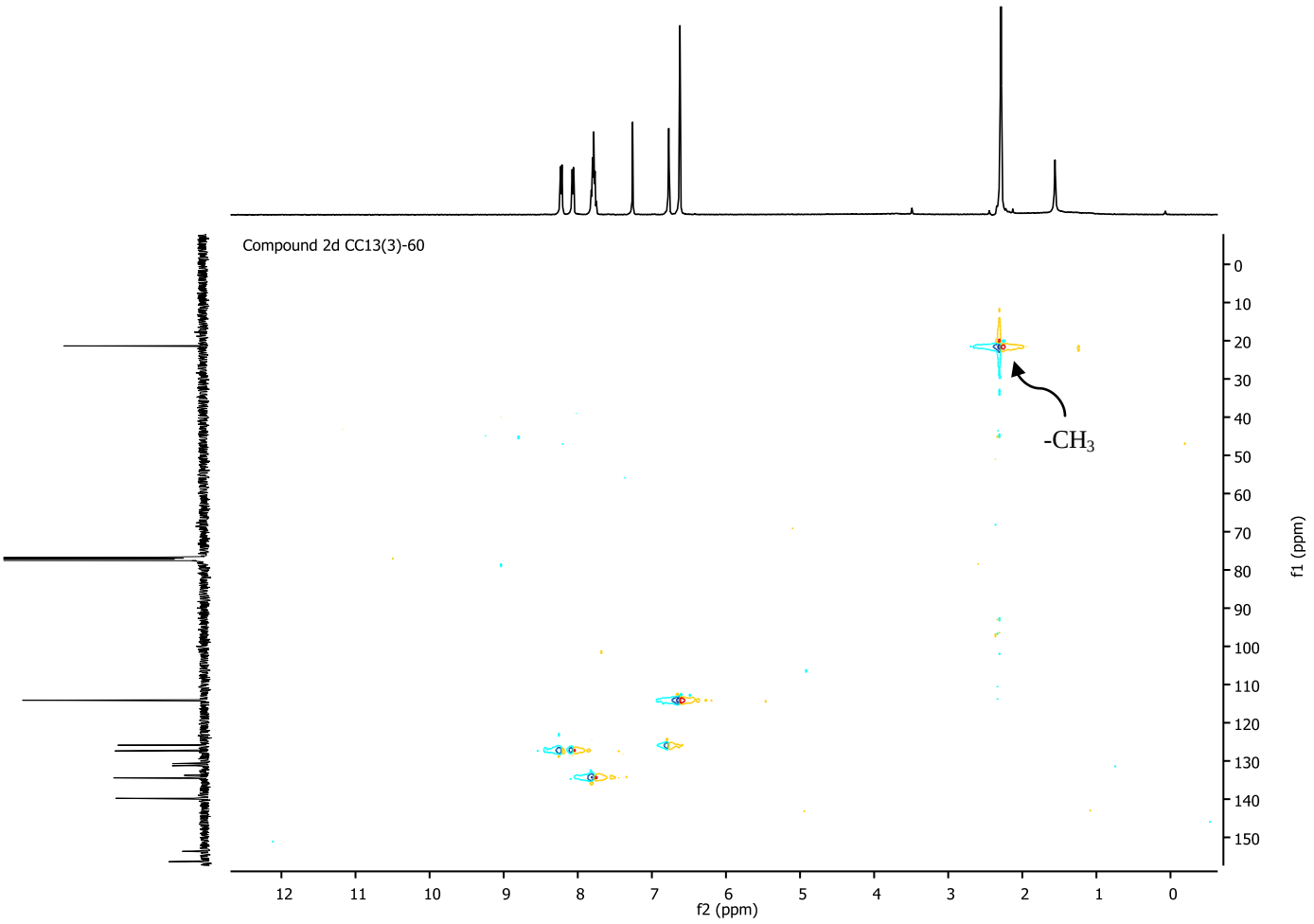
^{13}C NMR (100MHz, $CDCl_3$) spectrum of 2-chloro-3-(3,5-dimethyl-phenoxy)-1,4-naphthoquinone **2d**.



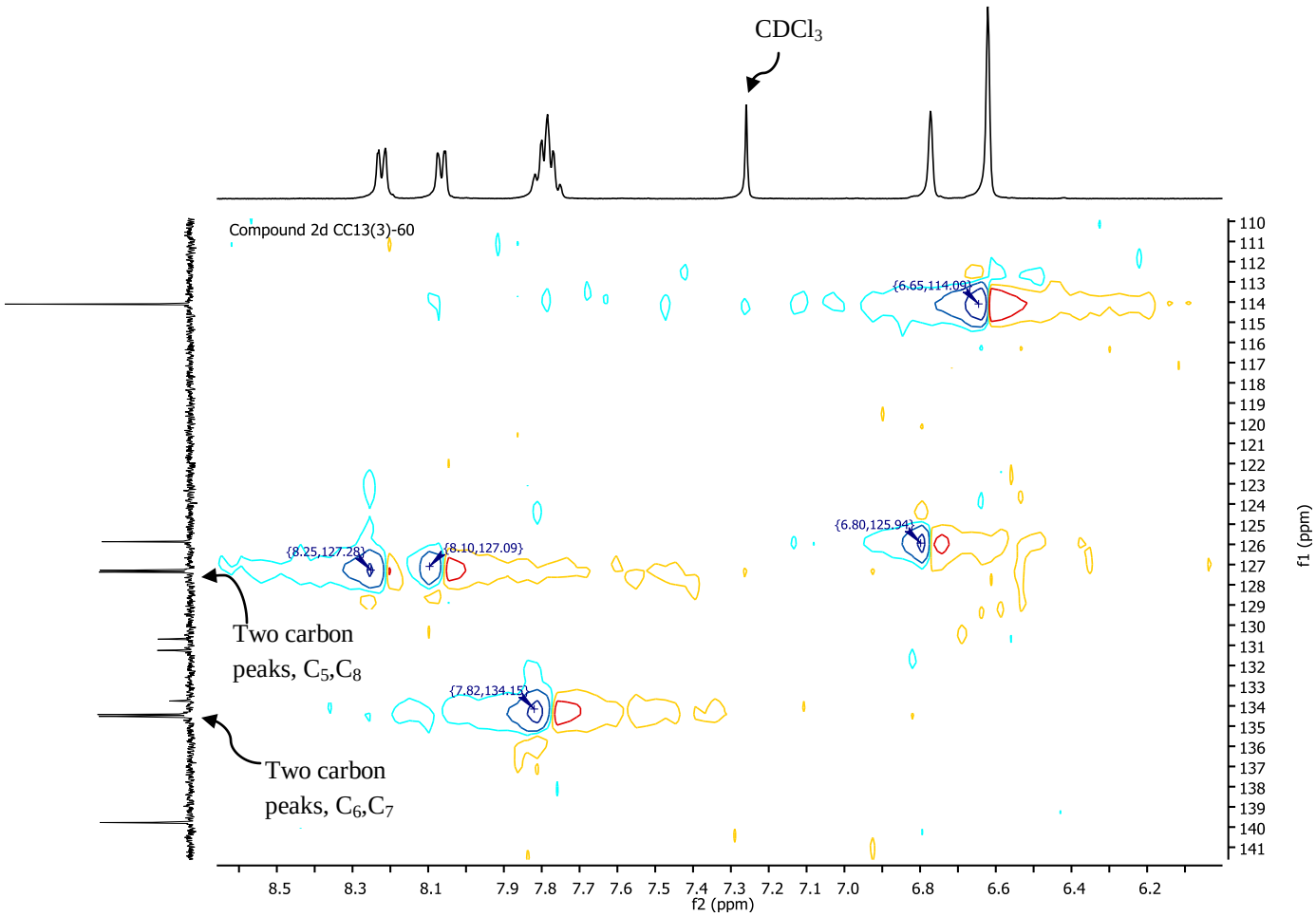
Full COSY NMR spectrum of 2-chloro-3-(3,5-dimethyl-phenoxy)-1,4-naphthoquinone **2d**.



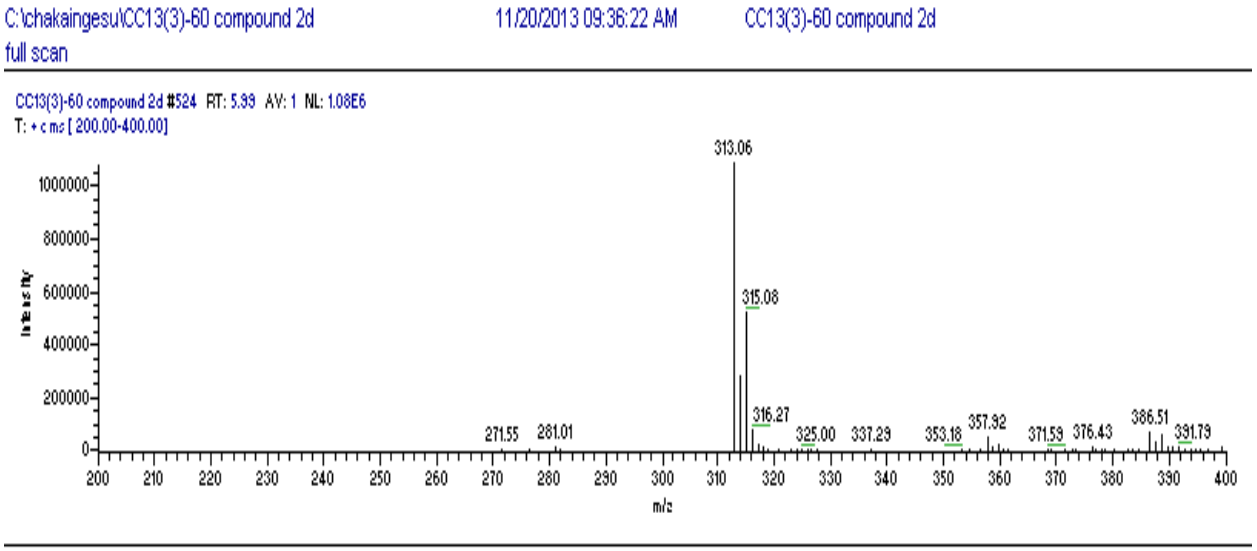
Expansion of the COSY NMR spectrum of 2-chloro-3-(3,5-dimethyl-phenoxy)-1,4-naphthoquinone **2d**.



Full HSQC NMR spectrum of 2-chloro-3-(3,5-dimethylphenoxy)-1,4-naphthoquinone **2d**.

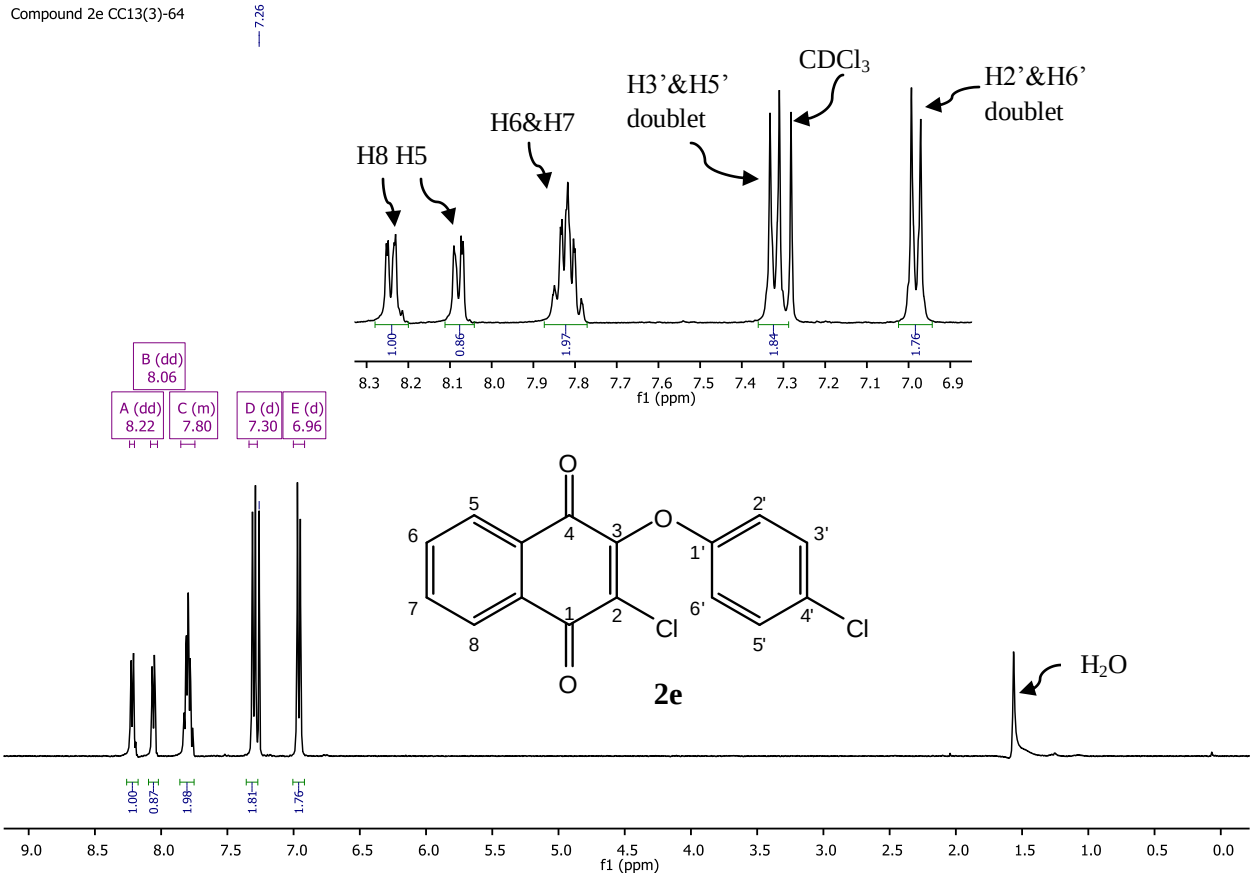


Expansion of HSQC NMR spectrum of 2-chloro-3-(3,5-dimethylphenoxy)-1,4-naphthoquinone **2d**. The methyl cross peak cannot be seen here.

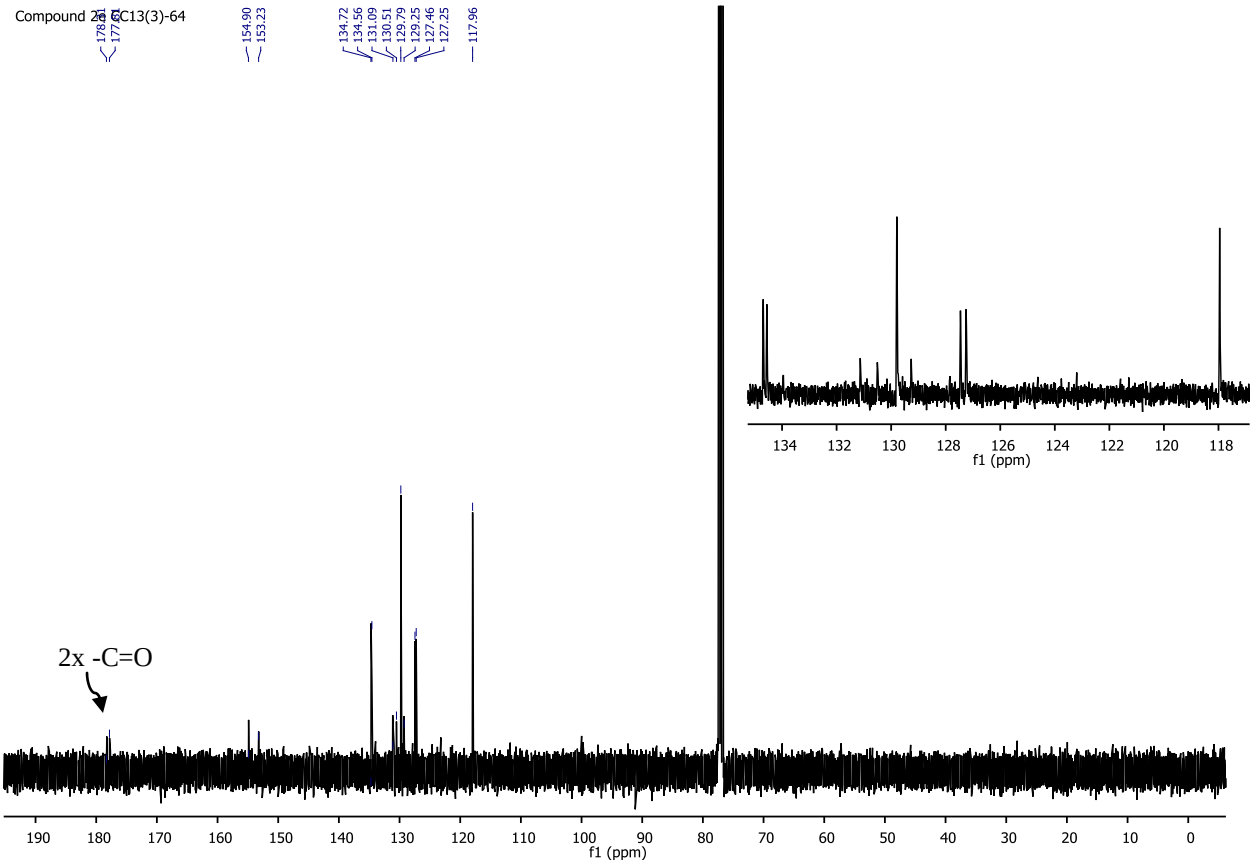


HPLC-ESI-MS spectrum of 2-chloro-3-(3,5-dimethylphenoxy)-1,4-naphthoquinone **2d**.

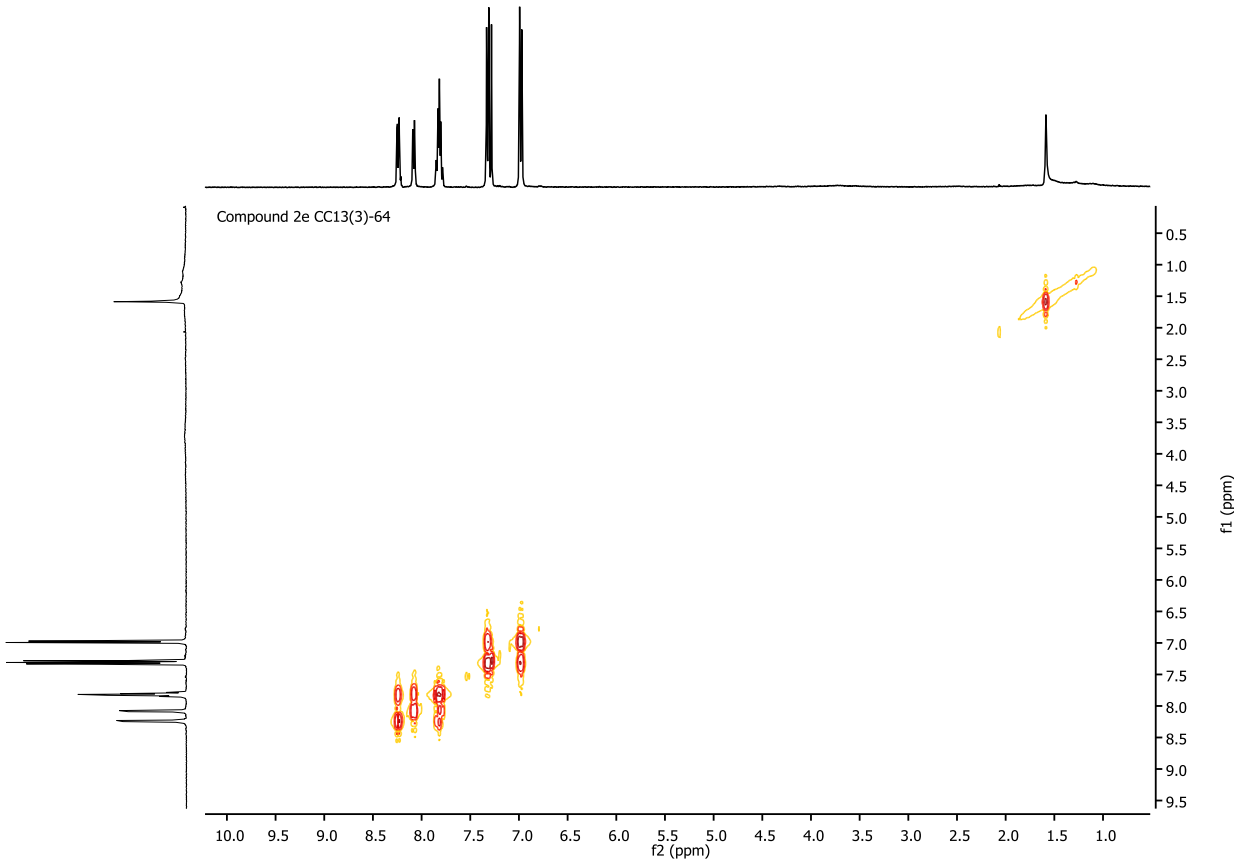
COMPOUND 2e



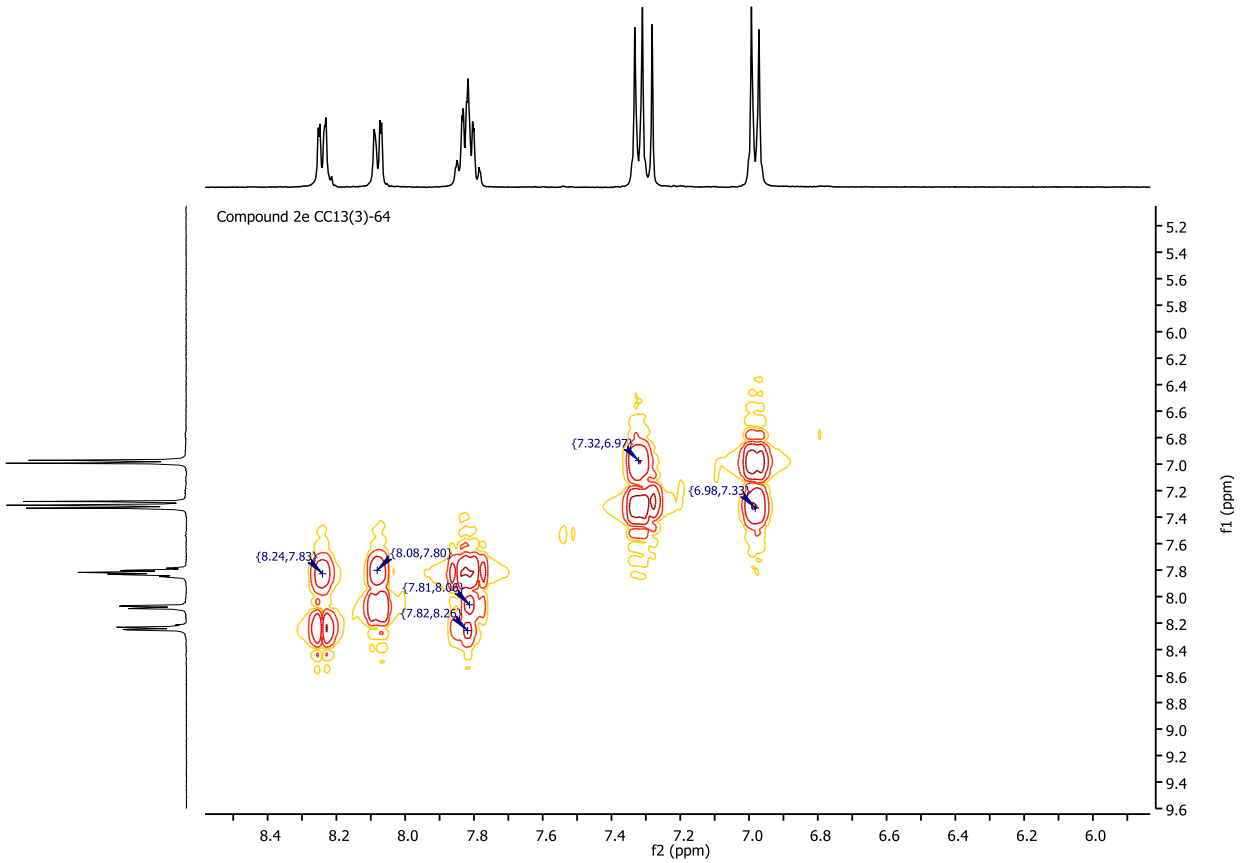
^{1}H NMR (400MHz, $CDCl_3$) spectrum of 2-chloro-3-(4-chlorophenoxy)-1,4-naphthoquinone **2e**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 4-chlorophenol. Expansion of the aromatic region is shown in the off-set plot.



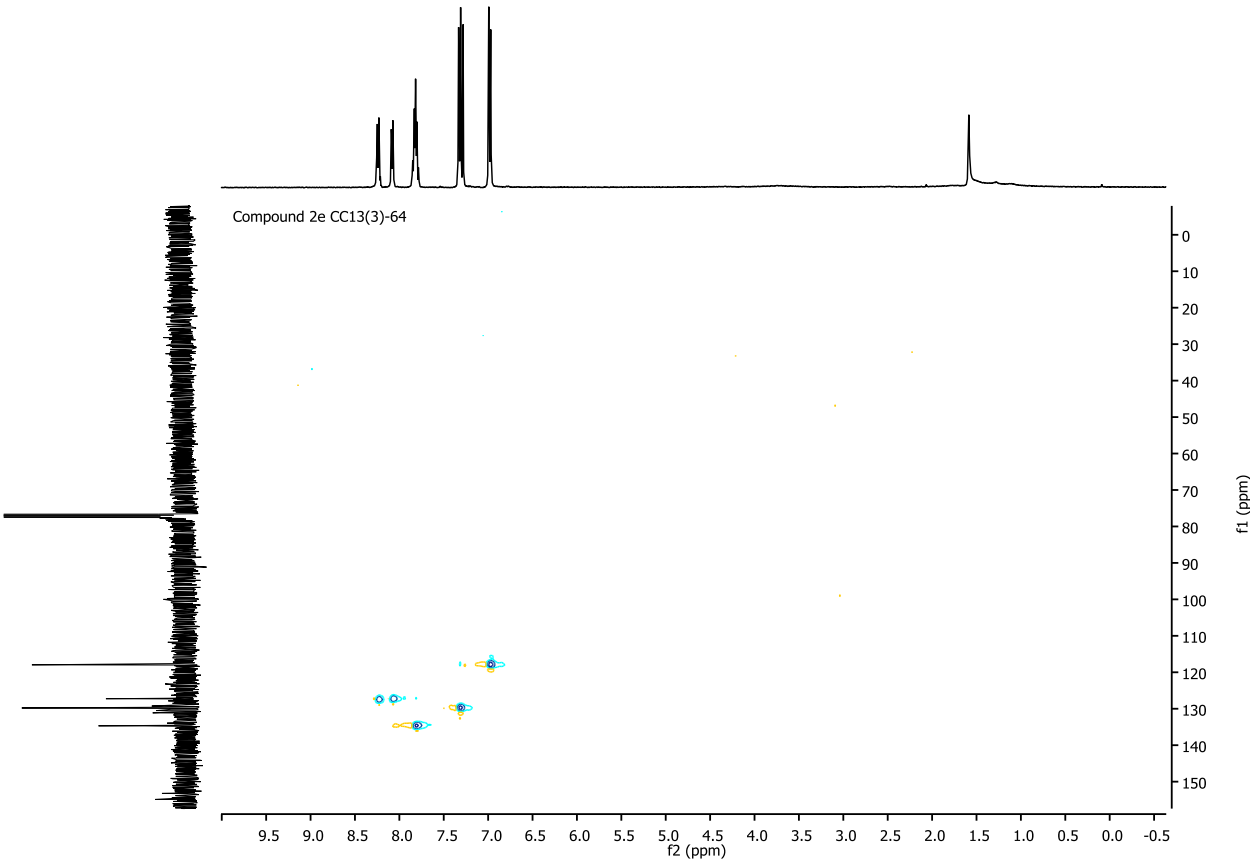
^{13}C NMR (100MHz, $CDCl_3$) spectrum of 2-chloro-3-(4-chlorophenoxy)-1,4-naphthoquinone **2e**. Expansion of the signals between 118 ppm and 136 ppm are shown in the off-set plot.



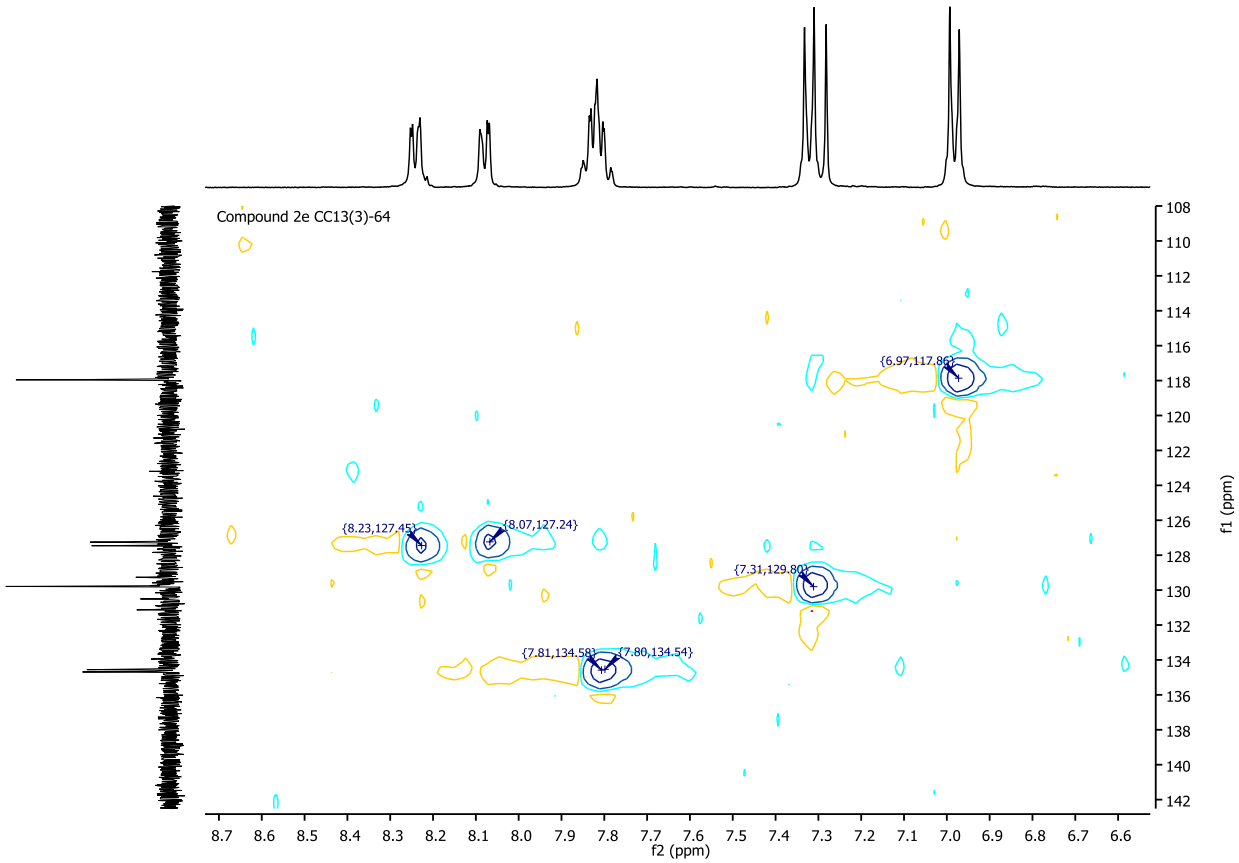
Full COSY NMR spectrum of 2-chloro-3-(4-chlorophenoxy)-1,4-naphthoquinone **2e**.



Expansion of the COSY NMR spectrum of 2-chloro-3-(4-chlorophenoxy)-1,4-naphthoquinone **2e**.

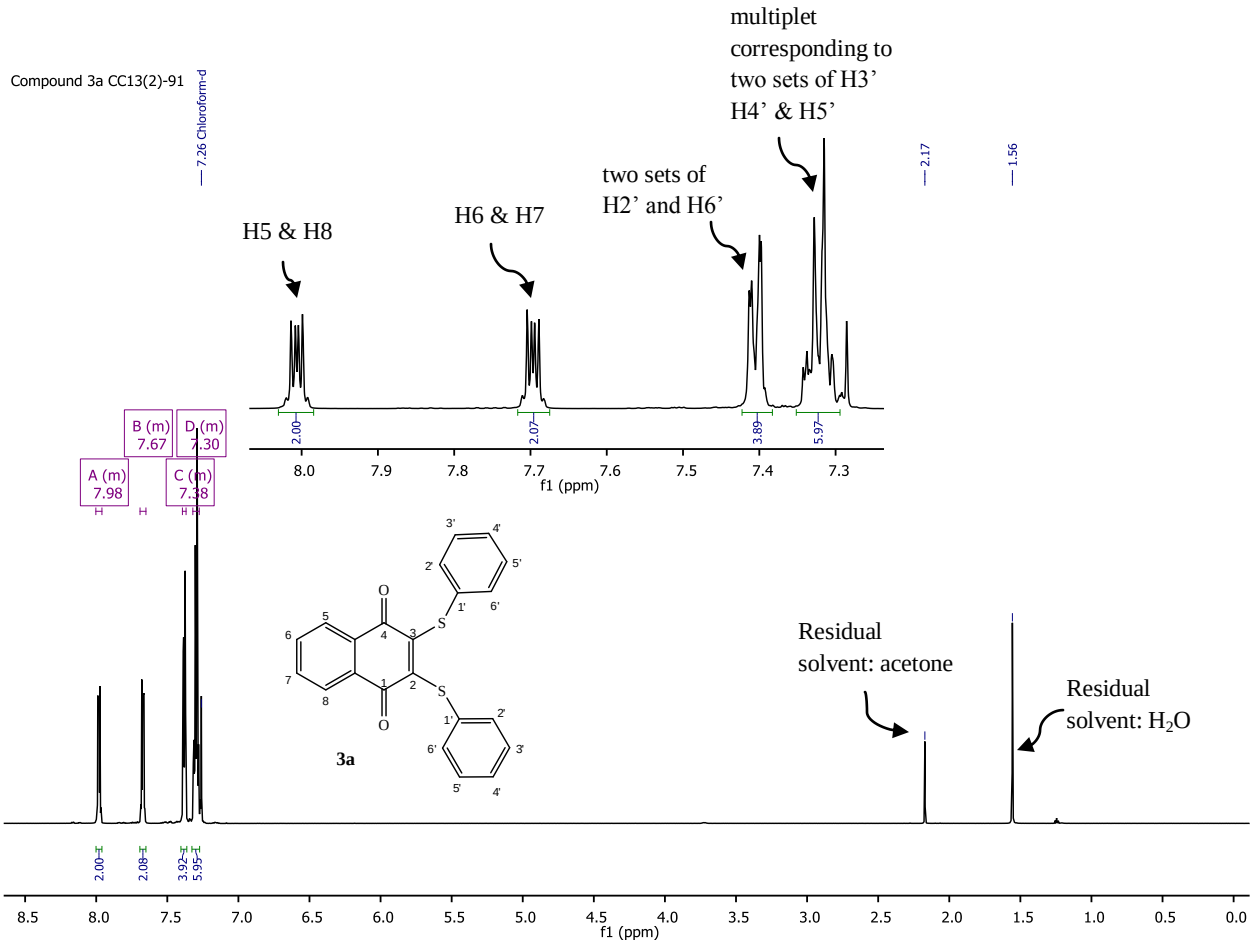


Full HSQC NMR spectrum of 2-chloro-3-(4-chlorophenoxy)-1,4-naphthoquinone **2e**.

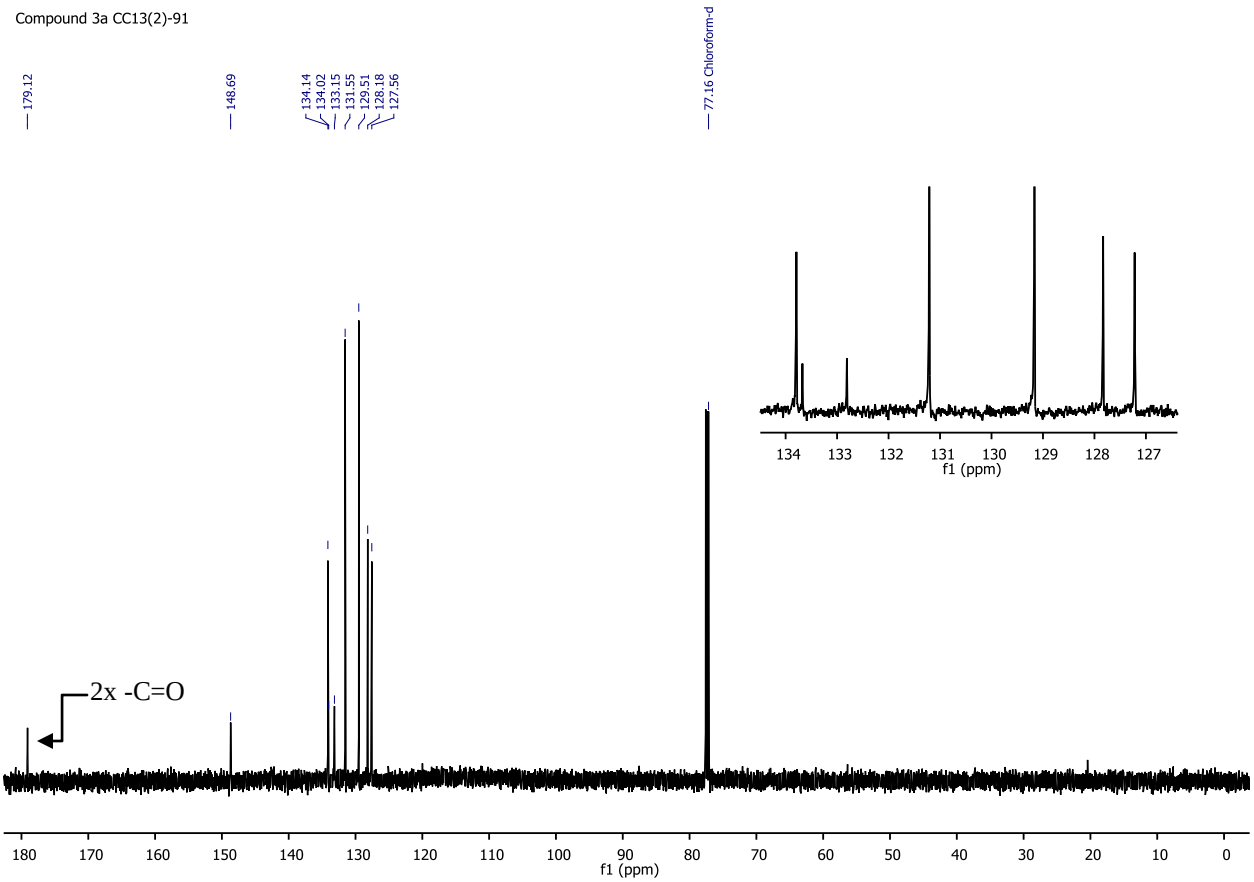


Expansion of the HSQC NMR spectrum of 2-chloro-3-(4-chlorophenoxy)-1,4-naphthoquinone **2e** showing the aromatic region only.

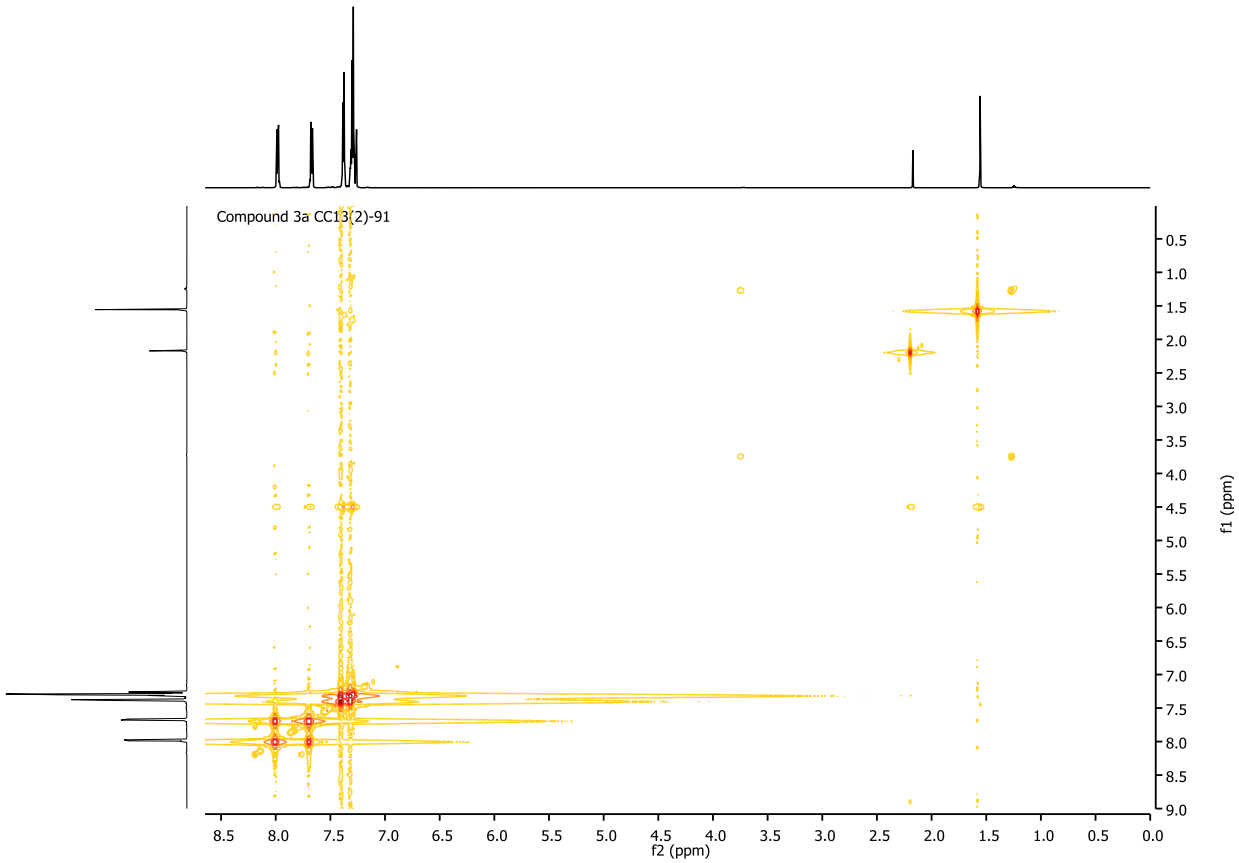
COMPOUND 3a



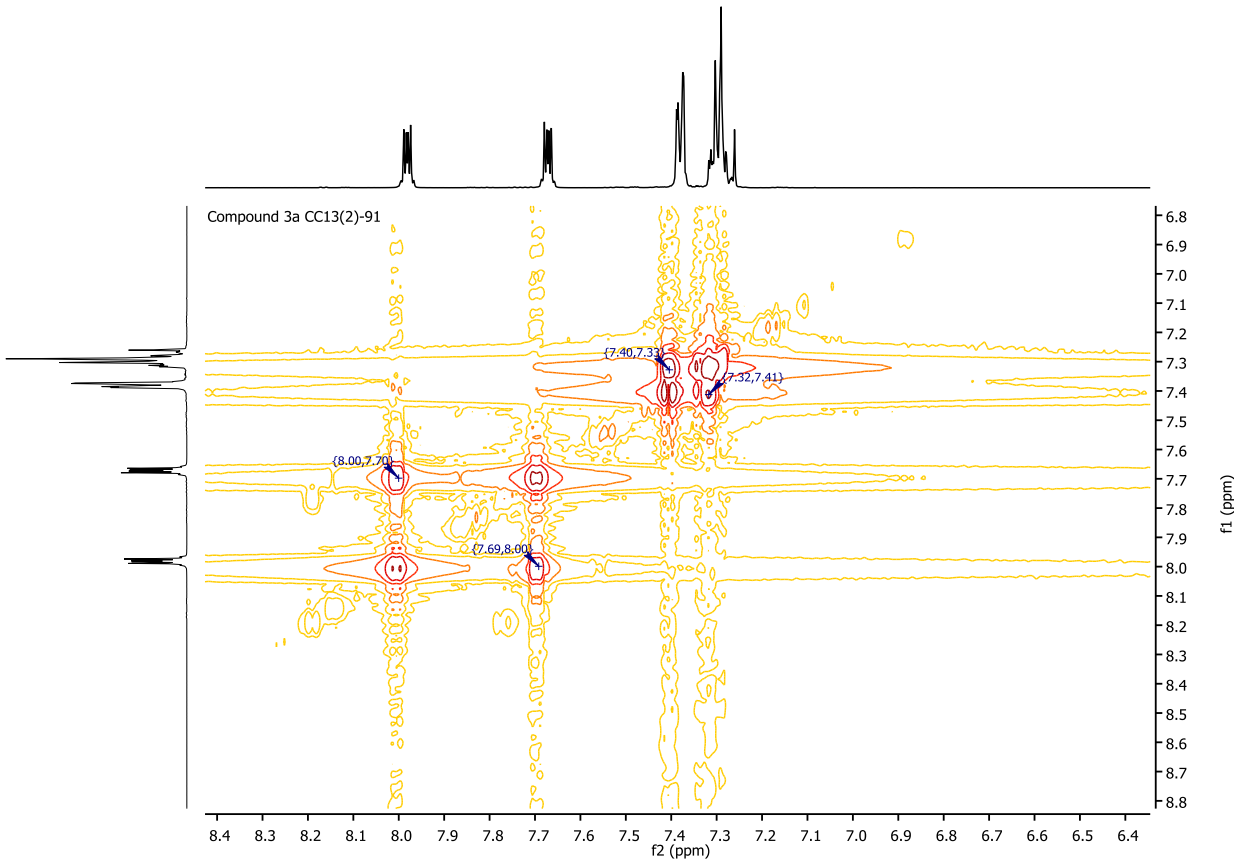
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2,3-bis(phenylthio)-1,4-naphthoquinone **3a**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with thiophenol. Expansion of the aromatic region is shown in the off-set plot.



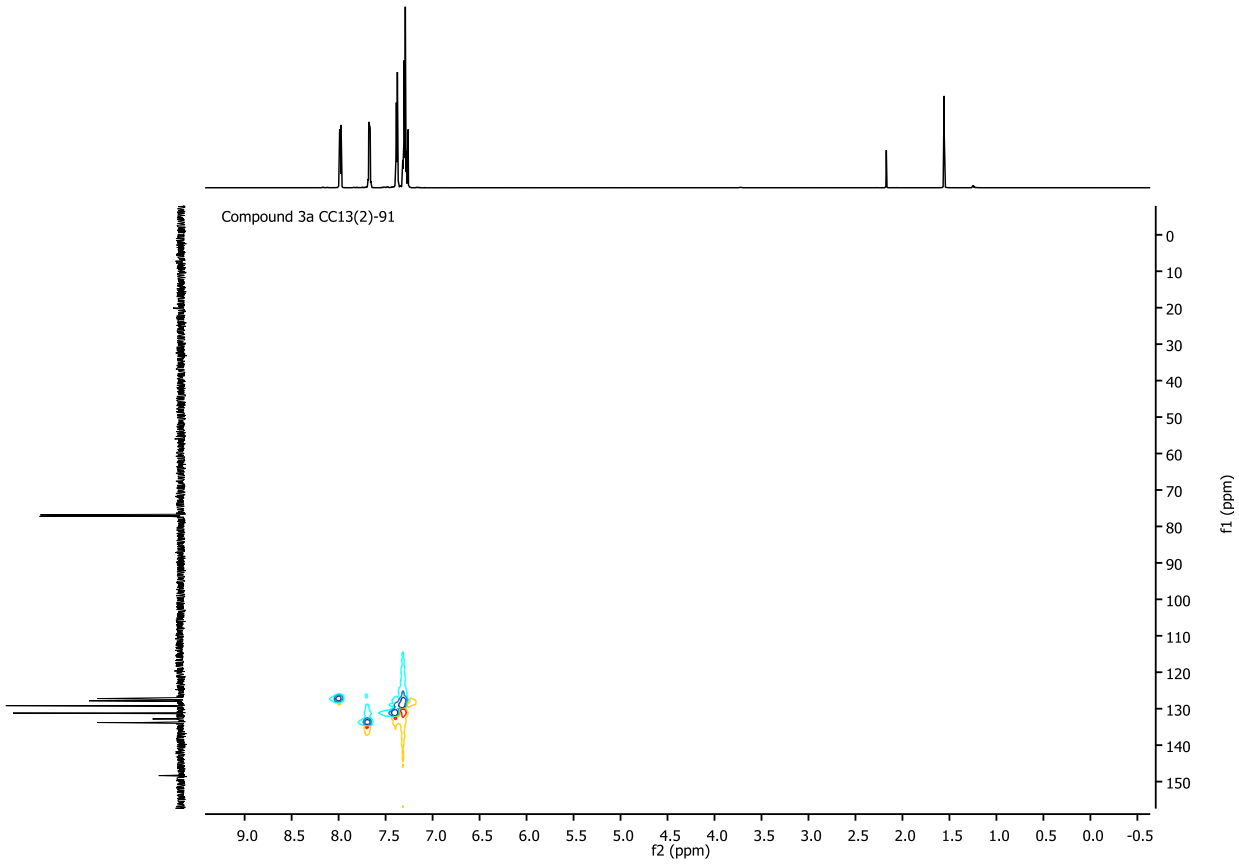
^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2,3-bis(phenylthio)-1,4-naphthoquinone **3a**. Expansion of the signals between 127 ppm and 134 ppm are shown in the off-set plot.



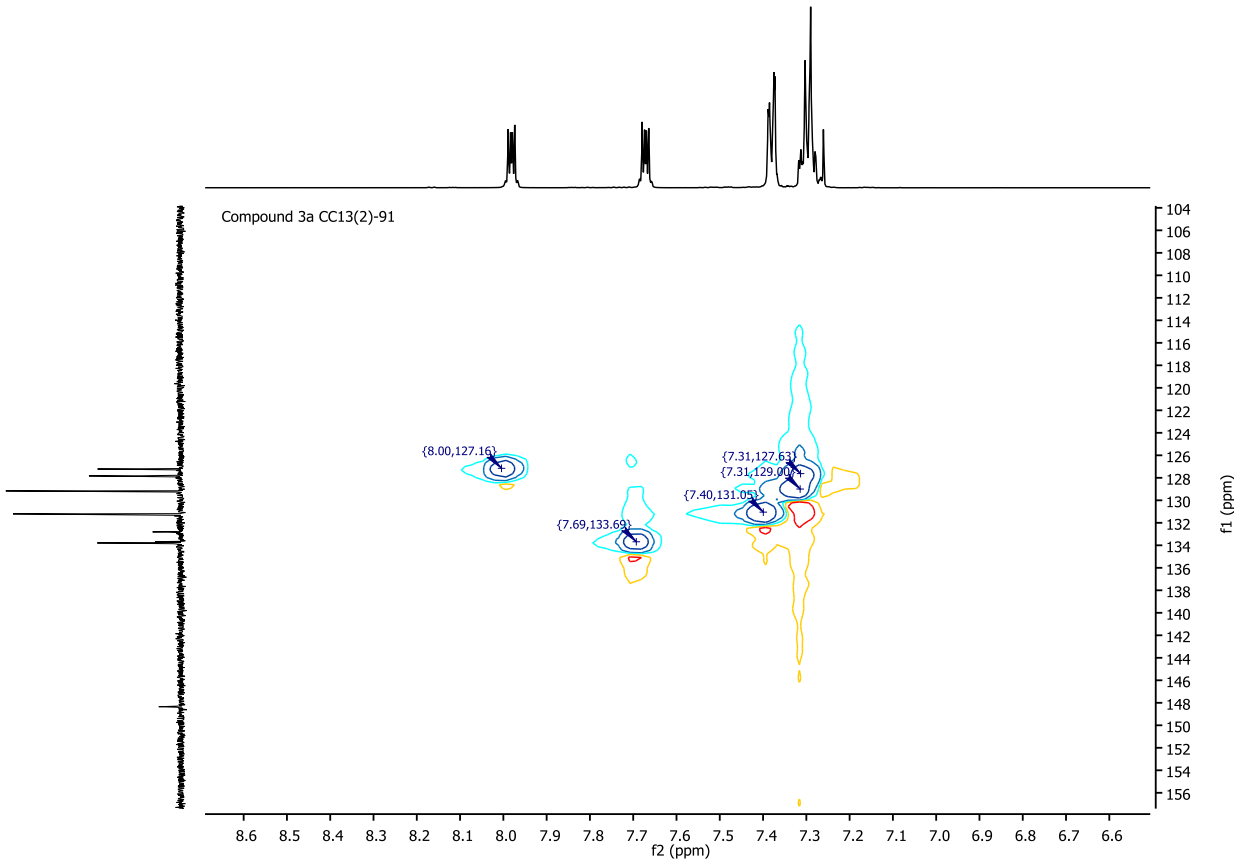
Full COSY NMR spectrum of 2,3-bis(phenylthio)-1,4-naphthoquinone **3a**.



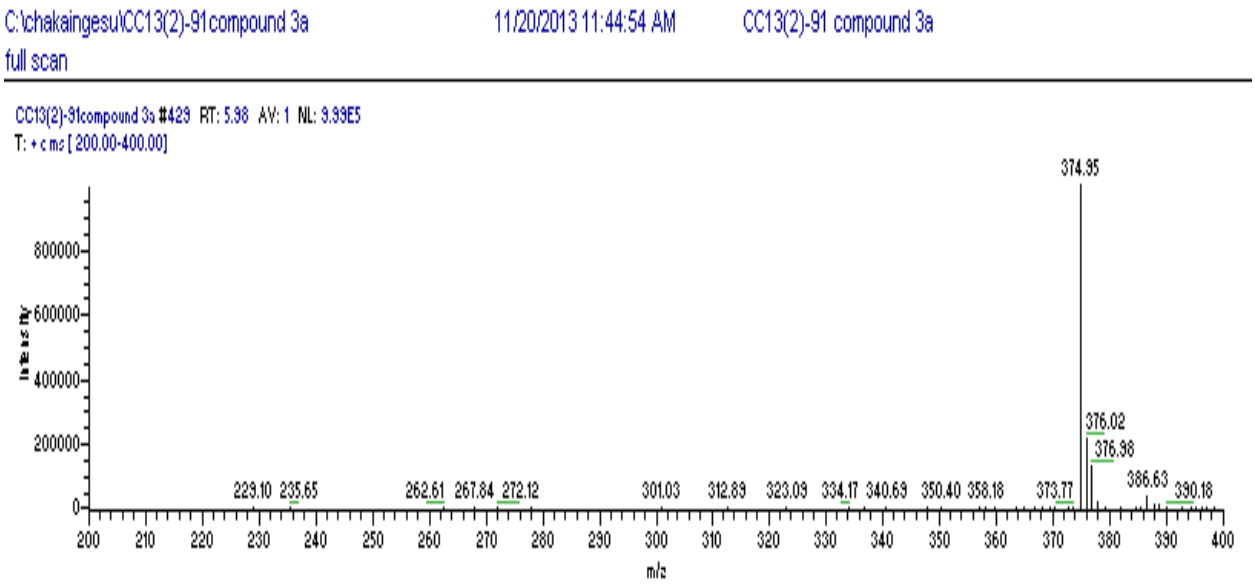
Expansion of the COSY NMR spectrum of 2,3-bis(phenylthio)-1,4-naphthoquinone **3a**.



Full of the HSQC NMR spectrum of 2,3-bis(phenylthio)-1,4-naphthoquinone **3a**.

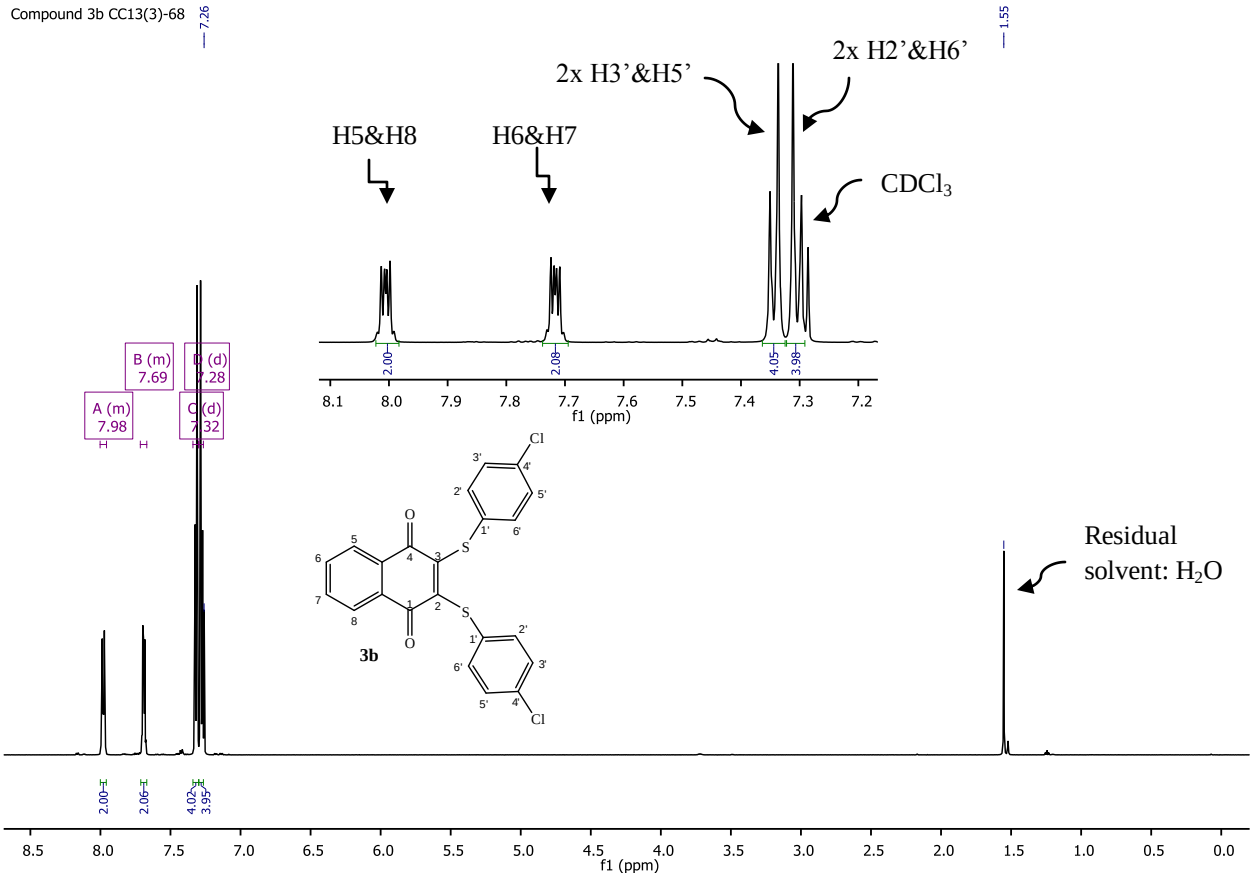


Expansion of the HSQC NMR spectrum of 2,3-bis(phenylthio)-1,4-naphthoquinone **3a**.

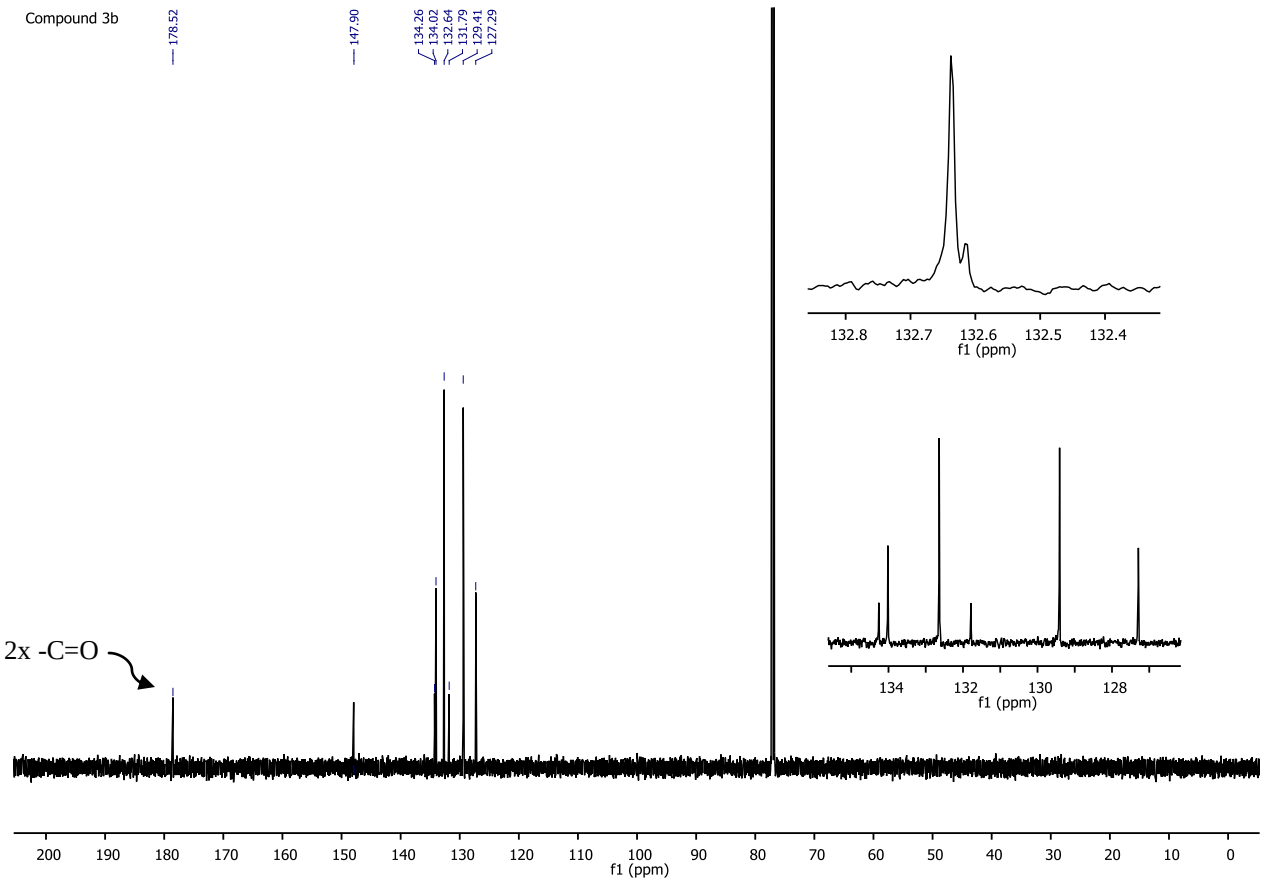


HPLC-ESI-MS spectrum of 2,3-bis(phenylthio)-1,4-naphthoquinone **3a**.

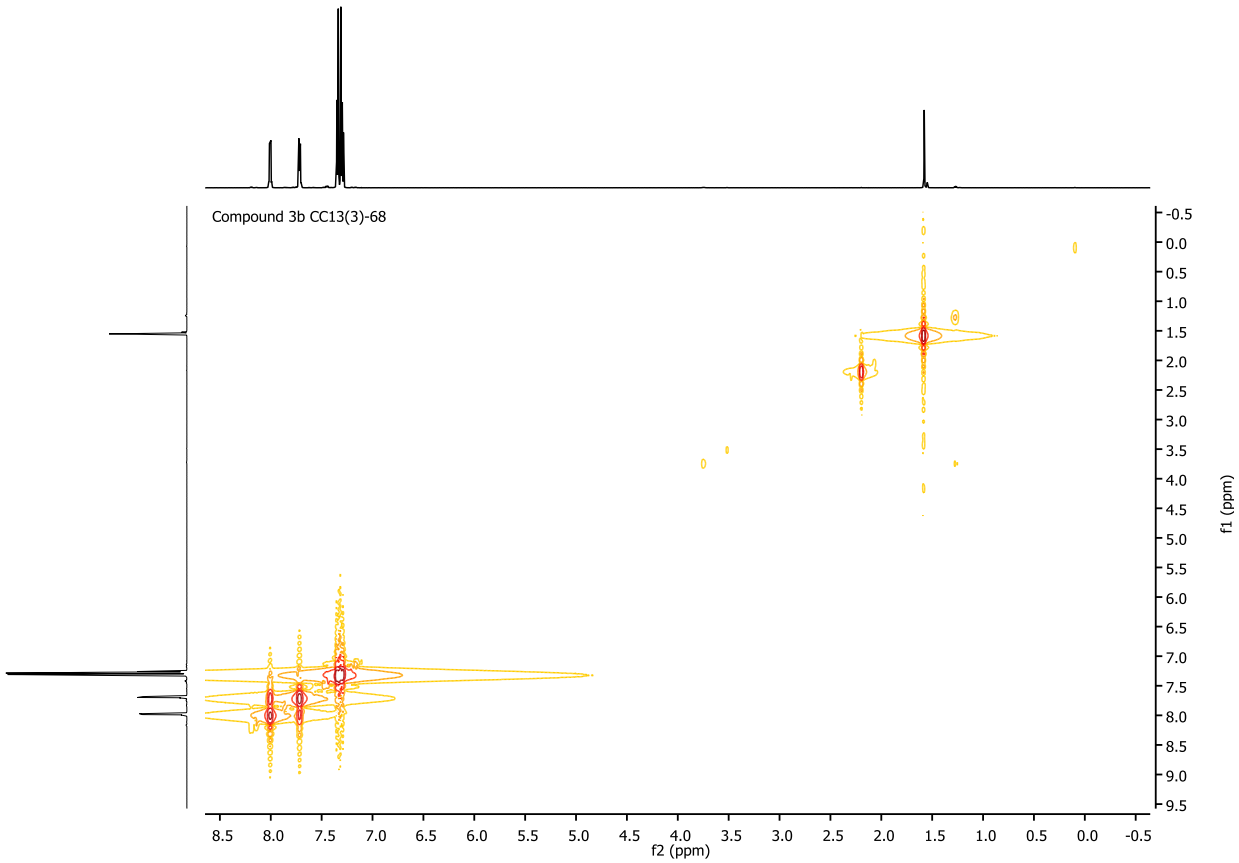
COMPOUND 3b



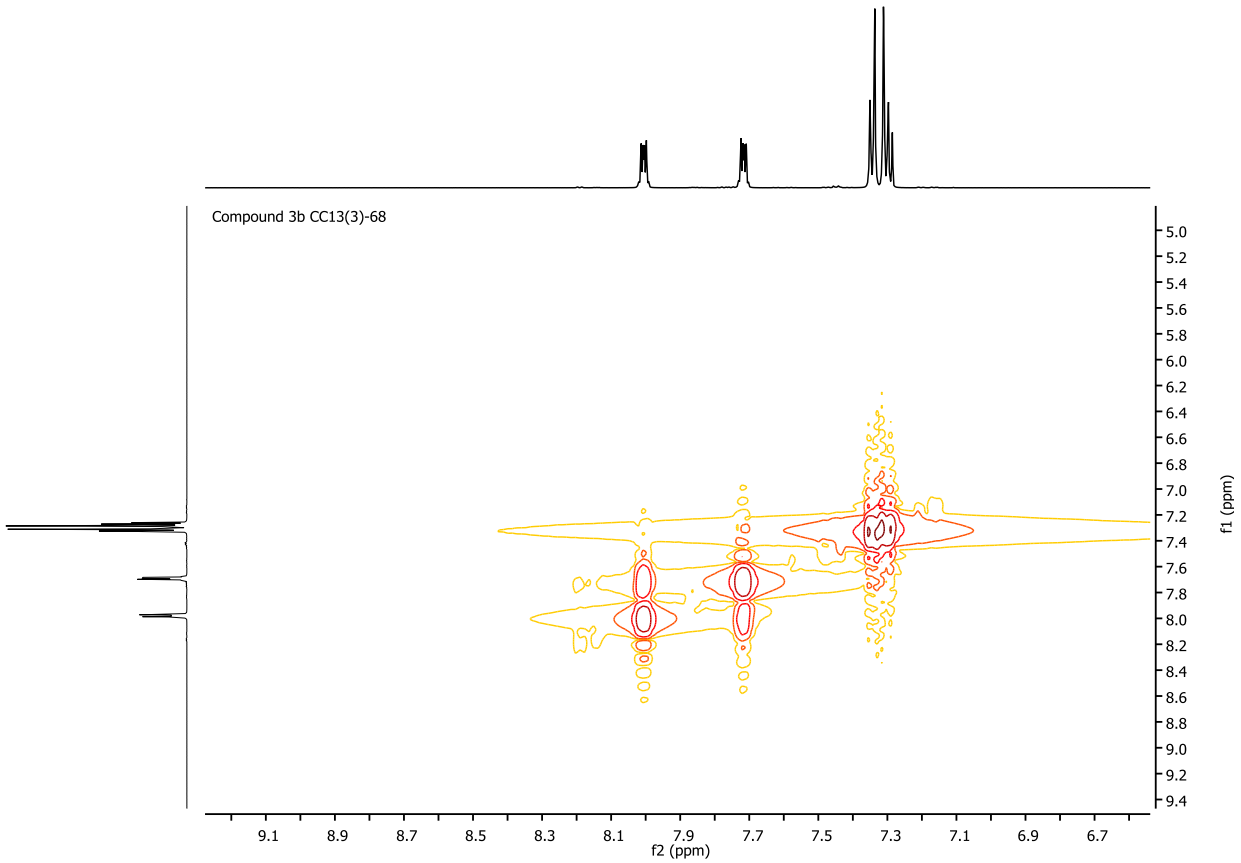
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2,3-bis(4-chlorophenylthio)-1,4-naphthoquinone **3b**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 4-chlorothiophenol. Expansion of the aromatic region is shown in the off-set plot.



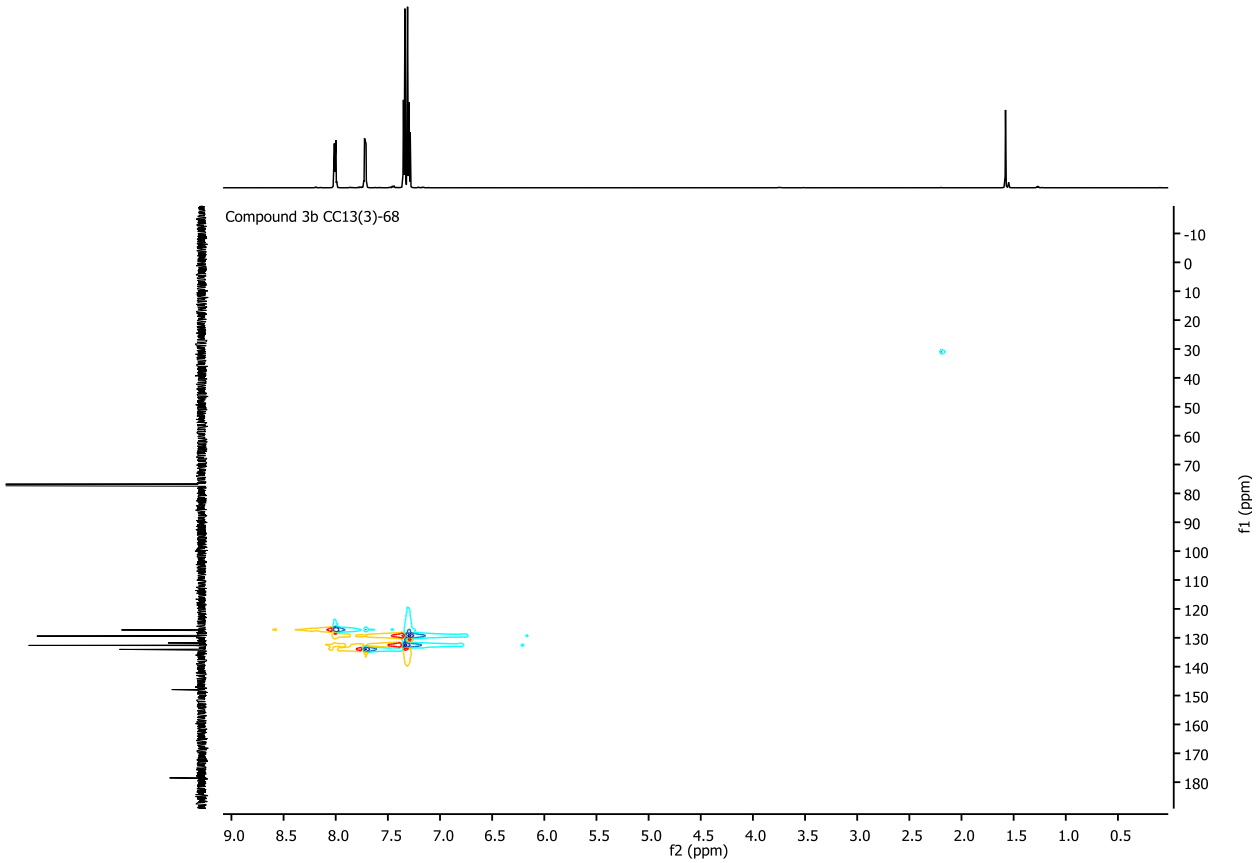
^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2,3-bis(4-chlorophenylthio)-1,4-naphthoquinone **3b**. Expansion of the signals between 124 ppm and 135 ppm are shown in the first off-set plot. The second of-set plot is highlighting the quaternary carbon signal at 132.6 ppm.



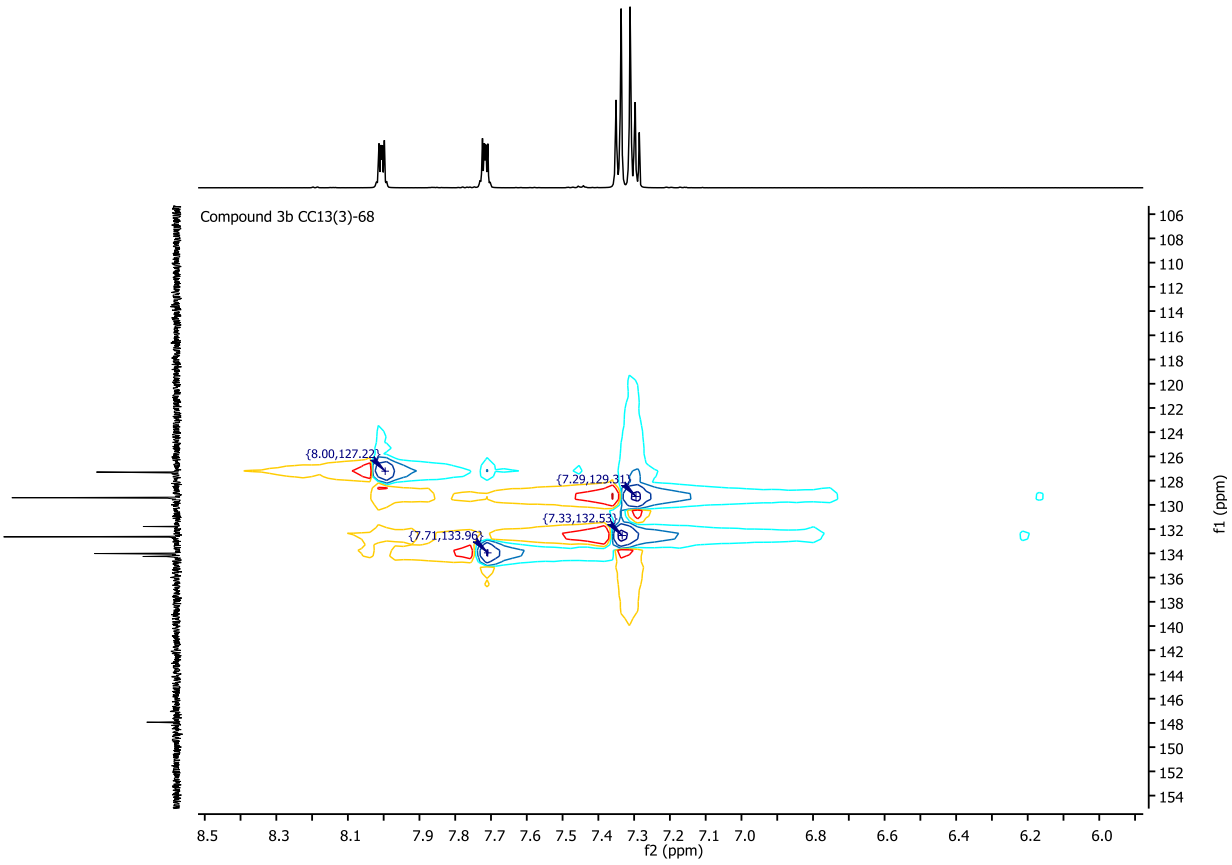
Full COSY NMR spectrum of 2,3-bis(4-chlorophenylthio)-1,4-naphthoquinone **3b**.



Expansion of the COSY NMR spectrum of 2,3-bis(4-chlorophenylthio)-1,4-naphtho-quinone **3b**.

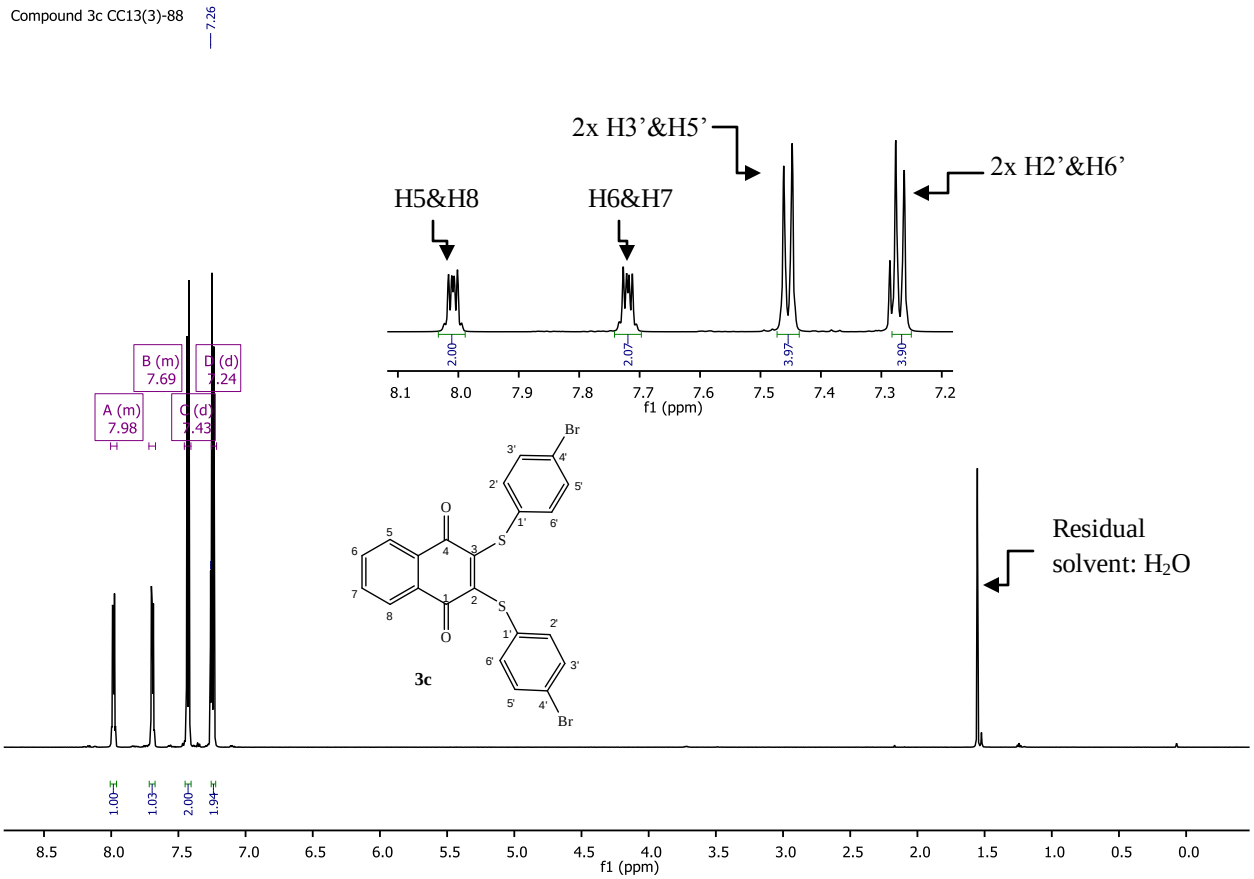


Full HSQC NMR spectrum of 2,3-bis(4-chlorophenylthio)-1,4-naphthoquinone **3b**.

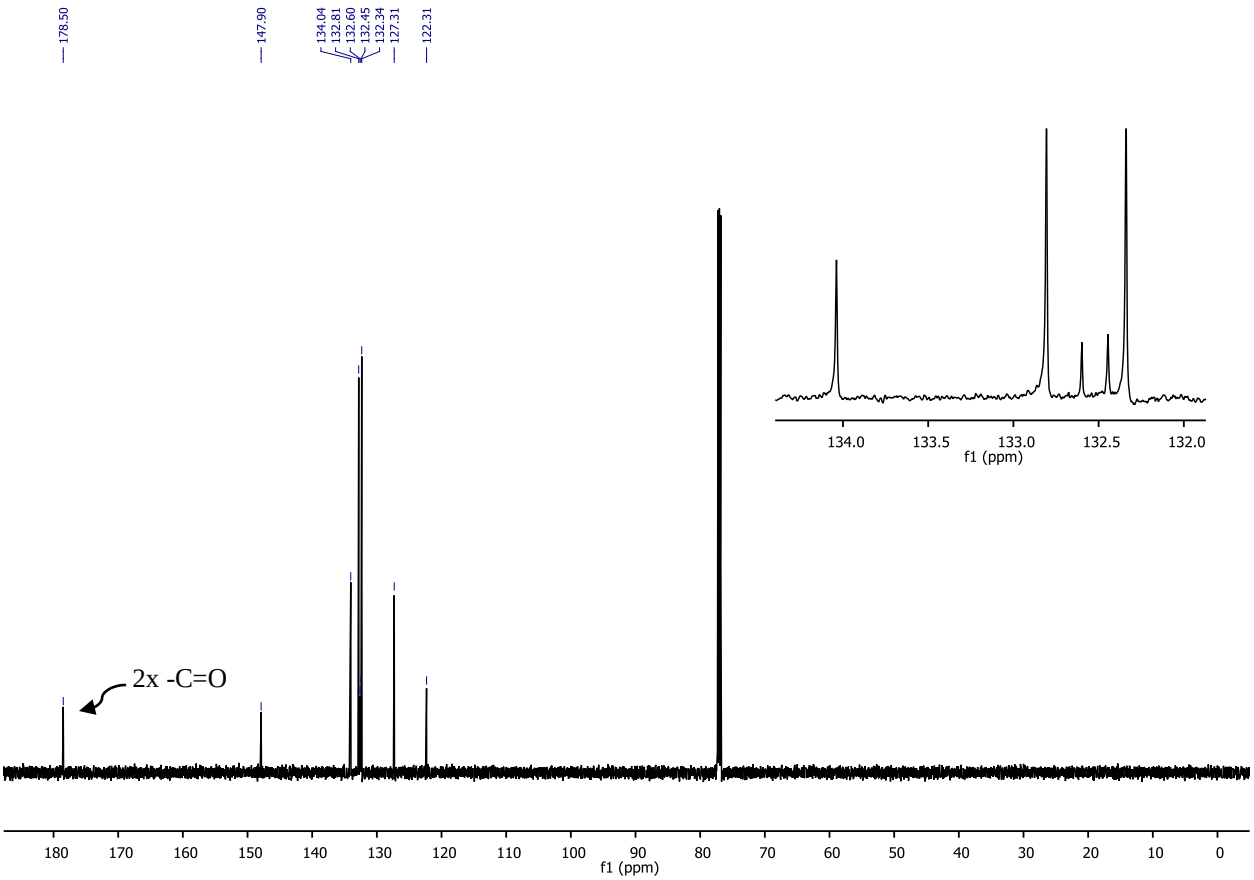


Expansion of the HSQC NMR spectrum of 2,3-bis(4-chlorophenylthio)-1,4-naphtho-quinone **3b**.

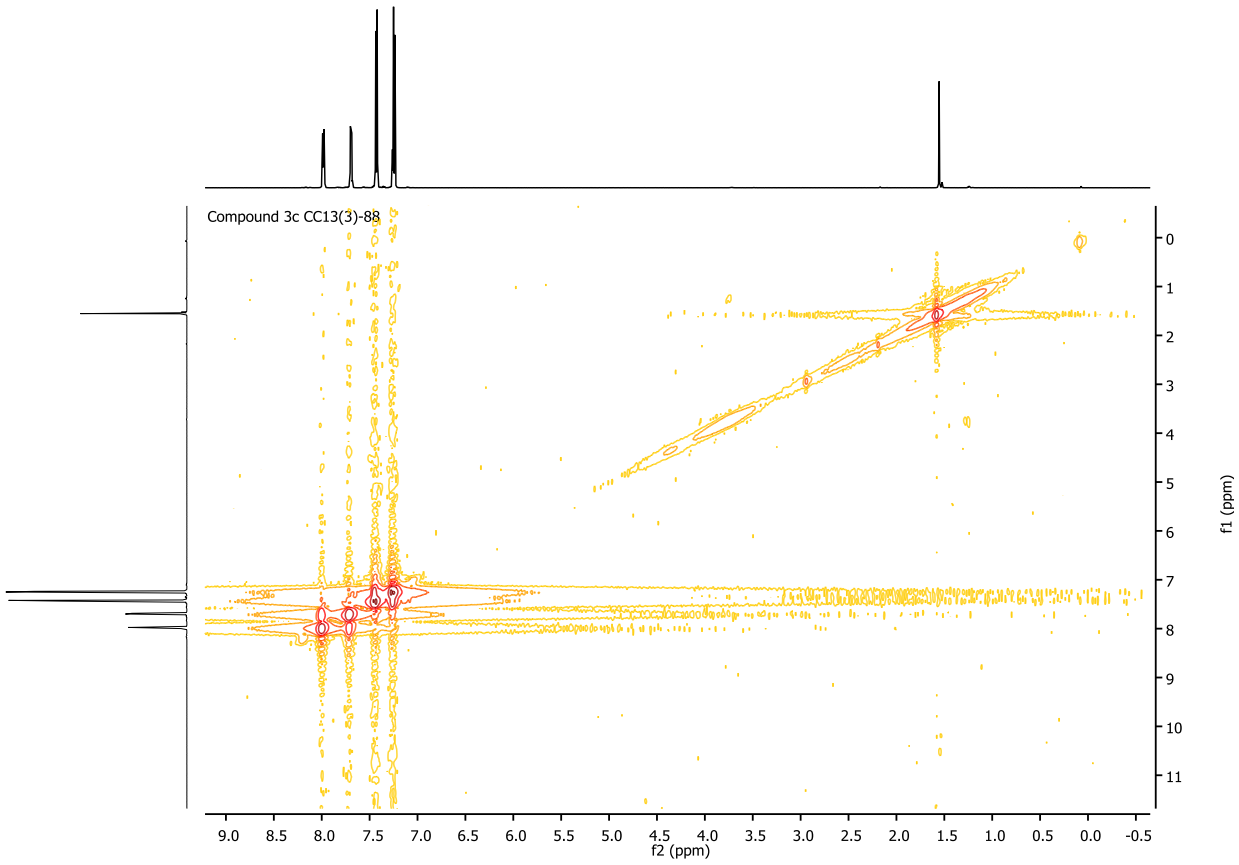
COMPOUND 3c



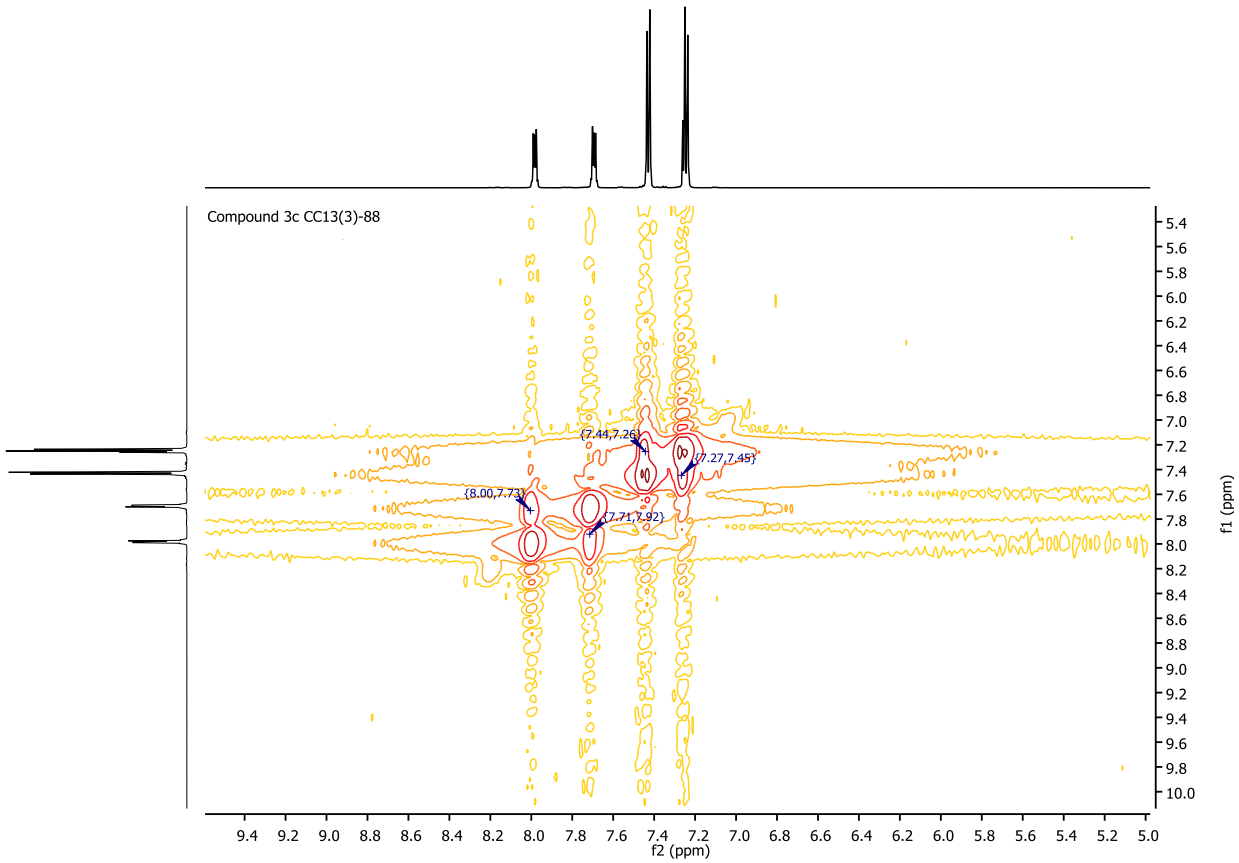
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2,3-bis(4-bromophenylthio)-1,4-naphthoquinone **3c**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 4-bromo-thiophenol. Expansion of the aromatic region is shown in the off-set plot.



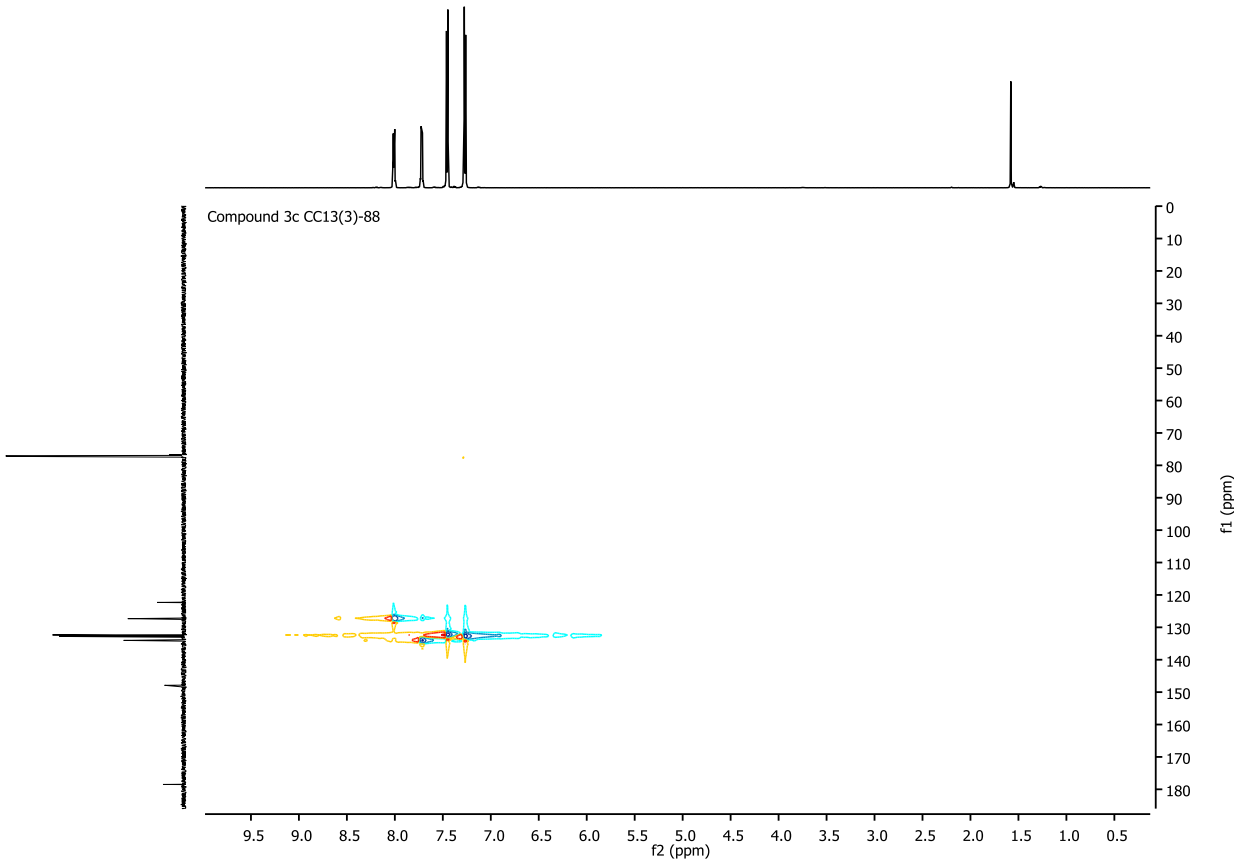
^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2,3-bis(4-bromophenylthio)-1,4-naphthoquinone **3c**. Expansion of the signals between 132 ppm and 134 ppm are shown in the off-set plot.



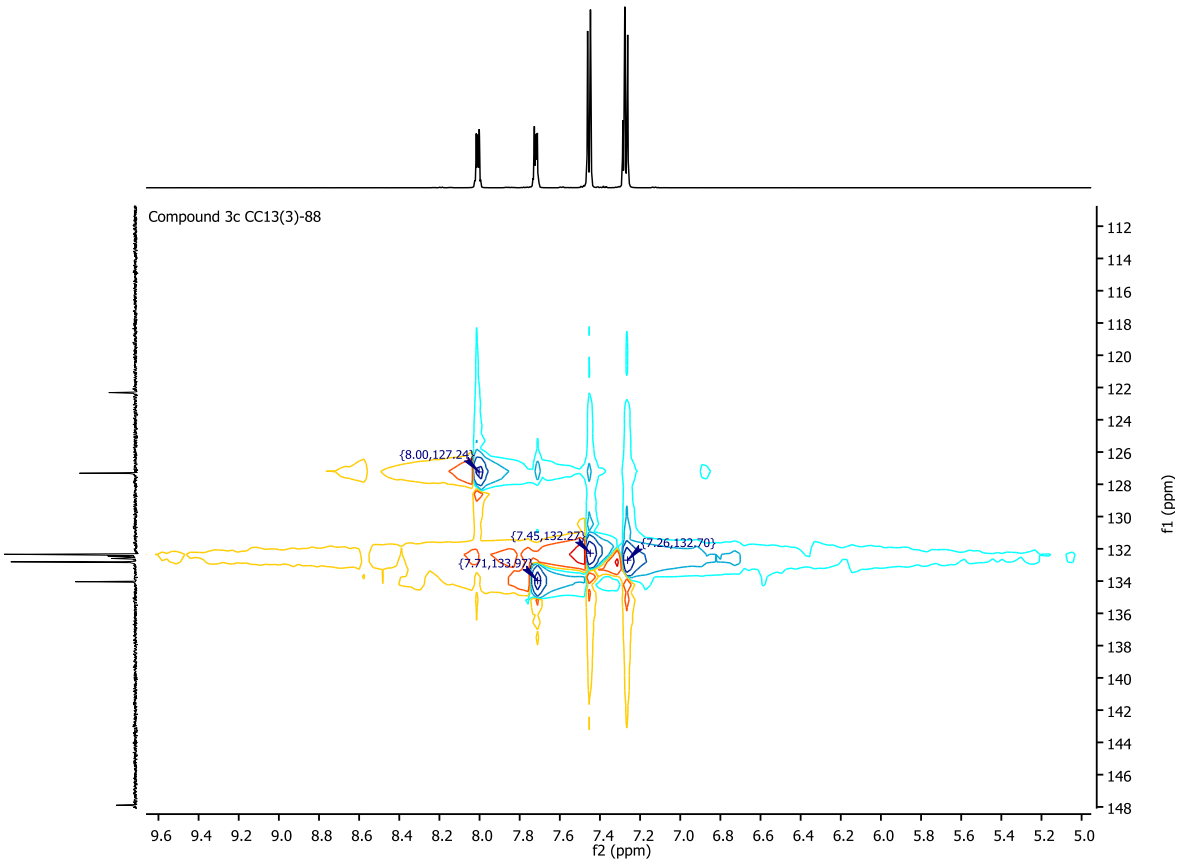
Full COSY spectrum of 2,3-bis(4-bromophenylthio)-1,4-naphthoquinone **3c**.



Expansion of the COSY spectrum of 2,3-bis(4-bromophenylthio)-1,4-naphthoquinone **3c**.

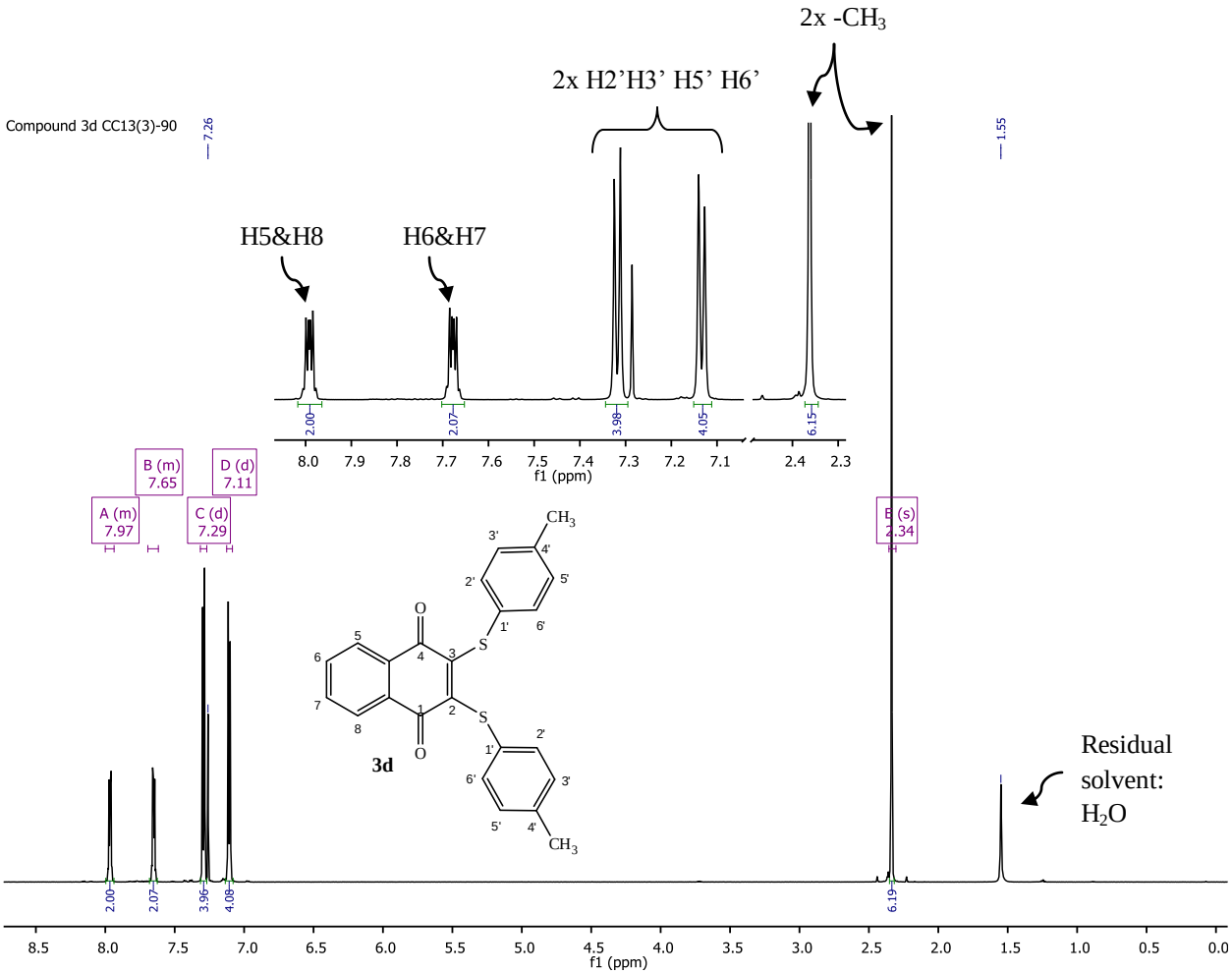


Full HSQC spectrum of 2,3-bis(4-bromophenylthio)-1,4-naphthoquinone **3c**.

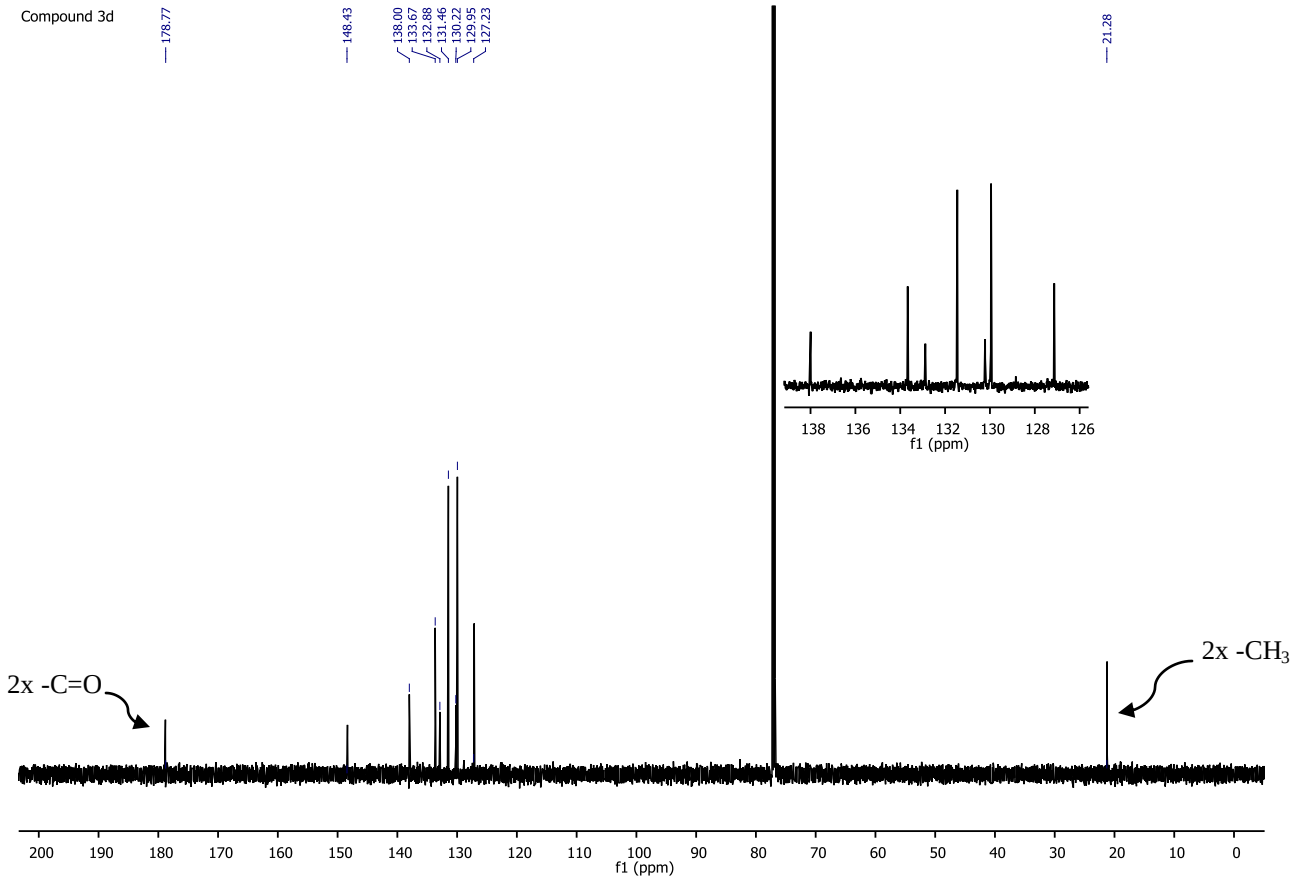


Expansion of the HSQC spectrum of 2,3-bis(4-bromophenylthio)-1,4-naphthoquinone **3c**.

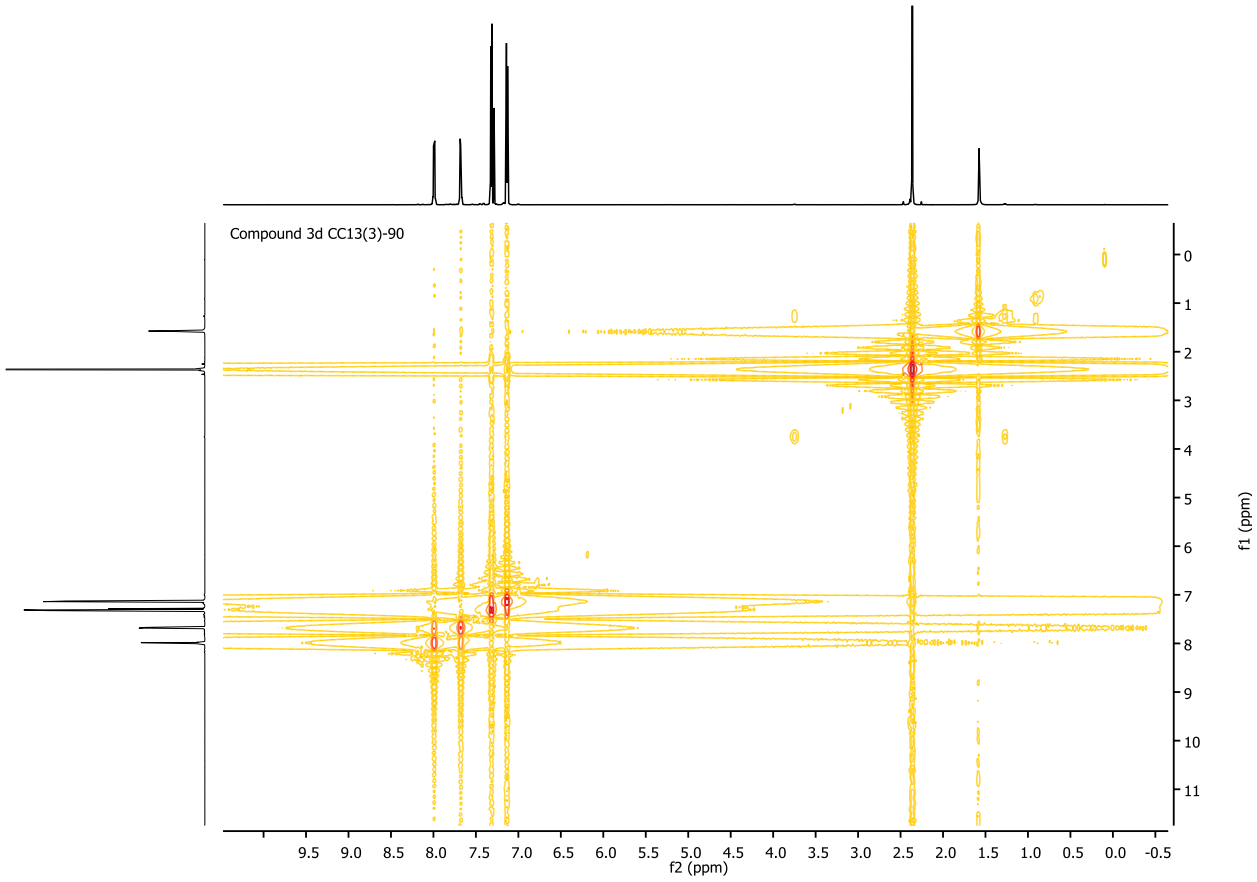
COMPOUND 3d



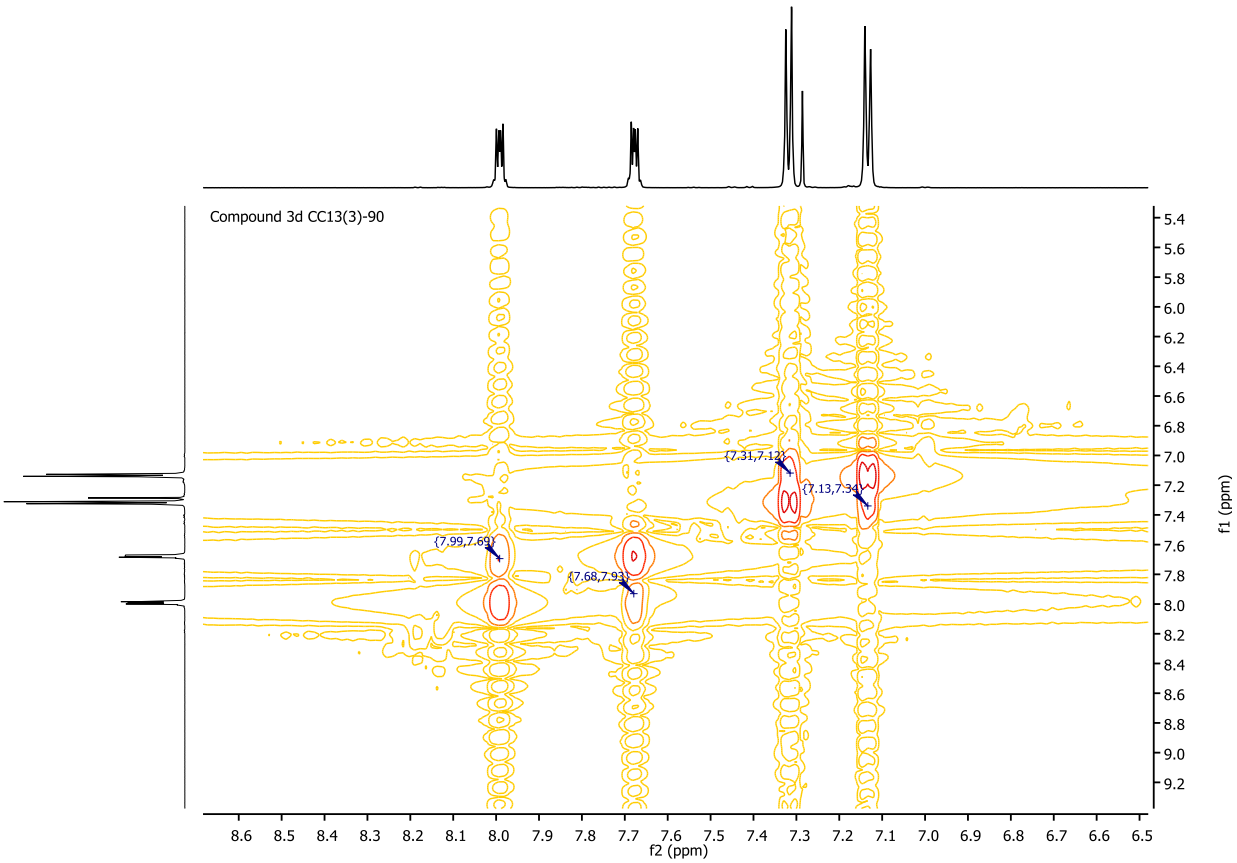
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2,3-bis(p-tolylthio)-1,4-naphthoquinone **3d**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 4-methylthiophenol. Expansion of the aromatic region is shown in the off-set plot.



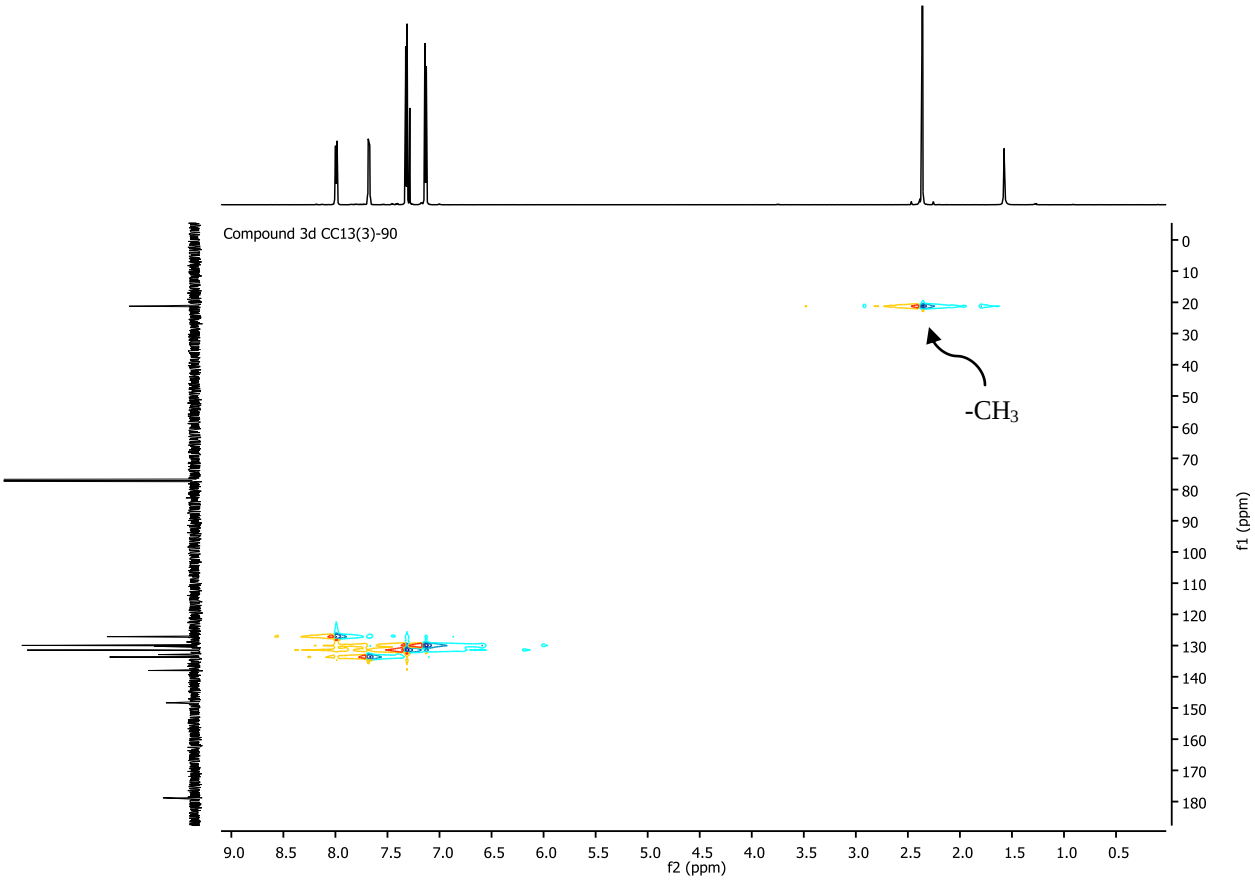
^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2,3-bis(p-tolylthio)-1,4-naphthoquinone **3d**. Expansion of the signals between 126 ppm and 138 ppm are shown in the off-set plot.



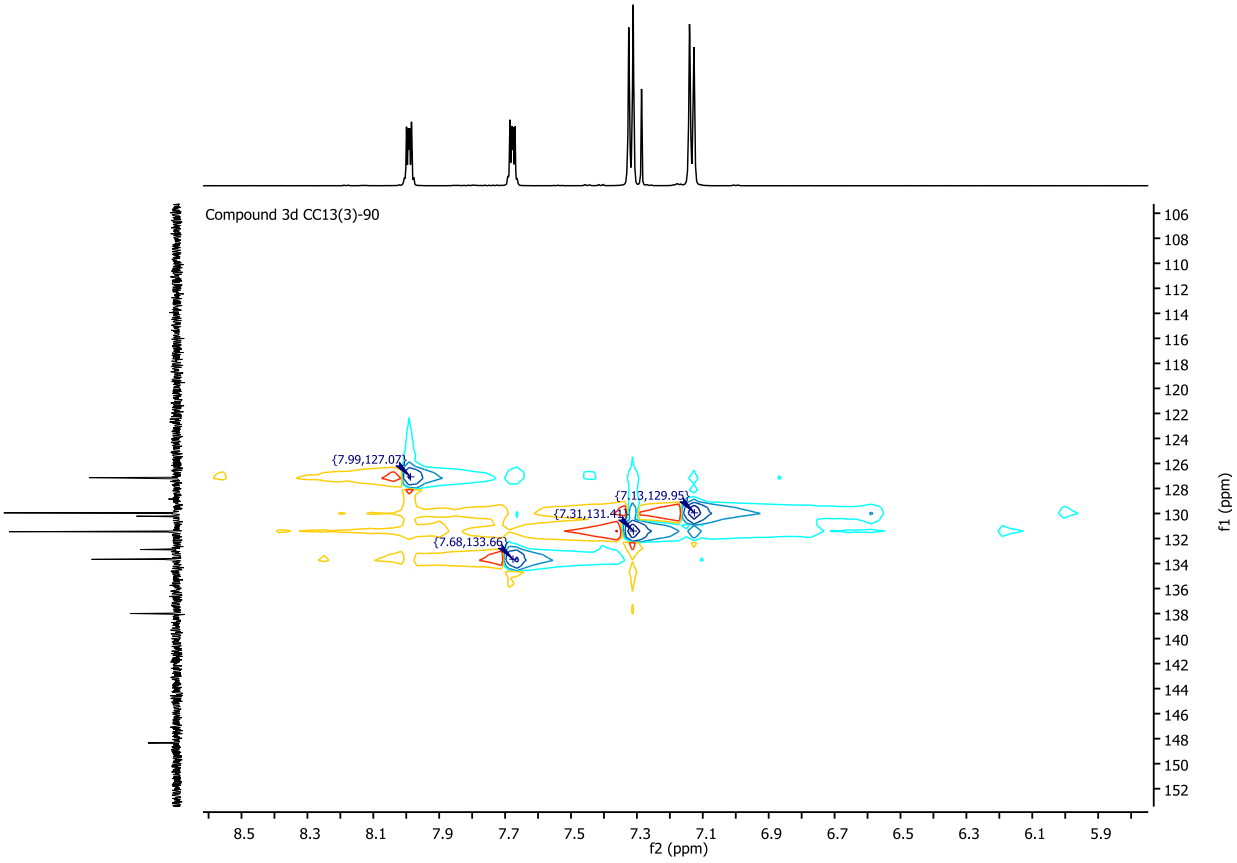
Full COSY NMR spectrum of 2,3-bis(p-tolylthio)-1,4-naphthoquinone **3d**.



Expansion of the COSY NMR spectrum of 2,3-bis(p-tolylthio)-1,4-naphthoquinone **3d**.

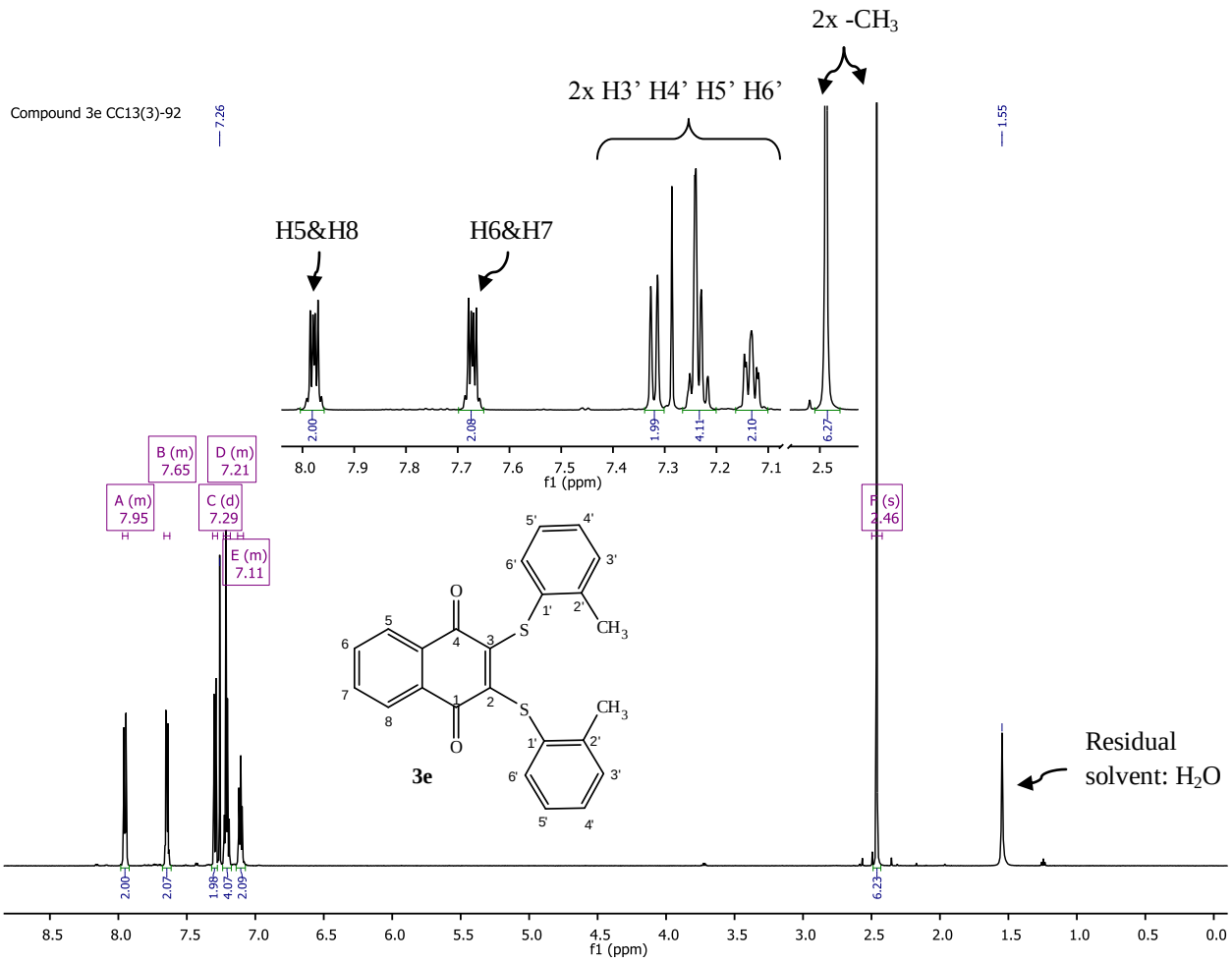


Full HSQC NMR spectrum of 2,3-bis(p-tolylthio)-1,4-naphthoquinone **3d**.

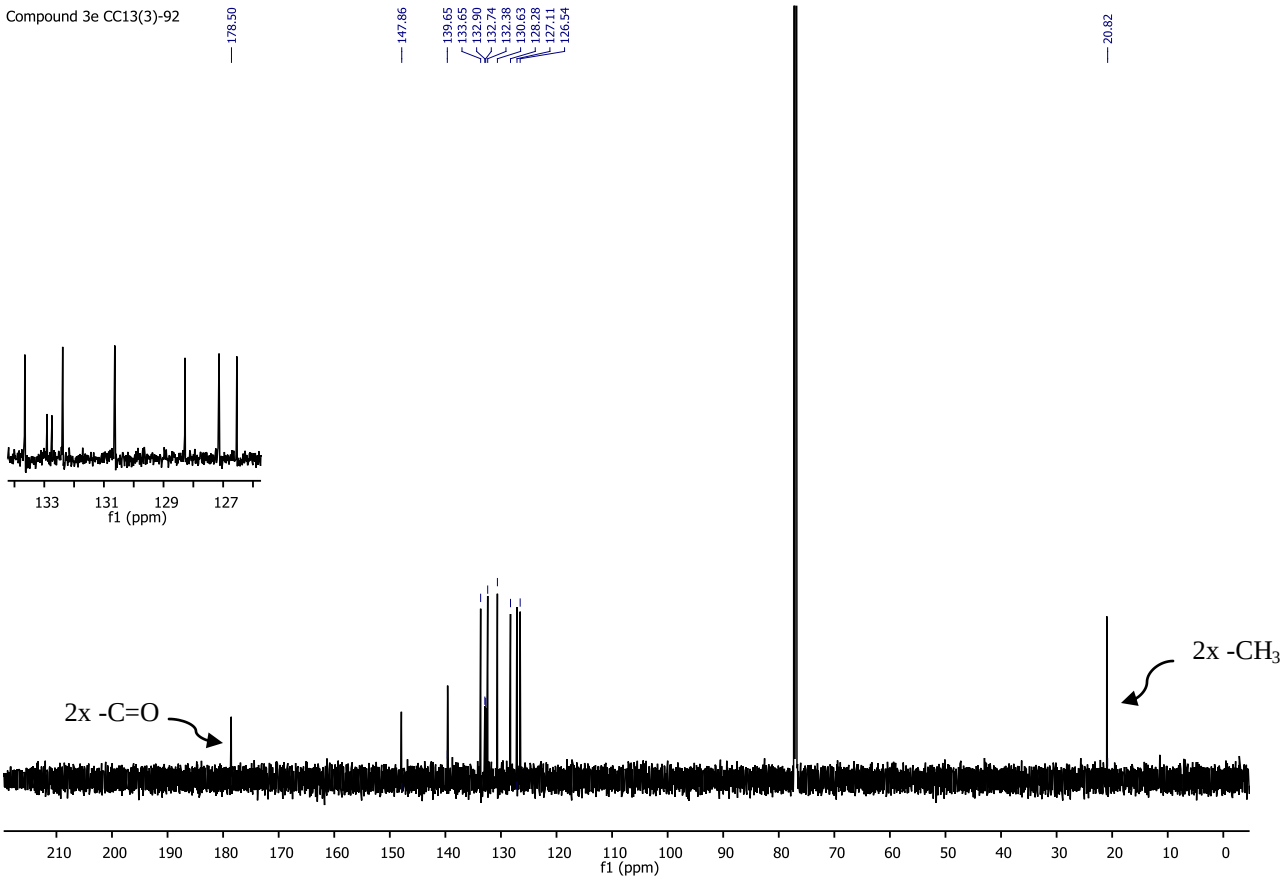


Expansion of the HSQC NMR spectrum of 2,3-bis(p-tolylthio)-1,4-naphthoquinone **3d**.

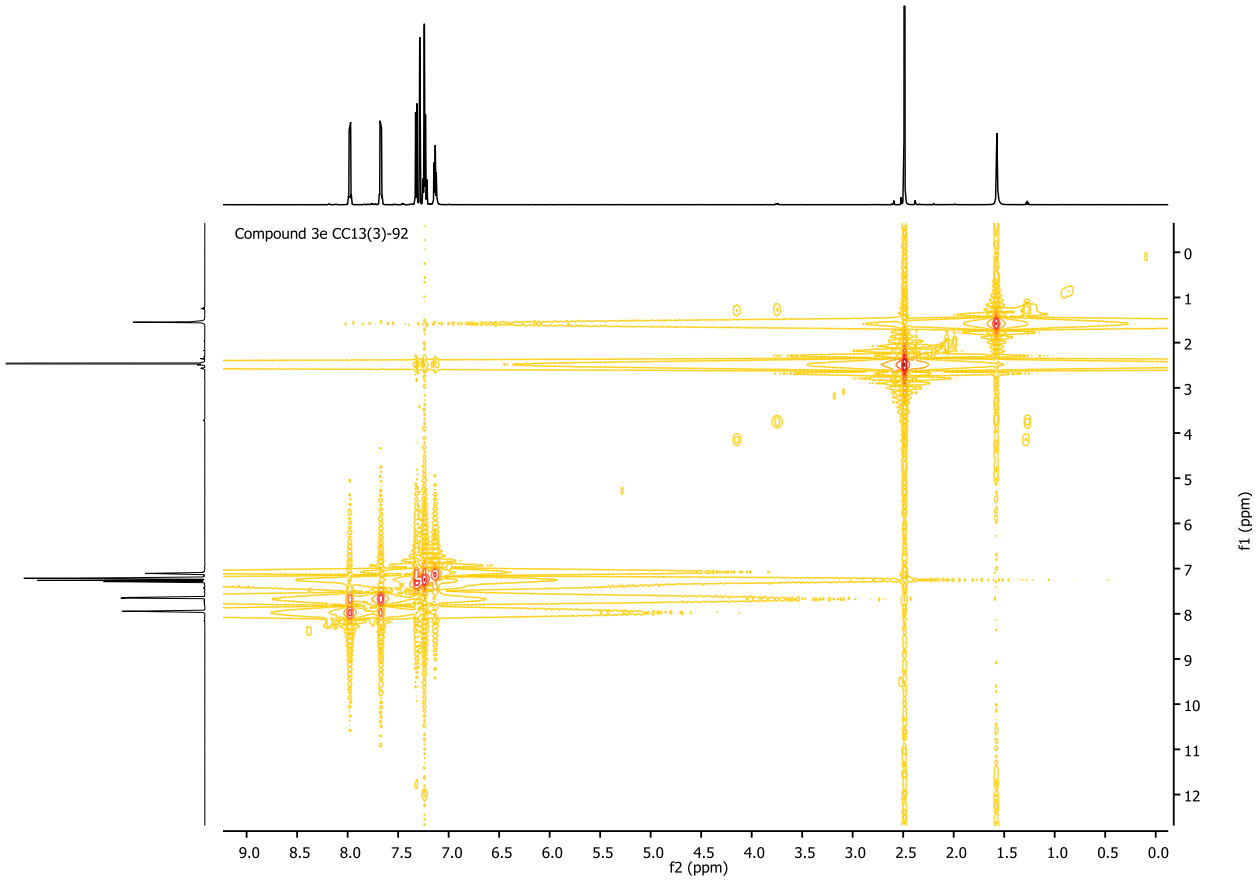
COMPOUND 3e



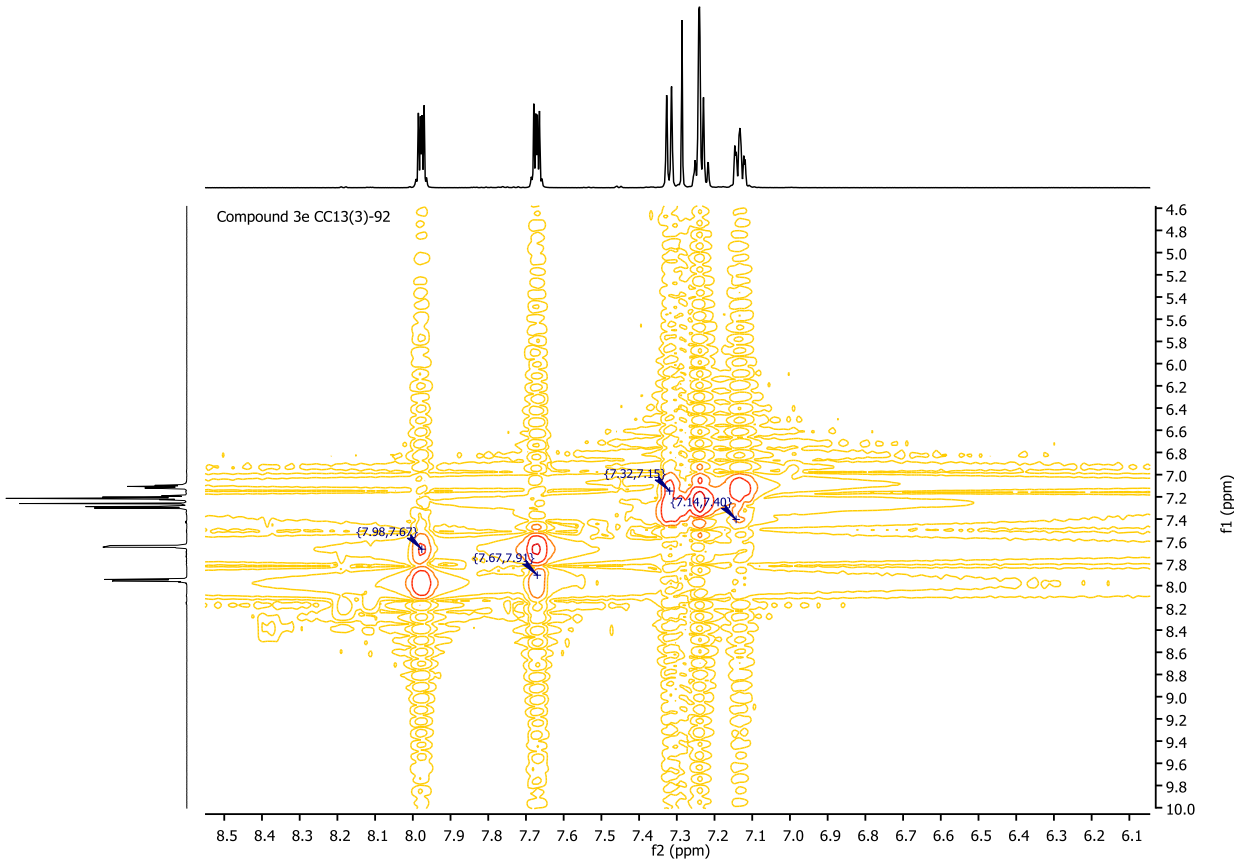
^{1}H NMR (600MHz, $CDCl_3$) spectrum of 2,3-bis(o-tolylthio)-1,4-naphthoquinone **3e**. The product was obtained from coupling 2,3-dichloro-1,4-naphthoquinone with 2-methyl-thiophenol. An expansion of the signals is shown in the off-set plot.



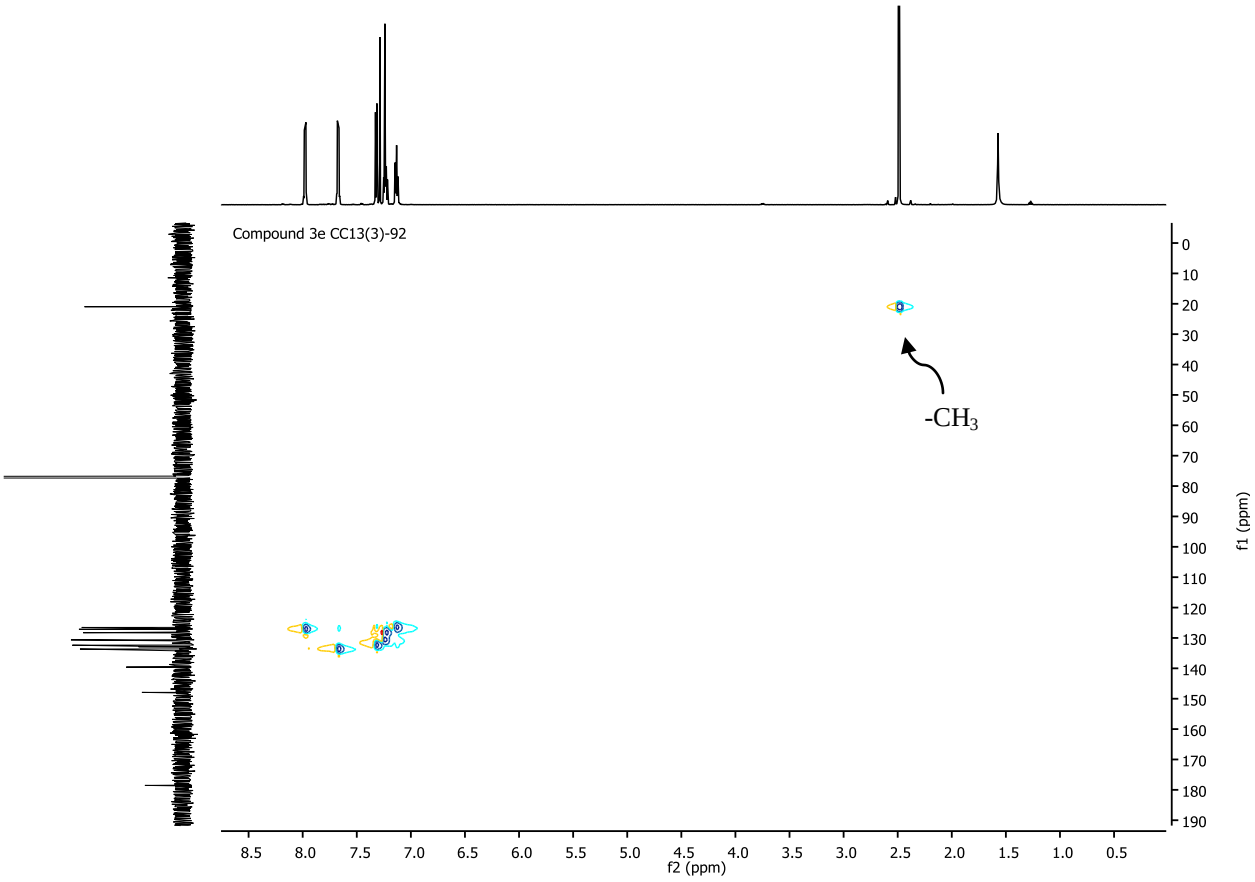
^{13}C NMR (150MHz, $CDCl_3$) spectrum of 2,3-bis(o-tolylthio)-1,4-naphthoquinone **3e**. Expansion of the signals between 126 ppm and 134 ppm are shown in the off-set plot.



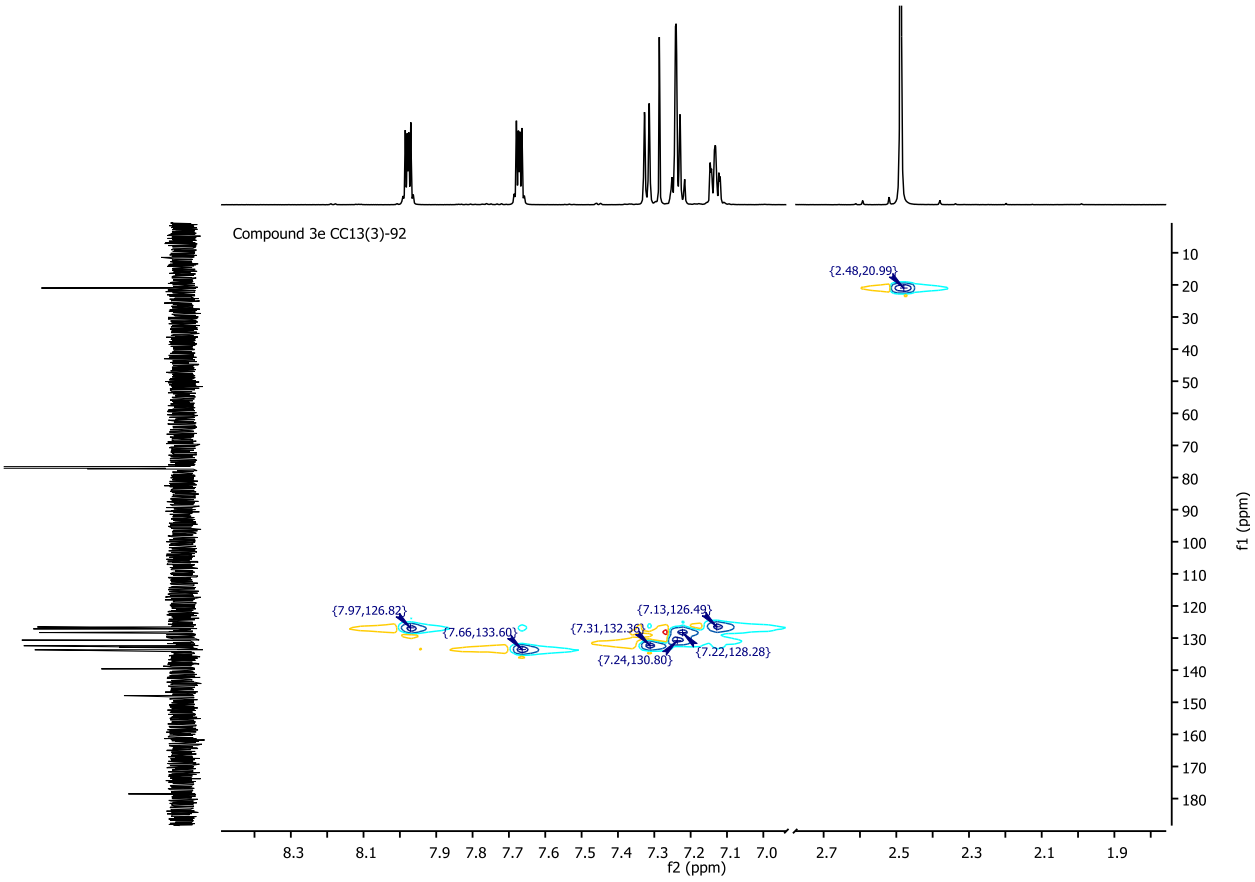
Full COSY NMR spectrum of 2,3-bis(o-tolylthio)-1,4-naphthoquinone **3e**.



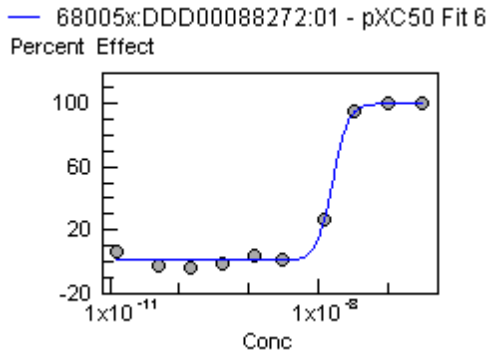
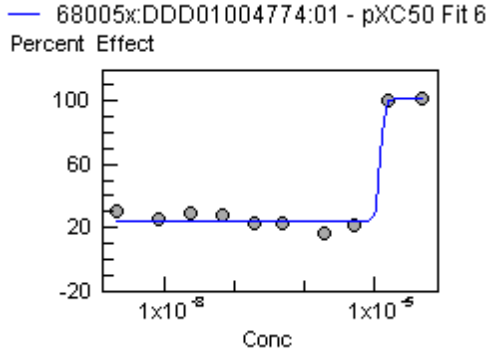
Expansion of the COSY NMR spectrum of 2,3-bis(o-tolylthio)-1,4-naphthoquinone **3e**.

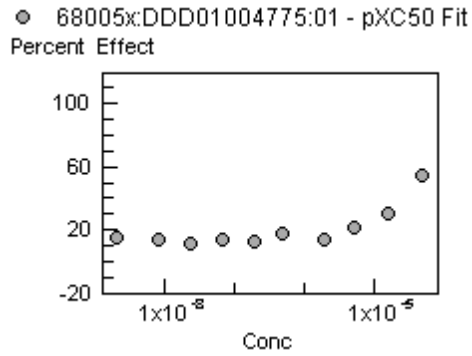
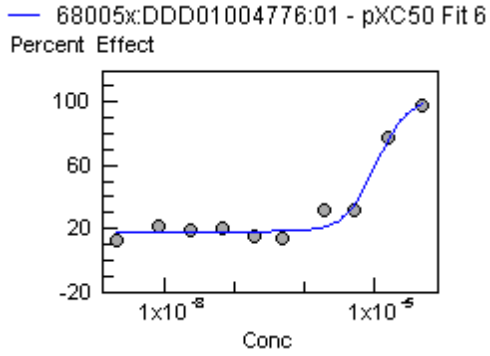


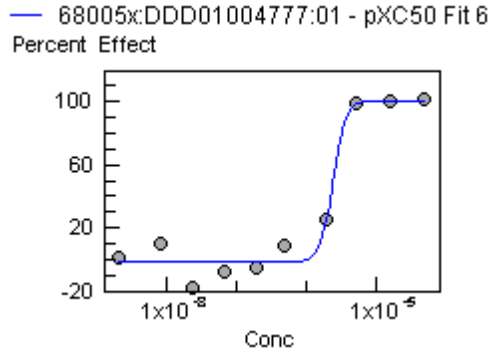
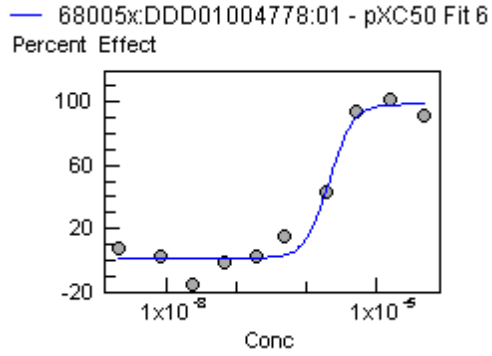
Full HSQC NMR spectrum of 2,3-bis(o-tolylthio)-1,4-naphthoquinone **3e**.

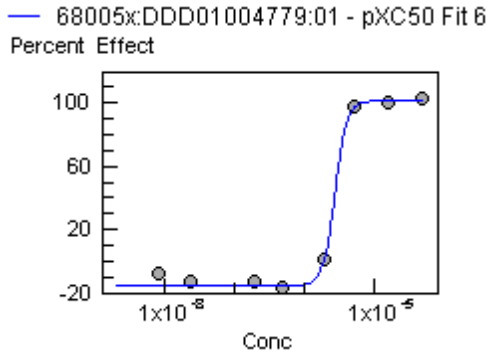
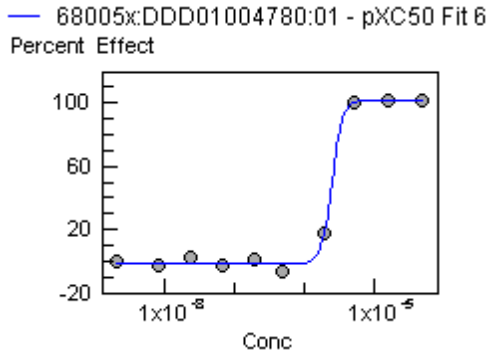


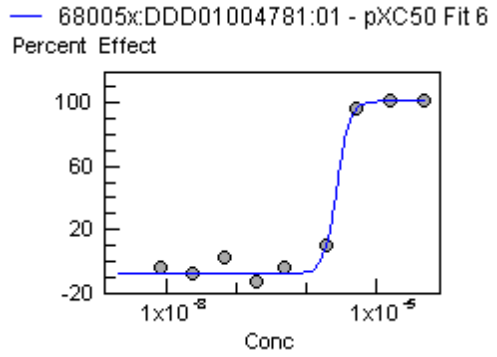
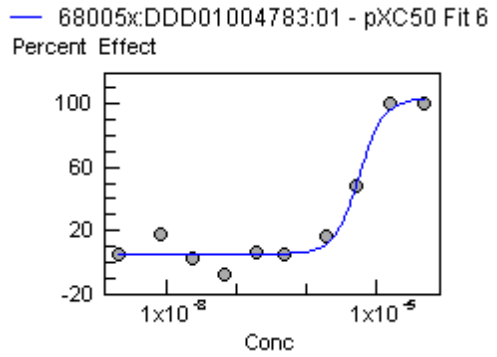
Expansion of the HSQC NMR spectrum of 2,3-bis(o-tolylthio)-1,4-naphthoquinone.

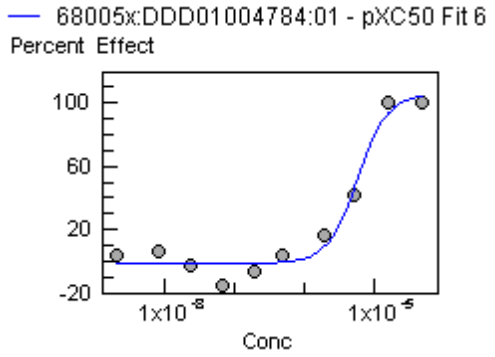
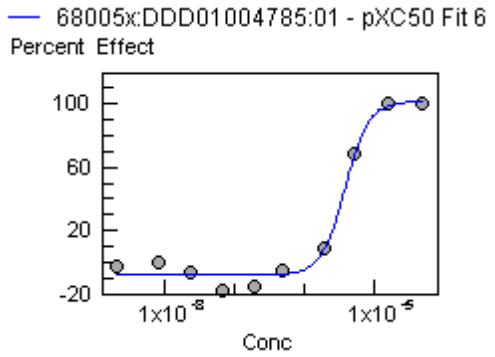
Compound Id	Supplier Id	Plate Id	pEC_{50}	Hill slope	Bottom	Top	Max Effect	Max Effect Conc	Top Confidence Interval	Chart
DDD00088272:01	Melarsoprol	68005	7.81	4.02	0.7	99.9	100.06	9.85E-08	6.07	
DDD01004774:01	CC13(3)-24 **1a**	68005	4.9	15.38	24.4	101.2	101.19	4.98E-05	12.07	

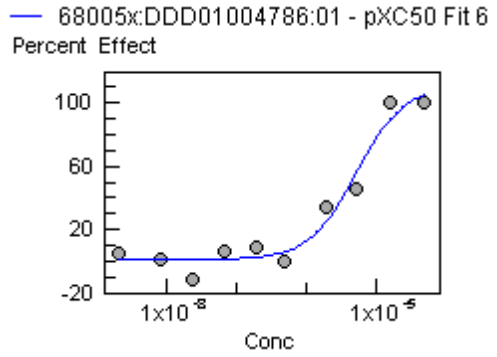
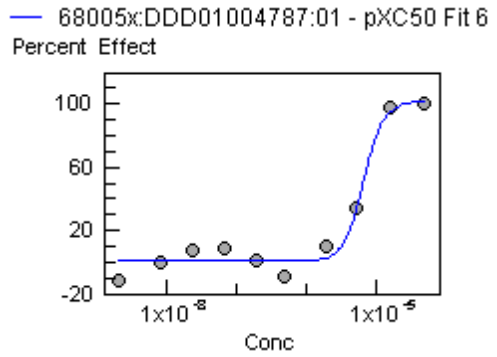
Compound Id	Supplier Id	Plate Id	pEC_{50}	Hill slope	Bottom	Top	Max Effect	Max Effect Conc	Top Confidence Interval	Chart
DDD01004775:01	CC13(3)-6 **1b**	68005	<4.3				54.88	4.98E-05	1224.55	
DDD01004776:01	CC13(3)-11 **1c**	68005	4.96	1.92	18.1	103.1	97.98	4.98E-05	22.77	

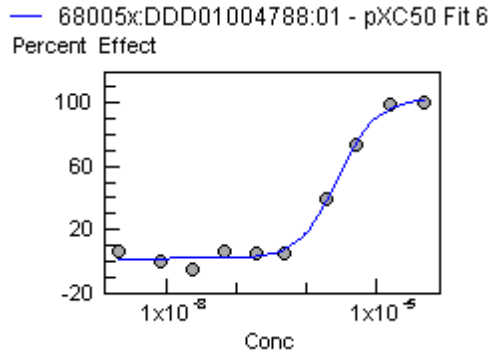
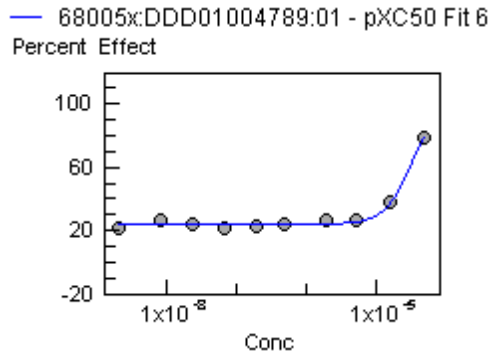
Compound Id	Supplier Id	Plate Id	pEC_{50}	Hill slope	Bottom	Top	Max Effect	Max Effect Conc	Top Confidence Interval	Chart
DDD01004777:01	CC13(3)-15 **1d**	68005	5.61	4.93	-1.7	100.9	101.06	4.98E-05	17.13	
DDD01004778:01	CC13(3)-40 **1e**	68005	5.67	2.59	1.1	98.5	102.15	1.64E-05	17.07	

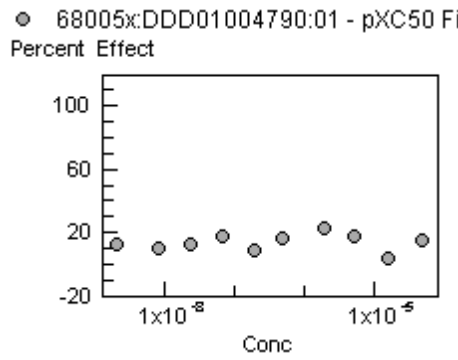
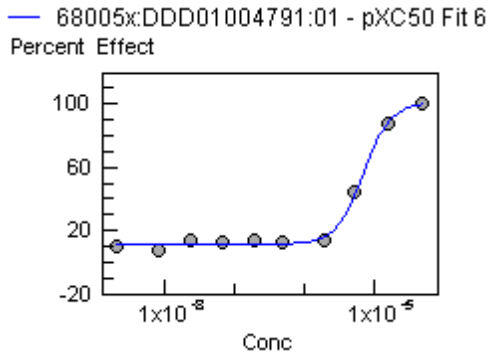
Compound Id	Supplier Id	Plate Id	pEC_{50}	Hill slope	Bottom	Top	Max Effect	Max Effect Conc	Top Confidence Interval	Chart
DDD01004779:01	CC13(3)-46 **1f**	68005	5.55	5.18	-15.5	101.3	102.36	4.98E-05	8.52	
DDD01004780:01	CC13(3)-53 **1g**	68005	5.6	6.08	-1.5	101.6	101.97	4.98E-05	5.4	

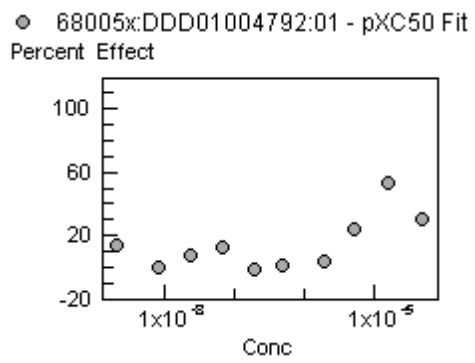
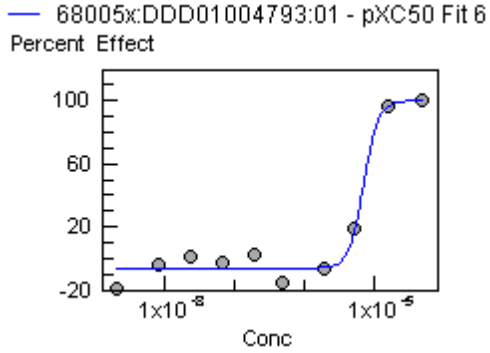
Compound Id	Supplier Id	Plate Id	pEC_{50}	Hill slope	Bottom	Top	Max Effect	Max Effect Conc	Top Confidence Interval	Chart
DDD01004781:01	CC13(3)-56 **1h**	68005	5.56	4.77	-8	101.1	101.17	4.98E-05	13.17	
DDD01004783:01	CC13(2)-96 **2a**	68005	5.23	2.45	5.1	103.7	100.35	1.64E-05	19.17	

Compound Id	Supplier Id	Plate Id	pEC_{50}	Hill slope	Bottom	Top	Max Effect	Max Effect Conc	Top Confidence Interval	Chart
DDD01004784:01	CC13(3)-74 **2b**	68005	5.22	1.99	-1.5	106.2	100.42	1.64E-05	24.72	
DDD01004785:01	CC13(3)-79 **2c**	68005	5.41	2.53	-8.3	101.7	100.49	1.64E-05	12.5	

Compound Id	Supplier Id	Plate Id	pEC_{50}	Hill slope	Bottom	Top	Max Effect	Max Effect Conc	Top Confidence Interval	Chart
DDD01004786:01	CC13(3)-60 **2d**	68005	5.27	1.28	0.8	111	100.16	1.64E-05	40.69	
DDD01004787:01	CC13(3)-64 **2e**	68005	5.17	3.06	0.6	101.8	99.65	4.98E-05	20.98	

Compound Id	Supplier Id	Plate Id	pEC_{50}	Hill slope	Bottom	Top	Max Effect	Max Effect Conc	Top Confidence Interval	Chart
DDD01004788:01	CC13(2)-91 **3a**	68005	5.54	1.57	1.6	102.7	99.95	4.98E-05	11.13	
DDD01004789:01	CC13(3)-68 **3b**	68005	4.49	2.23	24	100	79.01	4.98E-05	NaN	

Compound Id	Supplier Id	Plate Id	pEC_{50}	Hill slope	Bottom	Top	Max Effect	Max Effect Conc	Top Confidence Interval	Chart
DDD01004790:01	CC13(3)-88 **3c**	68005	<4.3				22.48	1.99E-06	5.21	
DDD01004791:01	CC13(3)-90 **3d**	68005	5.14	2.22	11.3	101.1	100.64	4.98E-05	8.29	

Compound Id[i]	Supplier Id[ii]	Plate Id	pEC_{50}	Hill slope	Bottom	Top	Max Effect	Max Effect Conc	Top Confidence Interval	Chart
DDD01004792:01	CC13(3)-92 **3e**	68005	<4.3				53.56	1.64E-05	NaN	
DDD01004793:01	NQ (**substrate**)	68005	5.14	4.12	-6.2	100	100.03	4.98E-05	20.47	

[i] Drug Discovery Unit, University of Dundee's compound identification (ID) codes.
[ii] Chikomborero Chakaingesu's supplier ID codes where CC = initials, 13 = the year 2013, (2)/(3) = laboratory note book number and the last digits denote the page number.