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MASTEROPPGAVE

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Tittel på masteroppgaven: Engelsk tittel: Naphthalene *trans*-dihydrodiols	
Studiepoeng: 30	
Emneord: Organic synthesis Laccase *Trametes versicolor* Naphthalene *trans*-dihydrodiols *Gadus morhua* Biliary metabolites GC-MS	Sidetall: ……57…… +vedlegg/annet: …17…… Stavanger, 01/09/18 dato/år

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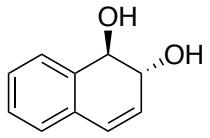
Preface

The aim of this Master project has been to synthesize C_0 – C_3 *trans*-naphthalene diol standards; intended for use as standards in gas chromatography–mass spectrometry (GC-MS) analysis of bile samples obtained from Atlantic cod (*Gadus morhua),* which have been exposed to dispersed crude oil. The currently used analytical method only studies phenols, therefore the results of this work will expand the current method to also include diols, and thus contribute to broaden the scope of knowledge related to the behavior of PAH metabolites.

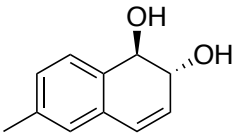
Abstract

The currently used GC-MS method for analysis of PAH metabolites in fish bile only focus on phenols; 1-naphthol, 2-naphthol, 1-hydroxyphenanthrene, and alkylated derivatives. 1-Hydroxypyrene is also included, but not alkylated forms. However, since *trans*-naphthalene dihydrodiol and methylated derivatives are formed *in vivo* and are known carcinogens, their presence in fish bile should be further investigated. Due to the absence of commercially available standards of *trans*-dihydro diols and their methylated derivatives, these compounds need to be synthesized.

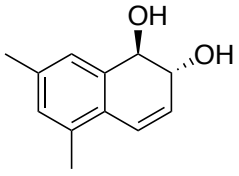
trans-1,2-Dihydronaphthalene-1,2-diol (**13±**), *trans*-6-methyl-1,2-dihydronaphthalene-1,2-diol (**19±**), *trans*-5,7-dimethyl-1,2-dihydronaphthalene-1,2-diol (**20±**) and *trans*-4,6,7-trimethyl-1,2-dihydronaphthalene-1,2-diol (**21±**) were synthesized from commercially available starting materials, and their overall yield were 40%, 59%, 28% and 3%, respectively. The compunds were prepared from 1,2-naphthoquinone, 6-methyl-1-tetralone, 5,7-dimethyl-1-tetralone, 1,2-dihydroxybenzene and 2,3-dimethyl-1,3-butadiene, and oxidation was performed by using 2-iodoxybenzoic acid (IBX) or Laccase-catalyzed Diels-Alder reaction, following reduction by sodium borohydride ($NaBH_4$) to give the *trans*-naphthalene dihydrodiols.



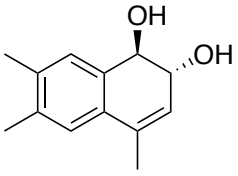
13±



19±



20±



21±

Fish bile samples obtained from Atlantic cod (*Gadus morhua),* exposed to dispersed crude oil, were prepared by de-conjugation, extraction and derivatisation, following standard procedure in order to prepare the samples for GC-MS analysis.

Selected Abbreviations

EPA	US Environmental Protection Agency
Equiv.	Mole equivalence
ESI	Electrospray ionization mode
GC IS	Gas chromatography internal standard
GC-MS	Gas chromatography mass spectrometry
GC SU	Surrogate internal standard
HMW PAH	High molecular weight polycyclic aromatic hydrocarbon
HPLC-F	High-performance liquid chromatography with fluorescence detection
IBA	Iodosobenzoic acid
IBX	2-Iodoxybenzoic acid
iNEXT	Indicators for Environmental Impact of Petroleum Activities: the Next Generation of Molecular Markers
IRIS	International Research Institute of Stavanger
LMW PAH	Low molecular weight polycyclic aromatic hydrocarbon
LRMS	Low resolution mass spectrometry
mp	Melting point [°C]
OH-PAH	Hydroxy polycyclic aromatic hydrocarbon
P450	Cytochrome P450
PAH	Polycyclic aromatic hydrocarbon
PAP	Polycyclic aromatic phenols
PAQ	Polycyclic aromatic quinones
PE	Petroleum ether
Py	Pyridine

R_f	Retardation factor
SET	Single electron transfer
SIM	Selected ion monitoring
TBACD	Tetrabutylammonium dichromate
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
TMS-O-PAH	Trimethylsilyl ether of OH-Polycyclic aromatic hydrocarbon

1. Introduction

1.1 Background and theory

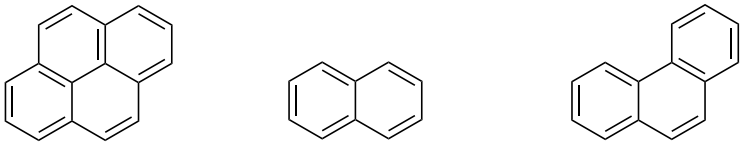
Environmental monitoring

The extraction and use of oil represent a potential source of marine pollution, and documentation of adverse environmental effects, which are related to these activities are increasing [1, 2]. Polycyclic aromatic hydrocarbons (PAHs) are related to this environmental concern because these substances are found in crude oil mixture, and undergo *in vivo* biotransformations into metabolites with carcinogenic and mutagenic properties [3]. Therefore, PAHs are high priority pollutants to be monitored in the marine environment, and their corresponding metabolites are currently used as biomarkers of oil exposure. In order to determine the environmental PAH exposure of organisms, PAH metabolites measurement in fish bile have been applied using gas chromatography-mass spectrometry (GC-MS) as preferential method.

In Norway, oil and gas activity operators are obliged to perform environmental monitoring of the water column and sediments nearby their installations, and detection of PAHs have been included in this program since 1988 [4]. The monitoring program involves the detection of pollutants in an area, and to evaluate their levels directly in water, sediment and in biological samples. Based on evaluation by the US Environmental Protection Agency (EPA), 16 PAHs have been targeted since the 1970s, and are named EPA-16 PAHs [5, 6]. Recently EPA has also included alkylated variants of the 16-PAHs, and suggests the detection of EPA-34 as a priority [7].

Structure and physiochemical properties of PAHs

The origins of PAHs are various, including oil seeps, incomplete combustion of organic matter, volcanic activity, forest fires, cigarette smoking, automobile exhaust, wood-treatment processes, waste incineration and the processing, production and spillage of petroleum [8-11]. Within the coastal zones, PAH mainly enters the water from sewage, run-off from roads, smelter industry and oil spills. In the offshore environment, PAHs enter the water column from installations by discharge of produced water, oil spills and oil seeps [12].



1 **2** **3**

Figure 1: Pyrene (**1**) belongs to the high molecular weight PAHs, while naphthalene (**3)** and phenanthrene **(3)** belongs to the low molecular weight PAHs.

PAHs consist of two or more fused benzene rings, which are configured in a linear, angular or cluster arrangement [13]. They are widespread contaminants found in the atmosphere, soil, water, and sediments [14]. PAHs are divided into two groups depending on their origin being from incomplete combustion of oil, namely pyrogenic and petrogenic PAHs, respectively. Pyrogenic PAHs are normally represented by larger ring systems compared to petrogenic PAHs [5].

PAHs are hydrophobic compounds and their persistence in the environment is due to their low aqueous solubility. Their bioavailability and biodegradation is influenced by adsorption onto soils and particulates [15]. The increase in the number of aromatic rings, structural angularity and hydrophobicity enhances the electrochemical stability, persistency, resistance against biodegradation and the carcinogenic index of PAHs, and decreasing the volatility [16, 17]. High molecular weight (HMW) PAHs have four or more fused benzene rings. Pyrene (**1**) in **Figure**

1 is an HMW PAH, and has an aromatic structure comprised of four benzene rings [16]. Low molecular weight (LMW) PAHs such as naphthalene (**2**) or phenanthrene (**3**), are easier to degrade and are more volatile compared to HMW PAHs [18].

PAH concentrations in crude oil

The concentration of PAHs, and the ratio between them in crude oil, varies based on the sources from which they have been retrieved [19, 20]. Petrogenic PAHs consist mainly of 2-3 ring PAHs and their corresponding alkylated forms. The alkylated structures may stand for 90% of the PAH components [7, 21].

The content of the 16 PAHs in 50 different crude oils, and its variations has been reported [5, 19]. **Table 1** projects an overview of the compounds and their concentrations in crude oil from the North sector [5].

Table 1: Minimum, maximum, and mean content of polycyclic aromatic hydrocarbons (PAH) in 50 different crude oils, and PAH content in two North European crude oil; North Sea crude oil and Goliat crude oil. (*Na*[a] = not detected; [b] = Goliat is situated in the Barents Sea; *Na*[c] = not analyzed for) [5].

Crude oil	48 different crude oils [19]			North Sea [22]	Goliat [23]
PAH	Minimum mg/kg oil	Maximum mg/kg oil	Mean mg/kg oil	mg/kg oil	mg/kg oil
Naphthalene	1.2	3700	427	1169	1030
Acenaphthene	0	58	11.1	18	12
Acenaphthylene	0	0	0	11	*
Fluorene	1.4	380	70.34	265	75
Anthracene	0	17	4.3	1.5	*
Phenanthrene	0	400	146	238	175
Fluoranthrene	0	15	1.98	10	6
Pyrene	0	9.2	-	20	*
Benzo[*a*]anthracene	0	16	2.88	11	*Na*[c]
Chrysene	4	120	30.36	26	*Na*[c]
Benzo[*b*]fluoranthrene	0	14	4.08	4.2	*Na*[c]
Benzo[*k*]fluoranthrene	0	1.3	0.07	*Nd*[a]	*Na*[c]
Benzo[*a*]pyrene	0	7.7	1.5	1.3	*Na*[c]
Dibenz[*a*,*h*]anthracene	0	7.7	1.25	*Nd*[a]	*Na*[c]
Benzo[*g*,*h*,*j*]perylene	0	1.7	0.08	1	*Na*[c]
Indeno[1,2,3-*cd*]pyrenec	0	1.7	0.08	*Nd*[a]	*Na*[c]

Naphtalene (**2**) has the highest concentration in crude oil, when including methylnaphthalens, the actual concentration is much higher (**Table 2**). **Table 2** presents the concentration of naphthalene and the three regioisomers of methylnaphthalene in crude oil from three different locations [5]. The alkylated forms of PAHs are known to be more toxic than the mother compounds [7, 21]. Being the compound of highest concentration, it is important to acquire more knowledge about its behavior in marine organisms, and improve the current analytical approach.

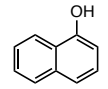
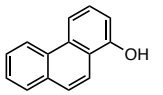
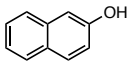
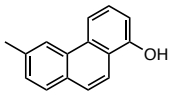
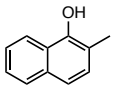
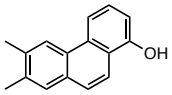
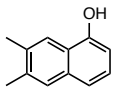
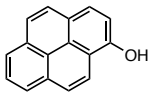
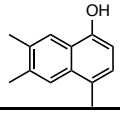
Table 2: Concentration of naphthalene and the three regioisomers of methylnaphthalene in crude oil from three different locations (*Na* = not analyzed) [5].

Crude oil	North Sea [22]	Goliat [23]	Exxon Valdez crude oil [24]
PAH	mg/kg oil	mg/kg oil	mg/kg oil
Naphthalene	1169	1030	720
1-Methylnaphthalene	2108	2700	1330
2-Methylnaphthalene	2204	4200	1020
3-Methylnaphthalene	1172	2800	*Na*

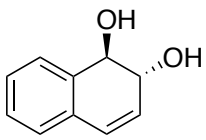
Current analytical approach

Currently, the analytical method related to detection of PAH metabolites by GC-MS, only target phenol metabolites; 1-naphthol (**4**), 2-naphthol (**5**), 1-hydroxyphenanthrene (**9**), and their alkylated derivatives. 1-Hydroxypyrene (**12**) is also included, but not the alkylated forms. Highlighted in **Table 3** are the compounds, which are included in the GC-MS protocol used by IRIS.

Table 3: The OH-polycyclic aromatic hydrocarbon (OH-PAH) metabolites, which currently are targeted in the protocol from IRIS.

Target OH-PAH metabolite			
1-Hydroxynaphthalene (**4**)		1-Hydroxyphenanthrene (**9**)	
2-Hydroxynaphthalene (**5**)		C1-Hydroxyphenanthrene (**10**)	
C1-Hydroxynaphthalene (**6**)		C2-Hydroxyphenanthrene (**11**)	
C2-Hydroxynaphthalene (**7**)		1-Hydroxypyrene (**12**)	
C3-Hydroxynaphthalene (**8**)			

However, the current method does not include the dihydrodiols, which are metabolites known to be formed *in vivo* (**Figure 2**). Implementation of a new protocol to include the dihydrodiols and their methylated derivatives would improve the existing approach by allowing for a broader identification of the metabolites present in fish bile from exposed fish.



13±

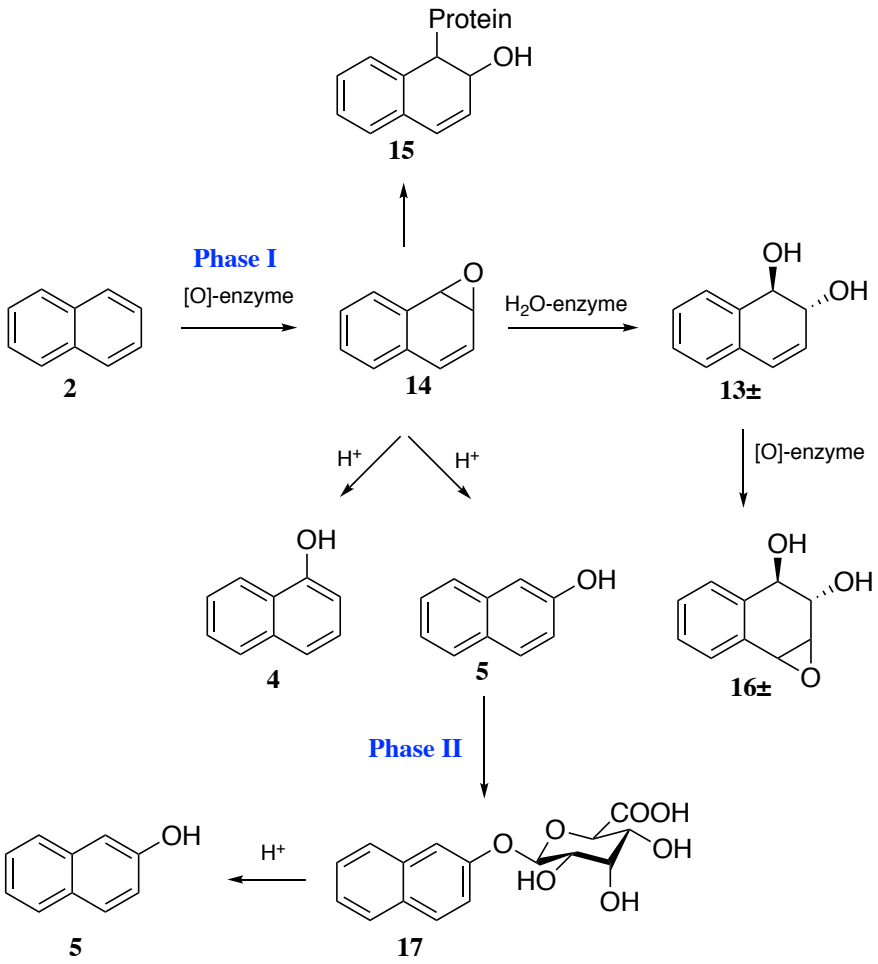
Figure 2: *trans*-1,2-Dihydronaphthalene-1,2-diol (**13±**) exemplifies a dihydrodiol PAH metabolite.

PAH toxicity, metabolites and naphthalene *trans*-dihydrodiols as target compounds

The structure of 1,2-dihydronaphthalene-1,2-diol, and its presence as a naphthalene metabolite was reported in 1947 by Young *et al*. [25]. The metabolite was identified in urine excreted by rats post treatment with naphthalene. Naphthalene was at that time the second example of a PAH to be identified to undergo metabolic change. Previously, Boyland and Levi had proved anthracene to behave in the same manner [26]. However, today it is known that PAHs form many types of metabolites *in vivo,* and the *trans*-dihydrodiol metabolites are of special concern because they are the starting point for the biosynthesis of the carcinogenic epoxide metabolites [5].

The toxicity of PAHs ranges from not being toxic to severely toxic, and depends on their structure, metabolic reactive state, dose and route of exposure and the organism, involved [2, 27, 28]. Another aspect is the effect of synergy caused by the combined effect of the mixture of various PAHs and other chemicals, referred to as the cocktail effect [29, 30].

In Atlantic cod (*Gadus morhua)*, the highest concentration of PAHs and their metabolites are normally found in the liver and the bile. The liver is the center of PAH metabolism, and uptake happens primarily through the gills or by ingestion of PAH contaminated food or sediments. Due to the hydrophobic nature of these compounds, bioaccumulation occurs in fatty tissues, such as the liver. Within the liver cells, the PAHs are oxidized and become more water-soluble and also more reactive. This is due to enzymes with aryl hydrocarbon hydroxylase activity, which form epoxide- and dihydrodiol metabolites (**14**, **16**), see **Scheme 1** [5, 27, 28]. The naphthalene epoxide (**14**) reacts with cellular macromolecules such as DNA and proteins and forms adduct (**15**). This latter mechanism makes them potentially genotoxic and therefore important to study [5].

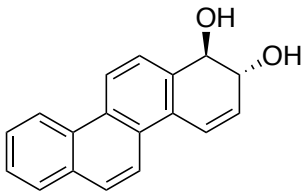


Scheme 1: Outline of the possible metabolic degradation of naphthalene by cytochrome P450 (CYP1A) enzyme which is most prominent in detoxification of xenobiotic in fish [5].

The first step of xenobiotic detoxification (Phase I) occurs within the hepatocytes by the cytochrome P450 enzymes. Phase I reactions introduce polar groups into the xenobiotic molecule such as naphthalene (**2**) and result in the formation of metabolites (**4**, **5**, **13±**, **14** and **16±**) with high affinity for nucleic acids and proteins. The interaction between epoxide **14** with proteins or DNA, may result in adduct such as compound **15**, see **Scheme 1**. Fish do not have a highly developed DNA or protein repair system, thus eventually DNA lesions and change in protein functions may lead to unfavorable effects within the organism [5, 27]. Metabolites from Phase I undergo glucosylation, sulfation or glucoronidation to form highly water-soluble conjugates such as glucuronides (**17**) **(Scheme 1)**. These compounds are readily excretable

products. However, the conjugated metabolites may be hydrolyzed due to pH change in the intestine, when bile is released into the alimentary tract to assist with the breakdown of lipids and other compounds. The latter causes the metabolites to become more hydrophobic, and this may result in reabsorption through the gut wall causing the metabolites to be returned to the liver. This is called enterohepatic circulation, and causes the PAH metabolites to stay longer within the organism and potentially cause more harm [27].

In fish, *trans*-dihydrodiols are the major PAH oxidation products formed and excreted to the bile [31], and for Atlantic cod, up to 88% of the chrysene metabolites in the bile is represented by the (1*R*,2*R*)-1,2-dihydrochrysene-1,2-diol (**18**) (chrysene diol) (**Figure 3)** [32]. Based on the latter, Pampanin *et al*. [33] have suggested that the case could be similar for naphthalene metabolites, in which (1*R*,2*R*)-1,2-dihydro-naphthalene-1,2-diol (**13**) would represent the majority of the naphthalene metabolites present in bile.



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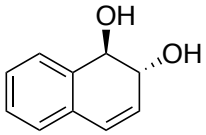

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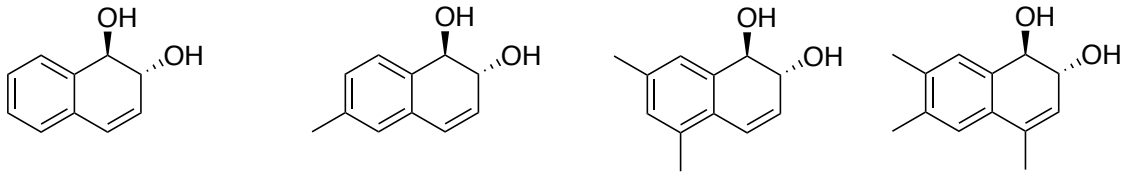
Figure 3: (1*R*,2*R*)-1,2-Dihydrochrysene-1,2-diol (chrysene diol) (**18**) and naphthalene with (1*R*,2*R*)-1,2-dihydro-naphthalene-1,2-diol (**13**) [33].

The PAH metabolite dihydrodiols with *R*,*R*-configuration and *R*,*S*-diol-*S*,*R*-epoxides have been shown to exhibit the most carcinogenetic activity [5, 34]. There are several studies related to PAH metabolite determination in fish bile and its use within environmental monitoring [32, 35-57]. However, studies with naphthalene diols (**Figure 3**) are limited. Pampanin *et al*. [33]

recently conducted a study to determine the biological responses of Atlantic cod to naphthalene, chrysene and two of their metabolites; (1*R*,2*R*)-1,2-hihydrodiol **18** and **13,** by an intraperitoneal injection exposure. The GC-MS method, in which targeted the naphthalene metabolites such as, 1-naphthol and 2-naphthol, showed a dose response in fish exposed to the parent compound naphthalene compared to the values of those injected with the naphthalene diol.

Naphthalene, methylnaphthalenes and dimethylnaphthalens are known to be the most abundant and accumulated aromatic compounds, along with being the most water-soluble components in petroleum [5, 58]. The toxicity of naphthalene derivatives has also been found to be related to the substitution pattern of the methyl groups [59]. It is therefore interesting to study the levels of the selected naphthalene *trans* diol metabolites; **13±**, **19±**, **20±** and **21±** in fish bile (**Figure 4**).



13± **19±** **20±** **21±**

Figure 4: Target compounds; *trans*-1,2-dihydronaphthalene-1,2-diol (**13±**), *trans*-7-methyl-1,2-dihydronaphthalene-1,2-diol (**19±**), *trans*-6,7-dimethyl-1,2-dihydronaphthalene-1,2-diol (**20±**) and *trans*-4,6,7-trimethyl-1,2-dihydronaphthalene-1,2-diol (**21±**).

GC-MS has been concluded to be the most appropriate method for the analysis of two and three ring PAH metabolites, and results have shown good selectivity and sufficient sensitivity [47, 60, 61]. Naphthalene diols **13±**, **19±, 20±** and **21±** in **Figure 4** are not readily available; they are therefore prepared in this study by organic synthesis.

GC-MS and determination of PAH metabolites in fish bile

The GC-MS system is composed of two analytical principles; the GC vaporizes and separates the individual components, whereas MS enables structural identification of the metabolites (**Figure 5**). The separation occurs as the various components within a sample travels through the column by an inert gas (mobile phase), usually helium. Within the column there's a stationary phase, which delays some compounds more than others, while others will pass more easily. PAH metabolites are detected by using a stationary phase with a polar film. This allows the lighter metabolites to pass quicker while larger molecules have a longer retention time on the solid phase, and along with different boiling points, the metabolites become separated. The different retention times provide peaks within the spectra, which represents the metabolites and the area of the peak represents the quantity (i.e. concentration). A known amount of internal standard, which is added during sample preparation, allows for quantification. Data from MS analysis enables the process of identification of the metabolites [62].

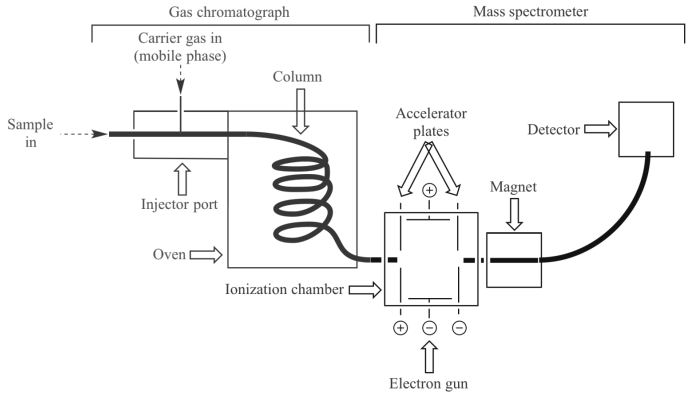


Figure 5: Principle schematic of a gas chromatography-mass spectrometry (GC-MS) system.

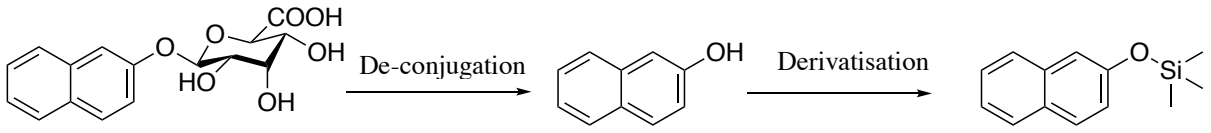
The MS ionizes the molecules from the vaporized sample. The analysis described herein uses electron impact (EI), which is a commonly used method. The ions are exposed to highly energetic electron beam, usually 70 eV, followed by acceleration through a magnetic field made of coiled copper. Within this field the ions are either repelled or attracted, and a receptor detects

the fragments of different charge by ion intensity and mass, from which a plot is produced. The MS provide peak identification, and from the GC spectra, concentration may be elucidated [62].

Preparation of bile samples

The efficient *in vivo* biotransformation of PAH in fish prevents the accumulation of these compounds in extrahepatic tissues, thus evaluation of tissue levels of parent PAHs is not a sufficient assessment method to detect the exposure level. However, hepatic PAH-metabolites accumulate in the gall bladder of fish before excretion, and its detection is an indication of recent exposure, thus bile sample is preferred [61].

Preparation of the bile samples for quantitative determination of OH-PAHs by GC-MS analysis involves; de-conjugation, extraction and derivatisation of the polar hydroxyl groups (**Scheme 2**). The first step, de-conjugation, involves enzymatic treatment of bile with β-glucoronidase and sulfatase to obtain free OH-PAHs (**5**). Extraction of the free OH-PAHs are performed by ethyl acetate (EtOAc) or other organic solvents, which are compatible with GC-MS. Derivatisation of the polar OH-groups with trimethylsilyl (TMS) (**22**), methyl or acetyl groups, is performed in order to prevent thermal decomposition and reduce peak tailing [61].



17 **5** **22**

Scheme 2: Conjugated metabolite hydroxynaphthalene glucuronide (**17**), free OH-PAH (**5**) after enzymatic treatment with β-glucoronidase and sulfatase, and TMS-derivatised OH-PAH (**22**) to be used in GC-MS analysis.

Prior to the de-conjugation step a surrogate internal standard (SU IS) must be added to the sample, which is the internal standard for quantification. The ratio of the surrogate standard to

other internal standards is used to calculate the recovery efficiency. The recovery is a percentage representing the true concentration of a substance recovered during the analytical procedure [63]. Herein, 2,6-dibromophenol (2,6-DBP) (**23**) and triphenylamine (TPA) (**24**) are used as SU IS and GC-MS performance standard (GC IS), respectively (**Figure 6**). TPA is added to the sample after preparation and just before injection.

23 **24**

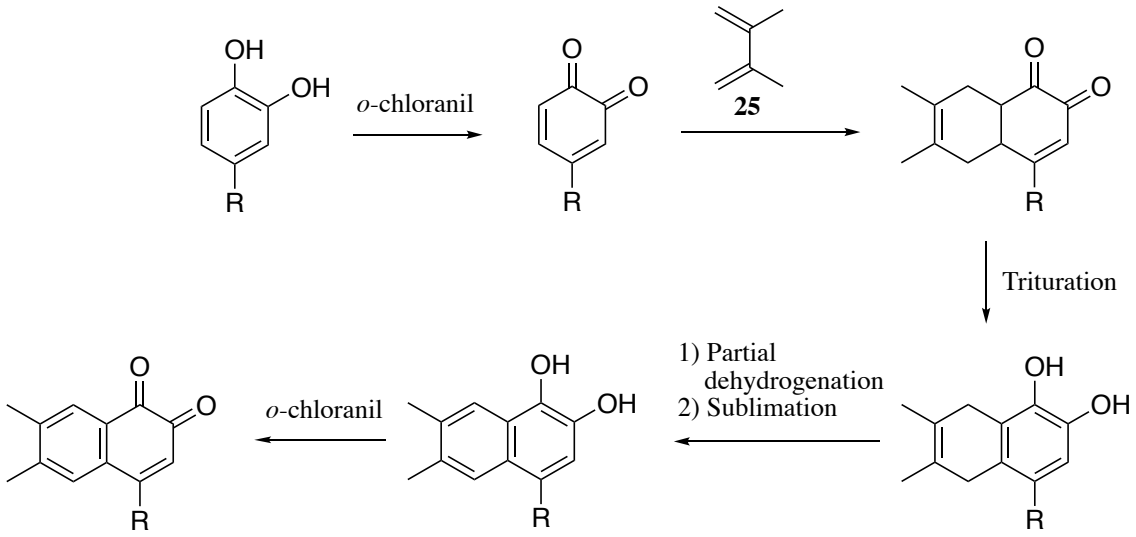
Figure 6: Structures of surrogate internal standard (SU IS) 2,6-dibromophenol (**23**) and triphenylamine (**24**) as gas chromatography-mass spectroscopy performance standard (GC IS).

1.2 Synthesis of the naphthalene *trans*-dihydrodiols

Synthesis of *o*-naphtoquinones by Diels-Alder reaction

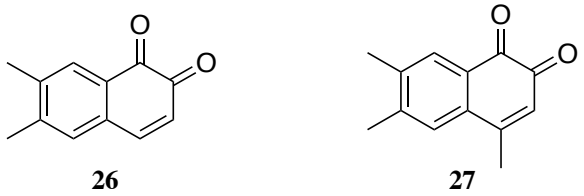
In 1928 Otto Diels and Kurt Alder introduced a reliable and efficient method to synthesize six-membered ring systems that was named the Diels-Alder [4+2] cycloaddition [64], for which they both were awarded the Nobel Prize in 1950. This method has previously been used to synthesize *o*-naphtoquinones, and will be introduced in the following section.

Barltrop and Jeffreys made a first attempt to apply the Diels-Alder reaction in order to synthesize *o*-napthoquinones, by trying to react *o*-benzoquinones with acyclic dienes in 1954, however without success [65]. Ansell *et al*. [66] reported two decades later, in 1971, on this matter, that the *o*-benzoquinone is known to be an unstable compound, which do exhibit some dienophilic reactivity towards cyclopentadiene and by its dimerization. It was therefore suggested that the lack of reactivity, in the case of Barltrop and Jeffereys, was connected to the rates of its decomposition and dimerization being much faster than the rate of the Diels-Alder reaction. Ansell *et al*. suggested that using a large excess of the diene would favor the Diels-Alder reaction and decrease the rate of dimerization reaction. Ansell managed to prove this hypothesis and showed that *o*-benzoquinone and substituted *o*-benzoquinones may undergo a Diels-Alder reaction with dimethylbutadiene **25**. The Ansell procedure (**Scheme 3**) commenced from recently made *o*-quinone, by oxidizing catechol using *o*-chloranil without purification.



Scheme 3: A five step reaction sequence resulting in the formation of target molecule **26** and **27** (R = H or CH_3) [66].

Dimethylbutadiene (25 equiv.) was added at room temperature and the reaction time was 6 hours. Yields for naphthoquinone (**26**) and (**27**) (**Figure 7**) were 51% and 27%, respectively, after a 5-step reaction path [66].



26 **27**

Figure 7: Dimethylated naphthoquinone **26** and trimethylated naphthoquinone **27**.

Weller and Stirchak investigated a potential path to quassinoid skeleton by the reaction of 3,5-disubstituted *o*-quinones with simple dienes in 1983 [67], and Paquet and Brassard studied reaction of polar dienes towards halogenated *o*-quinones in 1988 [68]. Following Paquet and Brassard, another group consisting of Wozniak and co-workers used Diels-Alder reaction to make lignin derived quinones in 1989 [69].

Nair and Kumar conducted a series of reaction experiments between *o*-quinones and electron-rich dienes in 1996 [70]. They focused on the substituted *o*-benzoquinones; 3,5-di-*tert*-butyl-*o*-benzoquinone, 4-*tert*-butyl-*o*-benzoquinone and 3-methoxy-*o*-benzoquinone with various acyclic dienes. More recently, in 2007, Witayakran and co-workers reported the first and still the only Laccase-catalyzed Diels-Alder reaction to synthesize naphthoquinones [71].

Laccase catalyzed *o*-naphthoquinones and Diels-Alder reaction

Laccases (benzenediol:oxygen oxidoreductase, EC1.10.3.2) belongs to the family of multicopper oxidases; proteins which contain four copper ions in the active site. These enzymes are found in plants, fungi, bacteria, insects and algae. Their physiological functions vary with source and they are diverse; fruiting body formation, pigment formation, lignin degradation and -biosynthesis, detoxification, morphogenesis, plant pathogenesis and fungal virulence, and synthesis of humic substances [71-73].

Originally, laccases were found in the sap of the Japanese lacquer tree *Rhus vernicifera* in 1883 [74], and later in 1896, the first fungal laccases were identified in various species of *Russula fungi* [75]. These multicopper oxidases are able to form bonds under environmentally friendly conditions, requiring solely oxygen and produces water as by product, and have therefore become an application within green chemistry [73]. Basically, laccases couple the four-electron reduction of dioxygen to water by oxidizing various substrates such as; methoxyphenols, phenols, *o*- and *p*-diphenols, aminophenols, polyphenols, phenols polyamines and ligning-related compounds [72, 76]. The enzyme structure of Laccase *Trametes versicolor* is illustrated in **Figure 8**. The three-dimensional structure is to the left, where copper ions are shown as spheres (magenta), and carbohydrates (green/blue/red) and protein backbone (blue). The next image shows the active site of the enzyme as a 3D image and by a schematic representation.

Substrate oxidation takes place at T1 Cu whereas oxygen reduction occurs at the T2 and T3 Cu sites [73].

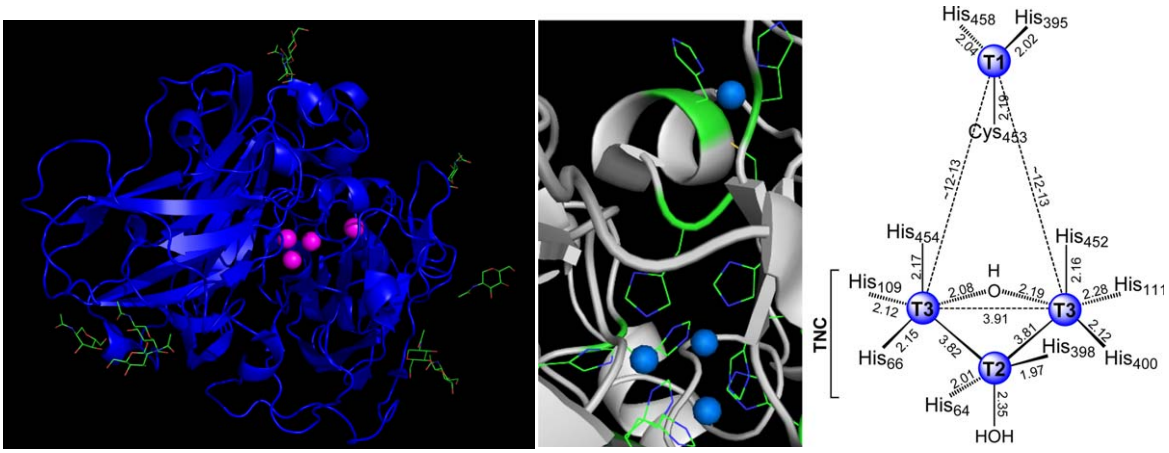
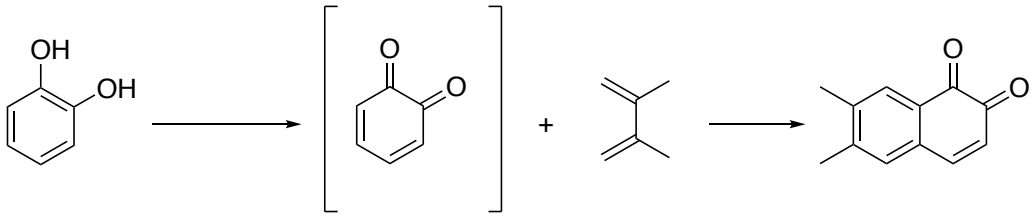


Figure 8: Three-dimentional structure of a *Trametes vesicolor* laccase is displayed to the left; Copper ions are shown as spheres (magenta), and carbohydrates (green/blue/red) and protein backbone (blue) [73].

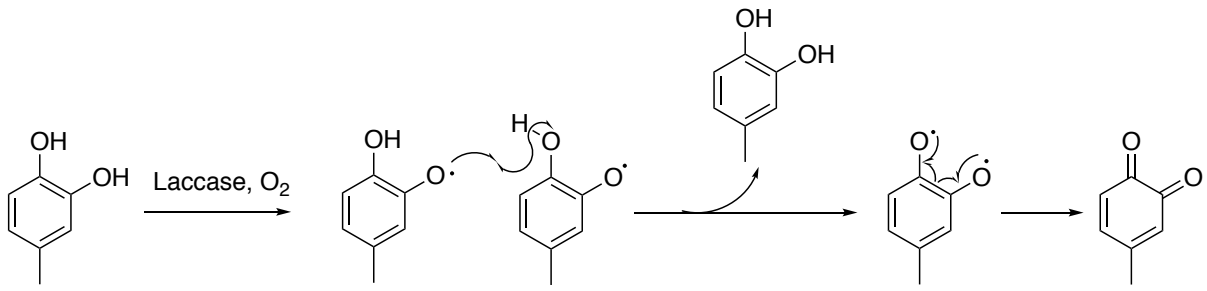
As previously mentioned, Witayakran and co-workers reported a tandem synthesis of naphthoquinones in 2007 by the reaction of laccase-generated benzoquinones and acyclic dienes via Diels-Alder reaction performed in sodium acetate buffer solution with various catechols and diene substrates, and **Scheme 4** represents a general reaction route [71].



25 **26** **27** **28**

Scheme 4: Laccase-initiated cascade synthesis of substitute naphthoquinones via Diels-Alder reaction.

The benzoquinone is formed *in situ* from the corresponding catechol via disproportionation shown in **Scheme 5** [73].

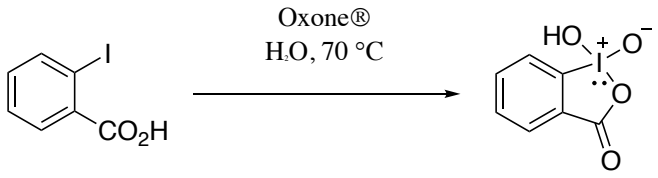


29 **30**

Scheme 5: Laccase-initiated disproportionation of 4-methylcatechol (**28**) into 4-methyl-*o*-benzoquinone (**29**).

Oxidation by 2-iodoxybenzoic acid (IBX)

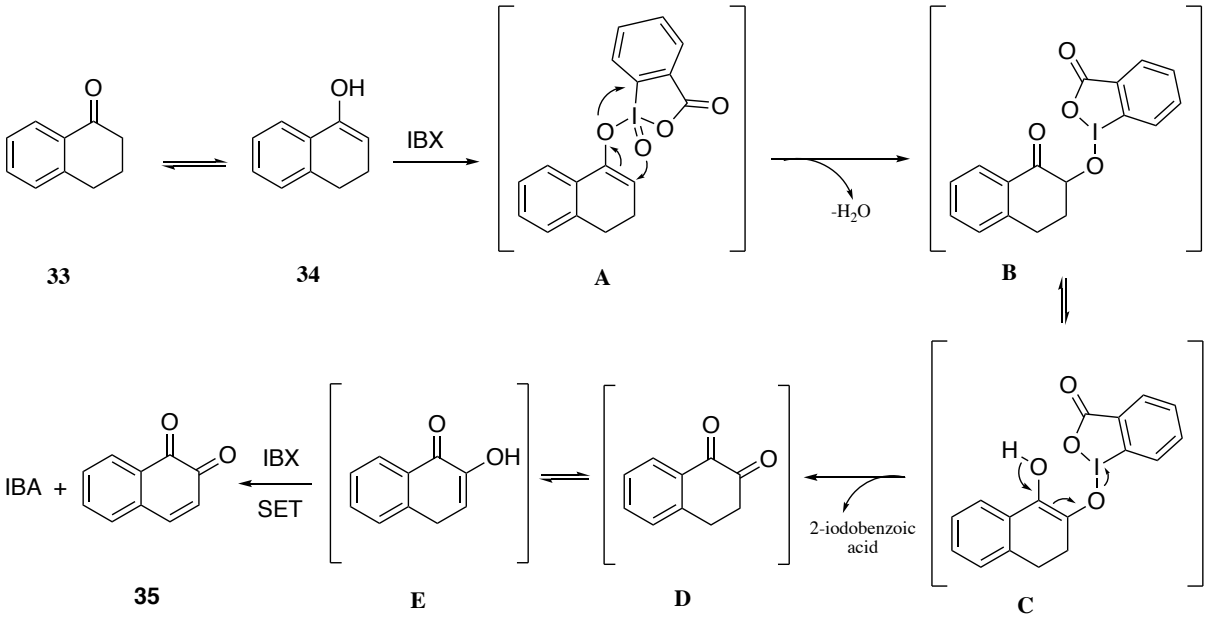
2-Iodoxybenzoic acid (IBX) (**31**) is a hypervalent iodine reagent, which is used for regioselective oxidation of polycyclic aromatic phenols (PAP) to isomers of polycylic aromatic quinones (PAQ). Frémy's salt [$(KSO_3)_2NO$] has been the most frequently used compound when oxidizing PAPs [77], but the use of this reactant may often result in mixtures of *otho-* and *para-*quinone isomers along with byproducts [78]. IBX (**31**) may be synthesized from 2-iodobenzoic acid (**32**) and Oxone (**Scheme 6**) [79].



32 **31**

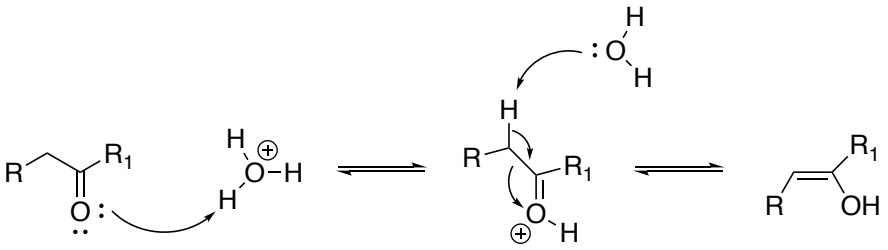
Scheme 6: The structure of 2-iodoxybenzoic acid (IBX) (**31**).

Ren *et al*. reported the oxidation of 1-tetralone derivatives into 1,2-naphtoquinones by IBX (**31**) [80]. The proposed mechanism for this reaction is outlined in **Scheme 7** [81].



Scheme 7: Proposed mechanism of 1,2-oxidation by 2-iodoxybenzoic acid (IBX) (**31**) (Single electron transfer (SET).

The mechanism in Scheme 7 is based on the proposed mechanism by Quideau and associates for IBX-mediated oxidation of 1-naphthol (**4**) [82]. The oxidation starts from the acid-catalyzed tautomerization between 1-tetralone (**33**) into its enol form (**34**) (**Figure 8**).



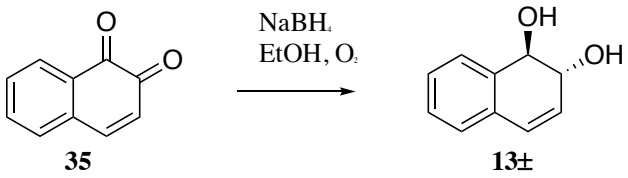
Scheme 8: Mechanism of acid-catalyzed enolization of ketones.

Intermediate complex **A** is formed by interaction with IBX (**31**) and loss of water (**Scheme 7**). Oxygen is removed from complex **A**, caused by a sigmatropic transfer from the iodine(V) to the adjacent carbon with concomitant reduction to iodine(III) which forms complex **B**. Through

tautomerization of **B** into **C** and a two-electron displacement, intermediate **D** and the reduced 2-iodobenzoic acid is generated, respectively [78, 80]. Intermediate **E** is generated through a third tautomerization of **D**, and reacts with IBX (**31**) to give 1,2-naphthoquinone (**35**) along with the byproduct iodosobenzoic acid (IBA) through single electron transfer (SET) [80, 83].

Reduction of *ortho*-quinones into *trans*-dihydrodiols

Sodium borohydride ($NaBH_4$) was discovered by Schlesinger and Brown and their co-workers in 1943 [84]. Due to its characteristic chemoselectivity, it has become a widely used reducing agent within organic chemistry [85]. However, when Kundu *et al*. initially applied $NaBH_4$ to reduce non-K-region *o*-quinones of PAH to dihydrodiols, the procedure was not very successful at first. The olefinic bond had to be converted to dibromide in order for it to proceed [86], and then Platt and Oesch discovered in the 1983 that the presence of oxygen (**Scheme 9**) would give successful conversion to *trans*-dihydro diol [87].



35 **13±**

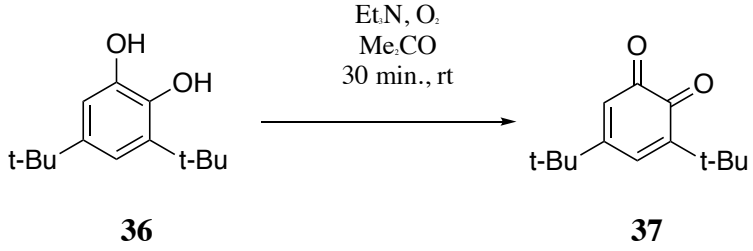
Scheme 9: Reduction of naphthoquinone (**35**) to *trans*-1,2-dihydronaphthalene-1,2-diol (**13±**) ($NaBH_4$) proposed by Platt and Oesch [87-89].

2. Results and discussion

Attempted synthesis of *o*-benzoquinones

The initial strategy to synthesize the target *trans*-napthalene dihydrodiol compounds **13±**, **19±**, **20±** and **21±** was to oxidize catechol into its corresponding quinone in order to perform a Diels-Alder [4+2] cycloaddition [64]. Oxidation of various *o*-catechols, phenols and tetralones into quinones has been described in the literature [80, 90-94], and several reactions, enlisted in **Table 4,** were pursued.

Hamann and co-workers [92] reported an elegant synthesis of 3,5-di-*tert*-butyl-*ortho*-quinone (**36**) from 3,5-di-*tert*-butylcatechol (**37**) within 30 minutes (min) at room temperature (rt), in acetone (Me_2CO) under O_2-atmosphere (**Scheme 10**). The yield was reported to be quantitative.



36 **37**

Scheme 10: Procedure from Hamman and co-workers' article on *o*-quinone synthesis [92].

The procedure was performed as described by Hamman and co-workers (**Table 4**), and after the addition of Et_3N a slight color change was observed, corresponding to previous observations.

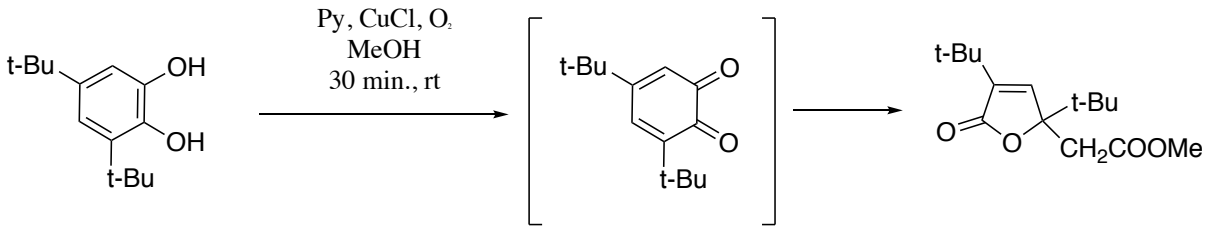
Table 4: Attempts to oxidize catechol into its corresponding *o*-benzoquinone at room temperature. (R = CH_3 or H)

Entry	R	OS[a]	Solvent	t [h]	Yield
1	H	Et_3N/O_2	Me_2CO	2	Nr[b]
2	H	Et_3N/O_2	Me_2CO	96	nr[b]
3	CH_3	Et_3N/O_2	Me_2CO	96	nr[b]
4	H	$Cu(I)Cl/Py/O_2$	MeOH	2[c]	-
5	H	Cu(I)Cl	MeOH	15[c]	-

[a]Oxidizing system. [b]no reaction. [c]min.

However, thin layer chromatography (TLC) did not display any conversion after 30 minutes, and the reaction was allowed to stir for 2 more hours, but the result was the same. The crude product from the reaction mixture was analyzed by nuclear magnetic resonance spectroscopy (NMR), and only starting material was identified. Two more experiments were conducted whereas the duration of the reaction was prolonged, but neither gave the oxidized catechol.

Speier and Tyeklár [95] studied the mechanism of oxidation by the system consisting of copper(I)chloride (CuCl) and pyridine (Py) in methanol (MeOH) (**Scheme 11**).



36 **37** **38**

Scheme 11: Speier and Tyklár suggested that the CuCl-assisted ring-cleavage in presence of pyridine in MeOH, would first produce the *o*-quinone intermediate.

This study suggests that catechol **36** is oxidized into the intermediate *ortho*-quinone intermediate **37** before CuCl promoted the ring cleavage form lactone **38**. Therefore, two experiments (Entry **4** and **5**) were conducted in order investigate whether it was possible to

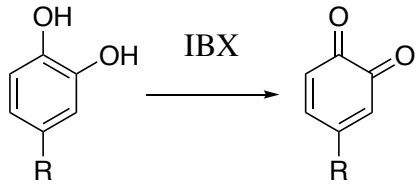
obtain the *o*-quinone intermediate **37**. Both experiments were conducted under similar conditions, by only changing the reduction time. Catechol was dissolved in MeOH and added to an oxygentated solution of pyridine and CuCl. TLC revealed a new spot below starting material (R_f = 0.21 in 50:50 v/v pet. ether/EtOAc). After aqueous workup the residue was dissolved in dimethyl sulfoxide-d_6 (DMSO-d_6), but there was nothing to interpret in the ^{1}H-NMR spectrum. Since the *o*-quionone is known to be unstable it might have decomposed and not entered the organic phase during workup. In the last attempt (**Entry 5**) only 1 mg was isolated, and characterization by NMR was not possible.

Neither of the procedures gave the desired *o*-quinone, however, these compounds are known to be very reactive and unstable, and therefore a challenging task to isolate. Decomposition, isomerization, or polymerization represents processes in which these compounds might undergo. Oxidation potentials of the reaction partners, thereof nucleophiles, frequently possess the same or lower potential compared to the catechol, thus preventing the catechol oxidation. In order to avoid these activities, *o*-catechols should be oxidized in the absence of organic substrate, and be used immediately in the following reaction [96]. Based on this information oxidation was targeted in a new manner.

Regioselective oxidation by IBX and immediate Diels-Alder reaction

Wu *et al*. [78] and Harvey *et al*. [94] have described a regiospecific oxidation of catechol and phenols by IBX (**31**), and based on the procedure from Paquet *et al*. [68] it was attempted to oxidize the catechol (**Table 5**) and proceed to Diels-Alder reaction without comprehensive purification.

Table 5: Regioselective oxidations by IBX (**31**) (R = CH_3 or H) under Argon atmosphere.



Entry	R	Solvent	T °C	t [h]	Yield	Ref.
1	CH_3	$CHCl_3$	rt	12	-	[97]
2	CH_3	DMSO	80	10	-	[12]
3	H	DMSO	80	10	Nd[f]	[80]

[a]Oxidizing agent. [b]min. [c]Not defined. [d]No reaction. [e]Conc. [f]MS from TLC confirmed the presence of target molecule. IBX (**31**) was synthesized from 2-iodobenzoic acid (**32**) and Oxone® in deionized water at 70 °C [79].

Indication of catechol oxidation could be seen from low-resolution mass spectrometry (LRMS). The mass of three adducts were observed, in which correlated to the mass of **26** (**Figure 9**); 141.1 ([M+CH_3OH+H]$^+$ = 108.7), 150.0 ([M+CH_3CN+H]$^+$ = 108.0) and 145.1 ([M+H]$^+$). The latter may correspond to the double hydrated form of **26**; 145.1 ([M+$(H_2O)_2$+H]$^+$) = 108.1. Based on this result, the crude mixture was only extracted and filtered through silica gel before the next step (**Scheme 12**).

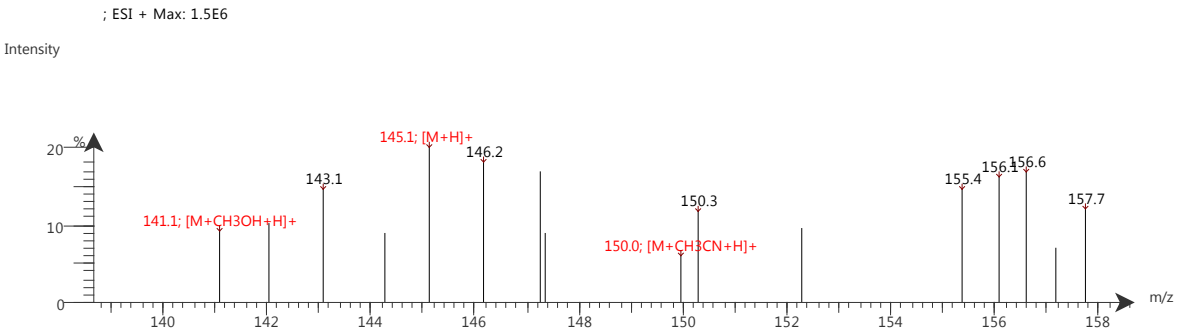
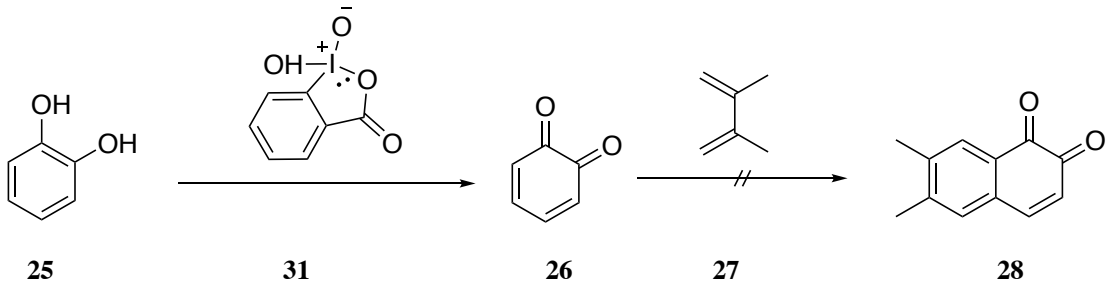


Figure 9: Low-resolution mass spectrometry (LRMS) indicating the presence of quinone **26** in crude mixture.

The residue from previous reaction was diluted in THF at 0 °C and kept on ice bath while adding 2,3-dimethyl-1,3-butadiene (**27)** to the mixture slowly (15-30 min) and stirring was continued at room temperature. After 48 h there was no sign of change in starting material.



Scheme 12: Oxidation of catechol by IBX in DMSO and immediate pursuing Diels-Alder reaction.

Enzymatic Diels-Alder reaction

While, having experimented with *o*-quinone oxidations, the laccase-catalyzed Diels-Alder reactions were investigated simultaneously. The next step in pursuing the methylated *o*-naphtoquinones involved a green chemistry approach by the application of enzymes; Laccase from *Trametes versicolor* (EC 1.10.3.2). Opposed to the abovementioned 5-step procedure introduced by Ansell *et al*. [66], Witayakran and co-workers had proposed a Laccase-catalyzed Diels-Alder reaction, in aqueous one-pot. They reported the synthesis of *o*-naphthoquinones and *p*-naphthoquinones from various catechols with dienes, and the yield of compound **28** and **39** (**Figure 10**) was 47% and 57%, respectively [71].

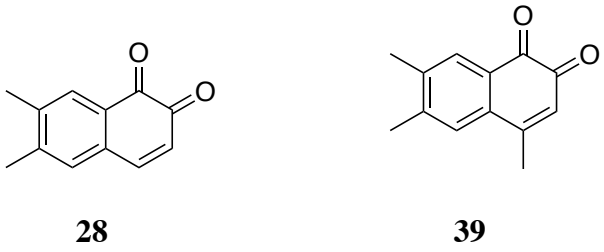
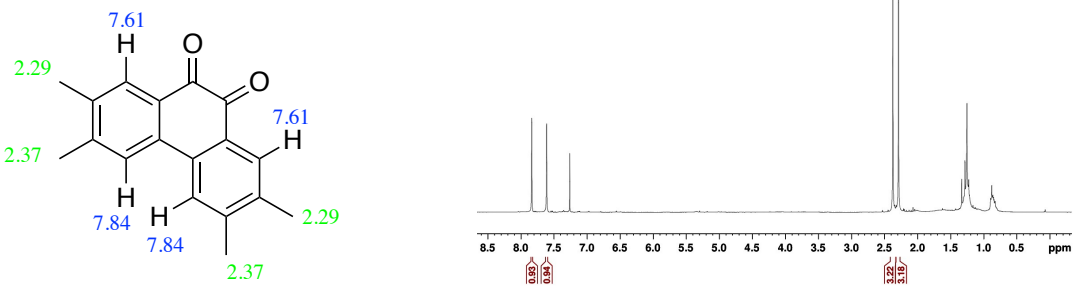


Figure 10: Compund **28** and **39** was isolated in presumable 47% and 57%, respectively. The results are based on Witayakran and co-workers' article from 2009 [71].

These results were a good starting point, and the first experiment (**Scheme 13**) (**Entry 1**, **Table 6**) was conducted as described in the procedure by Witayakran and co-workers. TLC was performed after 24 hours and revealed a spot, which had the same retention value (R_f) as the starting material, however when exposed to heat, it strongly discolored. During purification it was discovered that there were two spots, and both were isolated. The collected mass of the most polar compound was unfortunately not sufficient to interpret by NMR analysis, but LRMS confirmed the mass to be the target compound **28** (**Figure 10**). The less polar substance, which was in abundance, and had a strongly orange appearance, was identified as compound **40** (**Figure 11**).



40

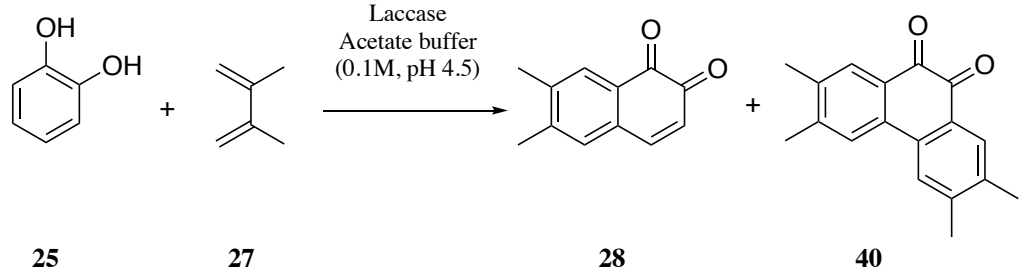
Figure 11: The most abundant compound in reaction mixture was interpreted to be 2,3,6,7-tetramethylphenanthrene-9,10-dione (**40**). ^{1}H-NMR spectrum of compound **40**.

The ^{1}H-NMR signals; 7.84, 7.61, 3.37 and 2.29 ppm were integrated to 2,2,6 and 6, respectively (**Figure 11**). ^{13}C-NMR- and LMRS analysis also supports the suggested structure **40**. This compound has been synthesized previously by Wozniak and his co-workers in 1989 [69], and

NMR data was in accordance with their findings. However, Witayakran and co-workers did not identify this compound in their study [71]. Compound **40** is formed by a double Diels-Alder reaction taking place on substrate **26**.

New attempts were pursued; reducing the reaction time from 24 to 5 hours (**Entry 2**, **Table 6**) and changing atmosphere to oxygen (Entry 3). Neither of these gave a positive outcome in terms of affording the target compound **28**. However, compound **40** was retrieved in all cases.

According to Ansell, compound **28** was synthesized by increasing the mole equiv. of diene **27** in order to prevent dimerization [66]. Witayakran and co-workers used 10 equiv. of diene **27**, and reports this amount to be ideal in order to synthesize **28** [71]. However, in this work, the previous reactions, mole equiv. were 10, and this might have had an unfavorable effect, thus causing the diene to react twice with the *o*-benzoquinone to give compound **40**. Therefore, another experiment was conducted by reducing mole equiv. (7 equiv.), and after purification 9% of compound **28** was isolated.



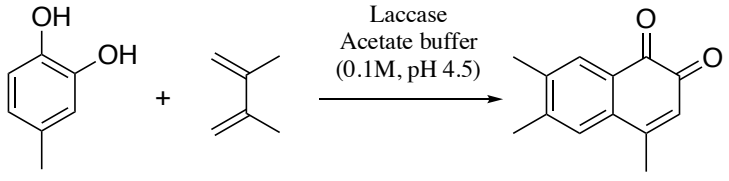
Scheme 13: Laccases enzyme-catalyzed Diels-Alder reaction to form 6,7-dimethylnaphthalene-1,2-dione (**28**) and byproduct 2,3,6,7-tetramethylphenanthrene-9,10-dione (**40**).

Table 6: Enzymatic reaction with catechol, Laccase *Trametes vresicolor i*n acetate buffer solution (0.1 M, pH 4.5) and 2,3-dimethyl-1,3-butadiene (10 equiv.) (**27**).

Entry	Equiv.(**26**)	atm	t [h]	Yield [%] (**28**)/(**40**)
1	10	Air	24	0/24
2	10	Air	5	0/5
3	10	O_2	48	0/12
4[a]	7	Air	24	9/6

While knowing that the reaction finally had worked, although the yield was frugal, performing the same reaction with 4-methylcatechol (**29**) was examined. Witayakran and co-workers report the reaction with compound **29** to be have been the most successful starting material to obtain the *o*-benzoquinone, 4,6,7-trimethylnaphthalene-1,2-dione (**39**) in 57% yield. The latter was proven to be a more cooperative affair (**Table 7**). The reaction (**Entry 1, Table 7**) was implemented in the same manner as with catechol (**25**), and gave the desired quinone **39** in 24% yield.

Table 7: Enzymatic reaction with catechol, Laccase *Tremetes vesicolor i*n acetate buffer solution (0.1 M, pH 4.5), 4-methyl-catechol (**29**) and 2,3-dimethyl-1,3-butadiene (10 equiv.) (**27**).



29 **27** **39**

Entry	atm	t [h]	Yield [%] **39**
1	Air	23	29
2	Air	5	23
3	Air	9	38
4	Ar	48	0

Two more experiments were performed with aims to improve the yield by adjusting the reaction time, **Entry 2** and **Entry 3** in **Table 7**. Reducing the reaction time to 5 and 8 hours resulted in yields of 23% and 38%, respectively. A final experiment was conducted under argon atmosphere, and as expected nothing from the target compound was seen according to TLC analysis, proving the importance of oxygen.

The laccase reactions provided quinones **28**, **39** and **40**, but only trace amount of **28** (<9%). Therefore, a new method was approached in order to retrieve the dimethylated *o*-benzoquinone, and the starting material was changed.

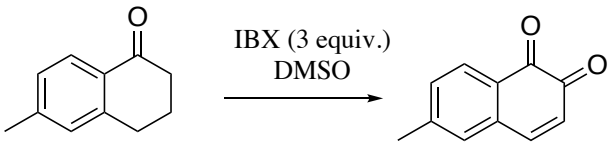
Selective oxidation of 1-tetralones to 1,2-naphthoquinones

Since IBX (**31**) already had been synthesized and the method of Ren *et al*. [80] had verified the catechol oxidation, by LRMS, oxidation of 1-tetralons (**41**, **42**) became the new approach **(Figure 12)**.

41 **42**

Figure 12: Starting material 6-methyl-3,4-dihydro-2*H*-naphthalene-1-one (**41**) and 5,7- dimethyl-1-tetralone (**42**) used in the synthesis of dione precursors to the *trans*-dihydrodiol naphthalens with one and two methyl groups, respectively.

Starting material **41** (**Entry 1, Table 8**) was dissolved in DMSO, and IBX (**31**) (3 equiv.) was added to the solution (**Scheme 14**).



41 **43**

Scheme 14: Selective oxidation of 6-methyl-3,4-dihydro-2*H*-naphthalene-1-one (**41**) into 6-methylnaphthalene-1,2-dione (**43**) by IBX in DMSO.

After 12 hours the TLC analysis indicated formation of a new product (R_f = 0.4 in 75:25 v/v pet. ether/EtOAc). There was also more starting material left, still purification was conducted in order to confirm target compound **43.**

^{13}C-NMR in **Figure 13** shows the two carbonyl signals to be 181.7 and 177.5 ppm, and the doublets at 7.35 and 6.43 ppm have a coupling constant of 10.1 Hz.

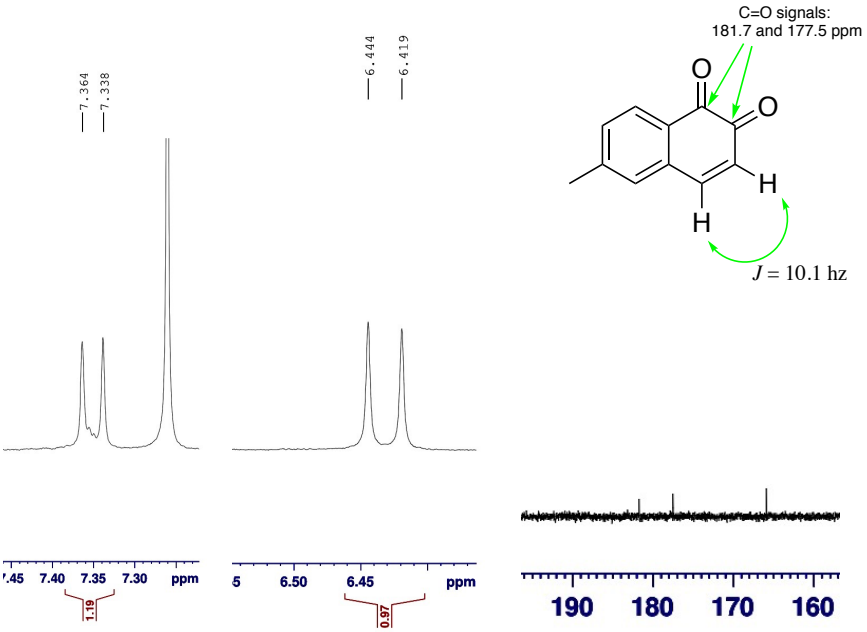
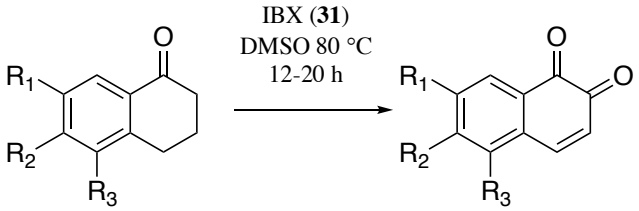


Figure 13: ^{1}H-NMR and ^{13}C-NMR analysis confirmed product **43**.

The yield of the reaction was low giving only 10% of target compound **43** and 53% recovery of starting material (**41**). Apparently, the reaction needed more IBX (**31**) in order to reach completion.

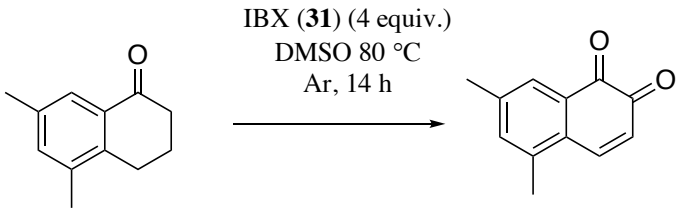
Table 8: Selective oxidation of 1-tetralone by IBX in DMSO at 80 °C under argon atmosphere.



Entry	R	Equiv. IBX	t [h]	Yield [%]
1	R_1=H, R_2=CH3, R_3=H	3.0	12	10[a]
2	R_1=CH_3, R_2=H, R_3=CH_3	4.0	14	86
3	R_1=H, R_2=CH3, R_3=H	4.4	20	Nd[b]

[a]Recovery of start material was 53%. [b]The crude mass was extracted, dried and weighed to perform reduction without other purification.

In **Entry 2**, the procedure was carried out in the same manner, however the starting material was changed to 5,7- dimethyl-1-tetralone (**42**) and 4 equiv. of IBX (**31**) were added in portions to the reaction mixture within 20 minutes (**Scheme 15**).



42 **44**

Scheme 15: Oxidation of 5,7- dimethyl-1-tetralone (**42**) into 5,7-dimethylnaphthalene-1,2-dione (**44**) by IBX (**31**) in DMSO.

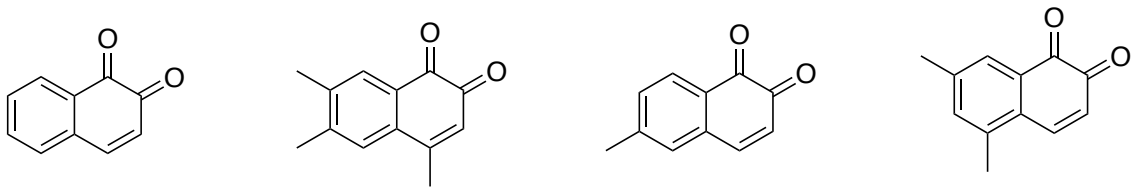
LRMS confirmed the target molecule 5,7-dimethylnaphthalene-1,2-dione (**44**), which was isolated in 86% yield. Since there were, according to TLC analysis, a small amount of starting material left, the reaction was allowed to stir longer. After 4 hours, starting material was left in solution, and there were no indications of change, thus the reaction mixture was quenched with distilled water.

Based on the last reaction, precursor, 6-methyl-3,4-dihydro-2*H*-naphthalene-1-one (**41**), to the methylated *trans*-dihydrodiol (**19**) was pursued by applying the method above, however the amount of IBX (**31**) was slightly increased (4.4 equiv.) to see if all the starting material would be converted.

According to TLC-MS analysis, the target compound **43** was formed. However, due to possible decomposition of unstable *o*-quinones, it was decided to reduce **43** immediately after extraction.

Reduction of *o*-naphthoquinones

In general, *o*-naphthoquinone products **35**, **39, 43** and **44** in **Figure 14** were reduced into the corresponding *trans*-dihydrodiols by the procedure introduced by Ray *et al*. [88] (**Table 9**).

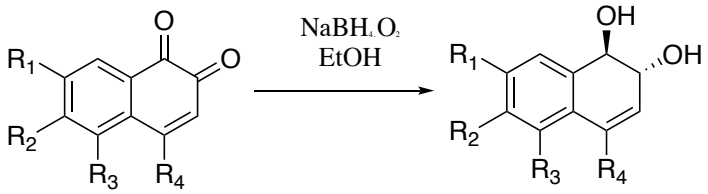


35 **39** **43** **44**

Figure 14: Precursor compounds **35**, **39, 43** and **44** to target compounds **13±**, **21±, 19±** and **20±**, respectively.

Naphthoquinones were dissolved in EtOH and $NaBH_4$ (11 equiv.) was added to solution in portions following the application of oxygen atmosphere.

Table 9: Stereoselective reduction of *o*-naphthoquinones by $NaBH_4$ (11 equiv.) under O_2 atmosphere in EtOH.



Entry	[a]SM	R	t [h]	Yield ([b]**TC**) [%]
1	**35**	R_1=R_2=R_3=R_4= H	12	(**13±**) 29
2	**35**	R_1=R_2=R_3=R_4= H	12	(**13±**) 39
3	**35**	R_1=R_2=R_3=R_4= H	48	(**13±**) 40
4[c]	**39**	R_3= H, R_1=R_2= R_4= CH_3	17	(**21±**) 7
5	**44**	R_2= R_4=H, R_1= R_3= CH_3	12	(**20±**) 32
6[d]	**43**	R_1= R_3=R_4=H, R_2= CH_3	10	(**19±**) 59

[a]Starting material. [b]Target compound. [c]The eluent was not suitable, and that may explain the low yield. [d]Elimination of purification step after oxidation.

Starting material in **Entry 1**-**3**, naphthalene-1,2-dione (**35**), was successfully reduced into target molecule *trans*-1,2-dihydronaphthalene-1,2-diol (**13±**) with moderate yields; 29%, 39% and 40%, respectively. The large coupling constant (J = 9.9 Hz) between H-1 and H-2 in ^{1}H-NMR gave the relative trans-stereochemistry of 1,2-diol **13±** (**Figure 15**).

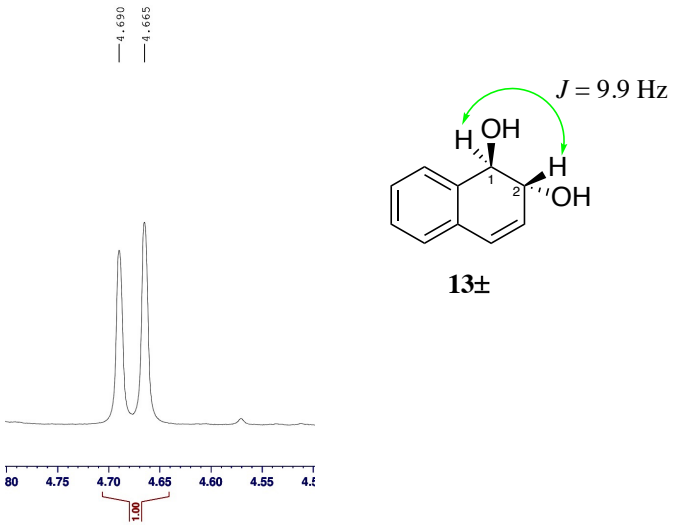


Figure 15: ^{1}H-NMR analysis of target compound **13±** indicate a *trans*-configuration instead of *cis*-configuration.

Platt and Oesch [87] made the same compound (**13)** in 1983, and reported the equivalent proton signal to have coupling constant of 10.5 Hz in Acetone-d_6/deuterium oxide.

4,6,7-Trimethylnaphthalene-1,2-dione (**39**) (**Entry 4**, **Table 9**) was reduced into *trans*-4,6,7-trimethyl-1,2-dihydronaphthalene-1,2-diol (**21±**) by the same procedure. However, after purification by flash chromatography (silica, pet. ether → 60:40 v/v pet. ether/EtOAc) only 7% of target compound was obtained. The low yield might be due to solubility in EtOAc and pet. ether eluent used for the purification by silica column chromatography. It was also conspicuously few fractions to be collected, perhaps indicating that this molecule is less prone to absorb UV light at 254 nm, and therefore fractions containing the molecule might have been omitted during purification.

Based on the experience from **Entry 4**, precautions were made when purifying target compound *trans*-4,6,7-trimethyl-1,2-dihydronaphthalene-1,2-diol (**21±)** (**Entry 5**, **Table 9).** The new eluent system was carefully chosen (silica, 97:3 v/v pet. ether/$CHCl_3$ → 40:57:3 v/v/v pet.

ether/EtOAc/$CHCl_3$), Puriflash Interchim was used in order to apply constant pressure and finer silica gel (mesh size 30 μm) to improve separation. After applying these measures, target molecule **20±** was afforded in sparingly 32%.

The last experiment (**Entry 6**, **Table 9**), reduction of 6-methylnaphthalene-1,2-dione (**43**) into *trans*-6-methyl-1,2-dihydronaphthalene **19±** was performed without other purification than extraction, drying, filtration and vacuum line (4 h). Due to the instability of the *o*-quinones reported previously, an attempt was made to increase the yield by eliminating one purification step. Purification in the same manner as for **Entry 5**, resulted in the isolation of 59% of target molecule (**19±**).

3. Conclusions

trans-1,2-Dihydronaphthalene-1,2-diol (**13±**), *trans*-6-methyl-1,2-dihydronaphthalene-1,2-diol (**19±**), *trans*-5,7-dimethyl-1,2-dihydronaphthalene-1,2-diol (**20±**) and *trans*-4,6,7-trimethyl-1,2-dihydronaphthalene-1,2-diol (**21±**) were synthesized, and their overall yield was 40%, 59%, 28% and 3%, respectively. 6,7-Dimethylnaphthalene-1,2-dione (**28**) and 2,3,6,7-tetramethylphenanthrene-9,10-dione (**40**) yielded 9% and 12%, respectively. Compunds were synthesized by oxidation using IBX or Laccase catalyzed Diels-Alder reaction, and reduction by $NaBH_4$ to give the *trans*-naphtalene dihydro diols. All target compounds were successfully obtained by synthesis, and are ready to be used as standards for GC-MS analysis.

4. Future perspective

With standards in hand; *trans*-1,2-dihydronaphthalene-1,2-diol (**13±**), *trans*-6-methyl-1,2-dihydronaphthalene-1,2-diol (**19±**), *trans* -5,7-dimethyl-1,2-dihydronaphthalene-1,2-diol (**20±**) and *trans*-4,6,7-trimethyl-1,2-dihydronaphthalene-1,2-diol (**21±**), and prepared bile samples, GC-MS analysis will be the next step. This will be done in order to broaden the overview of the overal exposure to naphthalene

5. Experimental

5.1 Synthesis

Chemicals

1,2-Naphthoquinone (**35**), 1,2-dihydroxybenzene (**25**), 5,7-dimethyl-1-tetralone (**42**) 2,3-dimethyl-1,3-butadiene (**27**), 4-methylcatechol (**29**) and 6-methyl-3,4-dihydro-2H-naphthal (**41**), sodium borohydride and Laccase from *Trametes Versicolor* (EC1.10.3.2) were obtained from Sigma Aldrich and used without further purification. DMSO, $CHCl_3$, triethylamine and pyridine were dried over 4 Å molecular sieves. IBX (**31**) was synthesized in laboratory.

Thin-Layer Chromatography (TLC) and Flash Chromatography

Separation and purification was performed by flash chromatography. Silica gel 60 (mesh size 0.040-0.063 mm) from Merck. Reactions were monitored by thin-layer chromatography (TLC) carried out on aluminium backed 0.2 mm thick silica gel 60 F_{254} sheets from Merck using 254 nm UV lamp for visualizing the spots. Automated flash chromatography was performed on an Interchim PuriFlash® 215 chromatography system. Detection at 254 nm.

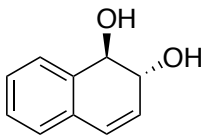
Spectroscopic analysis

Infrared absorption spectroscopy was performed on a Cary 630 FTIR from Agilent Technologies. Proton (^{1}H) and carbon (^{13}C) NMR spectra were conducted on an Ascend™ 400 NMR spectrometer from Bruker, which operated at 400 MHz and 100 MHz for proton and carbon, respectively. Chemical shifts (δ) are reported relative to residual DMSO (δ 2.50 ppm, ^{1}H; δ 39.52 ppm, ^{13}C), residual $CHCl_3$ in $CDCl_3$ (δ 7.26 ppm, ^{1}H; δ 77.16 ppm, ^{13}C) and residual CD_3OD (δ 3.31 ppm, ^{1}H; δ 49.0 ppm, ^{13}C) as references. ^{1}H-NMR data are reported by the following sequence: chemical shift (δ) [multiplicity, coupling constant(s) *J* (Hz), relative

integral] in which multiplicity is reported as: s = singlet; d = doublet; t = triplet; q = quartet; quint = quintet; dt = doublet of triplet; m = multiplet; bs = broad singlet. For ^{13}C NMR spectra, data is reported as chemical shift (δ). Other NMR spectra used to aid the structural interpretations has been heteronuclear single quantum correlation spectroscopy (HSQC).

Melting points (mp) were determined on a Stuart SMP20 melting point apparatus and are uncorrected. Low resolution mass spectra were recorded on an Advion expression[s] CMS mass spectrometer operating at 3.5 kV in electrospray ionization (ESI) mode.

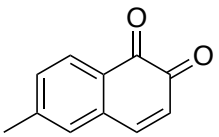
***trans*-1,2-Dihydronaphthalene-1,2-diol (13±)**



13±

EtOH (12 mL) was added to a flask containing 1,2-naphthoquinone (**35**) (100 mg, 0.63 mmol) and $NaBH_4$ (255 mg, 6.74 mmol, 10.7 equiv.). The atmosphere was changed to O_2, and the reaction mixture was left to stir overnight. The resulting reaction mixture was evaporated onto Celite®, and transferred to a prepacked flash column for purification (silica, pet. ether → 75:25 v/v pet. ether/EtOAc). Concentration of relevant fractions (R_f = 0.5 in 75:25 v/v pet. ether/EtOAc) resulted in compound **13±**, (40 mg, 39%) as a white solid, mp 105.7-106.0 °C (lit.[86] 105-106 °C) **IR** ν_{max} 3271, 3034, 2924, 2852, 2320, 2105, 1919, 1475, 1376, 1246, 1215, 1188, 1158, 1039, 975 cm^{-1}; ^{1}**H NMR** (400 MHz, CD_3OD) δ 7.53-7.51 (m, 1H), 7.25-7.19 (m, 2H), 7.09-7.07 (m, 1H), 6.43 (dd, *J* = 9.9 and 2.1 Hz, 1H), 5.92 (dd, *J* = 9.8 and 2.6 Hz, 1H), 4.68 (d, *J* = 9.9 Hz, 1H), 4.37-4.33 (m, 1H); ^{13}**C NMR** (100 MHz, CD_3OD) 138.3, 133.9, 131.7, 128.7, 128.7, 128.5, 127.3, 126.6, 75.5, 73.9. Spectroscopic data previously reported literature [87] did not provide ^{13}C NMR, and ^{1}H NMR was conducted in Acetone/D_2O, thus signals are not comparable. HRMS will be conducted.

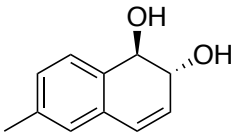
6-Methylnaphthalene-1,2-dione (43)



43

IBX (**31**) (105 mg, 0.37 mmol, 4.4 equiv.) was added in portions to a solution of 6-methyl-1-tetralone (**41**) (20 mg, 0.13 mmol) in dry DMSO (3 mL). After 12 h the reaction mixture was quenched with distilled water (10 mL) and extracted with EtOAc (3 x 15 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution (1 x 15 mL) and water (1 x 15 mL), dried ($MgSO_4$), filtered and evaporated onto Celite®. Purification was performed on a Puriflash Interchim 215 system (silica, 95:5 v/v pet. ether/EtOAc $\rightarrow$ 50:50 v/v pet. ether/EtOAc). Concentration of relevant fractions (R_f = 0.4 in 75:25 v/v pet. ether/EtOAc) resulted in recovery of starting material **41** (10.5 mg, 53%) and target compound **43**, (2.1 mg, 10%) as an orange solid, mp. 96-99°C. **IR** ν_{max} 2923, 2853, 1730, 1685, 1662, 1587, 1499, 1457, 1377, 1298, 1208, 1185, 1136, 1092, 1020, 957 cm^{-1}; ^{1}**H NMR** (400 MHz, $CDCl_3$) δ 8.11 (d, *J* = 8.6 Hz, 1H), 7.35 (d, *J* = 10.1 Hz, 1H), 6.95 (dd, *J* = 8.6 and 2.4 Hz, 1H), 6.83 (d, *J* = 2.4 Hz, 1H), 6.43 (d, *J* = 10.1 Hz, 1H), 3.94 (s, 3H); ^{13}**C NMR** (100 MHz, $CDCl_3$) 181.7, 177.5, 165.8, 144.9, 137.1, 133.4, 128.8, 125.2, 116,1, 114.9, 56.1. HRMS will be provided.

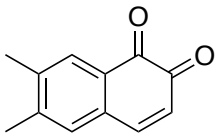
***trans*-6-Methyl-1,2-dihydronaphthalene-1,2-diol (19±)**



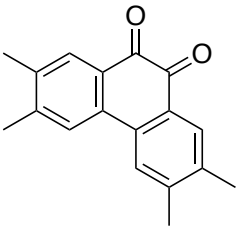
19±

IBX (**31**) (194 mg, 0.69 mmol, 4.4 equiv.) was added in portions to a solution of 6-methyl-1-tetralone (**41**) (25 mg, 0.16 mmol) in dry DMSO (3 mL). After 21 h the reaction mixture was quenched with distilled water (10 mL) and extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution (2 x 20 mL), dried (Na_2SO_4), filtered and concentrated under reduced pressure. The residue, which contained compound **43**, was placed on a vacuum line for 3 h prior to the next step. Residue was then dissolved in EtOH (15 mL) and $NaBH_4$ (60 mg, 1.6 mmol, 11 equiv.) was added to the solution in portions (3 x 20 mg). The atmosphere was changed to O_2, along with protection against light. After 17 h the reaction mixture was quenched with distilled H_2O (10 mL), and extracted with EtOAc (3 x 15 mL). The combined organic layers were washed with brine (15 mL). Solvent was evaporated onto Celite® followed by purification on a Puriflash Interchim 215 (silica, 97:3 v/v/v $CHCl_3$/EtOAc → 90:10 v/v $CHCl_3$/EtOAc). Concentration of relevant fractions (R_f = 0.2 in 85:15 v/v $CHCl_3$/EtOAc) resulted in compound **19±**, (16.1 mg, 59%) as a white solid, mp. 114-116 °C. **IR** ν_{max} 3295, 2923, 2852, 1717, 1600, 1574, 1498, 1462, 1443, 1375, 1310, 1252, 1168, 1093, 1045, 1029, 971 cm^{-1}; ^{1}**H NMR** (400 MHz, $CDCl_3$) δ 7.42 (d, *J* = 8.3, 1H), 8.74 (dd, *J* = 8.4 and 2.4, 1H), 6.60 (d, *J* = 2.3, 1H), 6.33-6.31 (m, 1H), 5.93 (dd, *J* = 10.0 and 2.0, 1H), 4.69 (d, *J* = 10.4, 1H), 4.38 (d, *J* = 10.4, 1H), 2.83 (s, 3H); ^{13}**C NMR** (100 MHz, $CDCl_3$) 159.4, 133.7, 131.2, 128.7, 127.6, 126.2, 112.5, 112.3, 74.6, 73.7, 55.4. Spectroscopic data were not found in literature. HRMS will be provided.

6,7-Dimethylnaphthalene-1,2-dione (28)



28



40

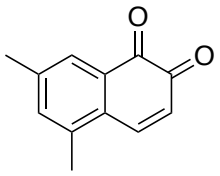
1,2-Dihydroxybenzene (**25**) (50 mg, 0.45 mmol) was dissolved in ice-cold acetate buffer (10 mL, 0.1 M, pH 4.5) containing Laccase (60 mg, 56.4 U). The mixture was added dropwise to a stirred solution of 2,3-dimethyl-1,3-butadiene (**27**) (260 mg, 3.1 mmol, 7 equiv.) in acetate buffer (10 mL) placed in an ice bath over a stirring plate, and exposed to air. The reaction mixture was protected from light, within the next three hours of reaction; Laccase (60 mg, 56.4 U) was added each hour and allowed to stir at room temperature after the last addition. The reaction mixture was extracted after 24 hours with EtOAc (4 x 6 mL) and centrifuged (4 min, 4000 rpm). The combined organic layers were evaporated onto Celite®, and transferred to a prepacked flash column for purification (silica, pet. ether $\rightarrow$ 90:10 v/v pet. ether/EtOAc). Concentration of relevant fractions of A (R_f = 0.5 in 75:25 v/v pet. ether/Et_2O) resulted in byproduct **40**, (6.8 mg, 6%) as a bright orange colored solid, 237 °C (decomposed) (lit. 250-254 °C with some decomposition [69]). **IR** ν_{max} 2924, 2854, 2237, 17.38, 1676, 1664, 1603, 1551, 1486, 1453, 1386, 1313, 1246, 1219, 1200, 1081, 979, 913 cm^{-1}; ^{1}**H NMR** (400 MHz, $CDCl_3$) δ 7.84 (s, 2H), 7.61 (s, 2H), 2.37 (s, 6H), 2.29 (s, 6H); ^{13}**C NMR** (100 MHz, $CDCl_3$) 180.5, 146.1, 138.2, 134.0, 131.4, 129.1, 124.9, 20.9, 19.4.

Spectroscopic data are in accordance with previously reported literature [69]

Concentration of relevant fractions of B (R_f = 0.3 in 75:25 v/v pet. ether/EtOAc) resulted in target compound **28**, (5.6 mg, 9%) as a light orange solid. **IR** ν_{max} 2955, 2853, 1737, 1690, 1664,

1598, 1557, 1458, 1414, 1376, 1283, 1260, 1231, 1183, 1140, 1081, 1022, 967, 934 cm^{-1}; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.37-7.34 (m, 1H), 7.10 (s, 1H), 6.35 (d, *J* = 10.1 Hz, 1H), 2.35 (s, 3H), 2.33 (s, 3H); **^{13}C NMR** (100 MHz, $CDCl_3$) 181.5, 179.1, 146.0, 145.6, 140.3, 131.8, 131.4, 129.8, 127.3, 124.6, 20.2, 19.8. Spectroscopic data was not found in literature, however reportet C=O IR signals correlate [66]. HRMS will be provided.

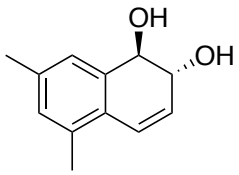
5,7-Dimethylnaphthalene-1,2-dione (44)



44

IBX (**31**) (0.96 mg, 3.4 mmol, 4 equiv.) was added in portions to a solution of 5,7-dimethyl-1-tetralone (**42**) (150 mg, 0.86 mmol) in dry DMSO (10 mL). After 21 h the reaction mixture was quenched with distilled water (10 mL) and extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution (2 x 20 mL), dried ($MgSO_4$), filtered and evaporated onto Celite®. Purification was performed on a Puriflash Interchim 215 (silica, 95:5 v/v pet. ether/EtOAc $\rightarrow$ 50:50 v/v pet. ether/EtOAc). Concentration of relevant fractions (R_f= 0.5 in 75:25 v/v pet. ether/EtOAc) resulted in compound **44**, (96.3 mg, 86%) as an orange solid, mp. 96-99 °C. **IR** ν_{max} 2921, 2853, 1659, 1611, 1380, 1298, 1260, 1211, 1163 cm^{-1}; ^{1}**H NMR** (400 MHz, $CDCl_3$) δ 7.77 (s, 1H), 7.70 (d, J = 10.44 Hz, 1H), 7.27-7.26 (m, 1H), 6.36 (d, J = 10.46 Hz, 1H), 2.44 (s, 3H), 2.36 (s, 3H); ^{13}**C NMR** (100 MHz, $CDCl_3$) 181.2, 179.9, 142.0, 141.5, 138.6, 137.8, 132.2, 130.2, 129.5, 126.2, 21.3, 18.8. Spectroscopic data were not found in literature. HRMS will be provided.

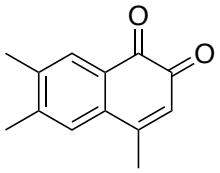
***trans*-5,7-Dimethyl-1,2-dihydronaphthalene-1,2-diol (20±)**



20±

5,7-Dimethylnaphthalene-1,2-dione (**44**) (85 mg, 0.46 mmol) was dissolved in dry EtOH (12 mL) and $NaBH_4$ (190 mg, 5.0 mmol, 11 equiv.) was added to the solution in portions (3 x 63.3 mg). The atmosphere was changed to O_2, along with protection against light. After 17 h the reaction mixture was quenched with distilled H_2O (10 mL), and extracted with EtOAc (3 x 15 mL). The combined organic layers were washed with brine (1 x 15 mL) followed by evaporation onto Celite® and purified on a Puriflash Interchim 215 (silica, 80:17:3 v/v/v pet. ether/EtOAc/$CHCl_3$ $\rightarrow$ 40:57:3 v/v/v pet. ether/EtOAc). Concentration of relevant fractions (R_f = 0.4 in 50:50 v/v pet. ether/EtOAc) resulted in compound **20±**, (28.3 mg, 0.15 mmol, 32%) as a white/light yellow solid, mp. 127-129 °C. **IR** ν_{max} 3347, 2922, 2854, 1609, 1454, 1376, 1302, 1253, 1166, 1064, 980 cm^{-1}; ^{1}**H NMR** (400 MHz, $CDCl_3$) δ 7.22 (s, 1H), 6.90 (s, 1H), 6.60 (dd, J = 10.06 and 2.00 Hz, 1H), 5.94 (dd, J = 10.08 and 2.14 Hz, 1H), 4.74 (d, J = 10.58 Hz, 1H), 4.46-4.42 (m, 1H), 2.61 (bs, 2H), 2.30 (s, 3H), 2.29 (s, 3H); ^{13}**C NMR** (100 MHz, $CDCl_3$) 137.4, 136.3, 133.9, 130.6, 128.9, 127.9, 124.6, 123.4, 75.6, 73.6, 21.5, 18.9. Spectroscopic data were not found in literature. HRMS will be provided.

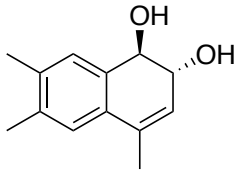
4,6,7-Trimethylnaphthalene-1,2-dione (39)



39

4-Methyl-1,2-benzenediol (**29**) (0.1 g, 0.81 mmol) was dissolved in cold acetate buffer (20 mL, 0.1 M, pH 4.5) containing Laccase (120 mg, 113 U). The mixture was added drop wise to a solution containing 2,3-dimethyl-1,3-butadiene (**27**) (0.7 g, 8.1 mmol, 10 equiv.) and acetate buffer (20 mL) placed in ice bath over a stirring plate, and exposed to air. The reaction mixture was protected from light, within the next three hours of reaction; Laccase (110 mg, 103 U) was added each hour and allowed to stir at room temperature after the last addition. After 10 hours the mixture was extracted with EtOAc (3 x 15 mL). The organic layers were evaporated onto Celite®, and transferred to a prepacked flash column for purification (silica, pet. ether → 75:25 v/v pet. ether/EtOAc). Concentration of relevant fractions (R_f = 0.44 in 80:20 v/v pet. ether/EtOAc) resulted in compound **39**, (61 mg, 38%) as an orange solid, mp 94-97 °C (decomposed) (lit. 112 C° (decomposed) [69]) **IR** ν_{max} 2921, 2854, 1902, 1740, 1685, 1654, 1599, 1550, 144, 1401, 1377, 1310, 1278, 1262, 1238, 1195, 1128, 1024, 968, 928, 900 cm^{-1}; ^{1}**H NMR** (400 MHz, $CDCl_3$) δ 7.80 (s, 1H), 7.20 (s, 1H), 6.24 (d, J = 0.76 Hz, 1H), 2.34 (s, 3H), 2.32 (d, J = 0.93 Hz, 3H), 2.29 (s, 3H); ^{13}**C NMR** (100 MHz, $CDCl_3$) 180.9, 179.7, 154.1, 145.6, 140.0, 133.5, 131.3, 129.2, 128.0, 126.8, 20.6, 20.6, 19.6. Spectroscopic data are in accordance with previously reported literature [69].

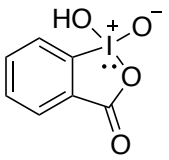
***trans*-4,6,7-Trimethyl-1,2-dihydronaphthalene-1,2-diol (21±)**



21±

4,6,7-Trimethylnaphthalene-1,2-dione (**39**) (46 mg, 0.23 mmol) was dissolved in dry EtOH and $NaBH_4$ (96 mg, 2.53 mmol, 11 equiv.) was added to the solution in portions (3 x 32 mg). The atmosphere was changed to O_2, along with protection against light, and after 17 h the reaction was complete. Solvent was removed under reduced pressure and crude residue was evaporated onto Celite®, and transferred to a prepacked flash column for purification (silica, pet. ether → 60:40 v/v pet. ether/EtOAc). Concentration of relevant fractions (R_f = 0.5 in 50:50 v/v pet. ether/EtOAc) resulted in compound **21±**, (3.3 mg, 10%) a white solid, mp. 129-131 °C. **IR** ν_{max} 3363, 2920, 2852, 1735, 1458, 1376, 1249, 1186, 1110, 1077, 1028, 969 cm^{-1}; ^{1}**H NMR** (400 MHz, CD_3OD) δ 7.26 (s, 1H), 7.03 (s, 1H), 5.67-5.66 (m, 1H), 4.54 (d, 1H), 4.24-4.20 (m, 1H), 2.27 (s, 3H), 2.25 (s, 3H), 2.04 (t, *J* = 1.72, 3H); ^{13}**C NMR** (100 MHz, CD_3OD) 136.8, 136.5, 135.7, 133.9, 132.9, 128.1, 127.3, 125.7, 75.4, 73.7, 19.7, 19.6, 19.1. Spectroscopic data were not found in literature. HRMS will be provided.

2-Iodoxybenzoic acid (IBX) (31)



31

2-Iodobenzoic acid (**32**) (5 g, 0.020 mol) was added to a solution of Oxone® (37.2 g, 0.061 mol, 3 equiv) and deionized water (200 mL). The suspension was set to 70 °C under argon atmosphere for 3 h. The reaction mixture was then cooled in ice bath for 1.5 h, and filtered through a glass sintered funnel, and the solid was washed with distilled water (6 x 50 mL), acetone (2 x 50 mL) and was allowed to dry at rt. for 16 h. The procedure resulted in compound **31** (2.62 g, 46%) as a white solid, mp. 237-239 °C (lit. 233 °C [79]). **IR** ν_{max} 3006, 2109, 1630, 1436, 1329, 1293, 1245, 1138, 1058 cm^{-1}; ^{1}**H NMR** (400 MHz, DMSO) δ 8.14 (d, *J* = 7.53 Hz, 1H), 8.04-7.98 (m, 2H), 7.86-7.82 (td, *J* = 7.44 and *J* = 0.89 Hz, 1H); ^{13}**C NMR** (100 MHz, DMSO) 167.4, 146.5, 133.3, 132.9, 131.4, 130.0, 125.0. Spectroscopic data are in accordance with previously reported literature [79].

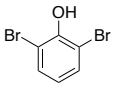
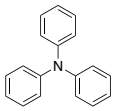
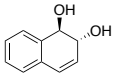
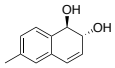
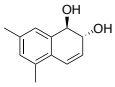
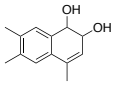
5.2 Preparation GC-MS

Chemicals

Triphenylamine (**24**) (98%) and 2,6-dibromophenol (**23**) (99%), were purchased from Chiron AS. *N,O*-Bis(Trimethylsilyl)trifluoroacetamid and β-glucuonidase and anhydrous sodium acetate were purchased from Sigma-Aldrich. Glacial acetic acid and EtOAc for GC analysis was purchased from VWR. *Trans*-dihydrodiol standards **13±**, **19±**, **20±** and **21±,** were synthesized at the University of Stavanger by Ingrid Caroline Vaaland.

Calibration standards, internal standard (GC IS) and surrogate standard (SU IS)

Table 10: Calibration standards; **13±**, **19±**, **20±** and **21±,** Internal standard (GC-IS) (**24**) and surrogate internal standard (SU IS) (**23**).

Diol-PAH metabolite information			TMS Ions	
Type	Full name	Structure	Diol-PAH	TMS-O-PAH (m/z)
SU IS	2,6-Dibromphenol (**23**)		249.86	321.90
GC IS	Triphenylamine (**24**)		245.12	
Diol-PAH	*trans*-1,2-Dihydronaphthalene-1,2-diol (**13±**)		162.07	306.15
Diol-PAH	*trans*-6-methyl-1,2-Dihydronaphthalene-1,2-diol (**19±**)		176.08	320.16
Diol-PAH	*trans*-5,7-dimethyl-1,2-Dihydronaphthalene-1,2-diol (**20±**)		190.10	334.18
Diol-PAH	*trans*-4,6,7-trimethyl-1,2-Dihydronaphthalene-1,2-diol (**21±**)		204.12	348.19

Fish bile samples and exposure experiment

Fish bile sample from Atlantic cod was obtained after exposing animals to dispersed crude oil [28]. Fish were caught with baited traps in Idsefjorden (Stavanger, Norway), and transferred to the laboratory facility and acclimatized in 1000 L glass-fiber tanks for approximately two weeks [28]. The exposure experiment was carried out in April 2015, with intention to simulate a field exposure to produced water by dispersed crude oil utilizing the continuous flow system (CFS) [98]. The PAH profile of the exposure is shown in **Table 11**. There were 65 fish divided into four groups: control, low (0.01 ppm), medium (0.05 ppm) and high (0.10 ppm). The first half

was sacrificed after 1 week of exposure, and the other half after four weeks. Three fish, in which belonged to the four-week high exposure group, died during the experiment. Fish were on average 2 years old (± 0.6), and there were 41 females, 18 males and 3 immature. Three fish that belong to the four-week group, died during the experiment. Samples of bile and liver were snap frozen in liquid nitrogen and stored at -80 °C until analysis [28].

Table 11: PAH profile for exposure experiment [28].

PAH	Conc. (µg/L)	PAH	Conc. µg/L
Naphthalene	6.7	C2-Dibenzothiophene	2.1
C1-Naphthalene	23	C3-Dibenzothiophene	0.05
C2-Naphthalene	29	Fluoranthene	0.06
C3-Naphthalene	44	Pyrene	0.14
Acenaphthylene	0.11	Benzo[*a*]anthracene	0.04
Acenaphthene	0.20	Chrysene	0.13
Fluorene	1.4	Benzo[*b*]fluoranthene	0.05
Phenanthrene	1.8	Benzo[*k*]fluoranthene	<0.01
Anthracene	0.02	Indeno[*1,2,3-c,d*]pyrene	<0.02
C1-Phenanthrene/Anthracene	5.0	Benzo[*g,h,i*]perylene	0.02
C2-Phenanthrene/Anthracene	8.7	Benzo[*a*]pyrene	0.03
C3-Phenanthrene/Anthracene	2.9	Dibenzo[*a,h*]anthracene	0.01
Dibenzothiophene	0.20	Sum 16 EPA-PAH	11
C1-Dibenzothiophene	0.77	Sum NPD	120

Fish bile sample preparation

The preparation of bile samples was performed as described in standard operating procedure (SOP) developed at IRIS, and is based on previous work by Krahn, Burrows *et al*. [99], Jonsson *et al*. [47] and Beyer *et al*. [100, 101]. In total 17 samples were prepared for analysis. Bile samples were obtained from -80 °C freezer and thawed on ice for about 30 minutes before hydrolysis. Buffer solution (1 L, 0.4 M, pH 5.0) was prepared by adding 500 mL distilled water to 33.14 g anhydrous sodium acetate, and pH was adjusted to 5.0 by adding concentrated acetic acid, followed by the addition of distilled water to reach a final volume of 1 L.

De-conjugation

β-Glucoronidase solution was made by diluting 1:10 by sodium acetate buffer (0.4 M, pH 5) and 300 µL was added to 1.5 mL eppendorf vials. Bile samples were mixed thoroughly on vortex before being transferred (300 µL) to the vials, and the weight was recorded. SU IS (40 µL) was added and the weight was recorded. The mixture was well mixed and placed in a heating cabinet for 2 h at 40 °C. The samples were then cooled on ice and protected against light from this point onwards.

Extraction

Ethyl acetate (2 mL) was added to 20 mL scintillation vials to prepare for extraction. Ethyl acetate (0.5 mL) was added to the cooled samples, mixed well followed by centrifuge at (4 °C, 2000 G, 3 minutes). The organic layer was transferred from eppendorf vial to the scintillation vial. The extraction procedure was repeated four times. (Since there was no trace of water in the samples, there was no need for sodium sulfate for drying purposes). Samples were stored in the fume hood overnight covered from light to evaporate the ethyl acetate to approximate 0.5 mL. The next day the samples were transferred to 2 mL amber vials with Teflon screw caps.

Derivatisation

N,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (200 µL, 0.19 g, 0.75 mmol) was added to the extracts, followed by incubation at 60 °C for 2 hours. The extracts were then cooled on ice for 15 minutes before GC IS (20 µL) was added. The weight of GC IS was recorded. The samples were mixed well and then transferred (100 µL) to 0.7 mL amber autosampler vials, and stored at 4 °C until analysis.

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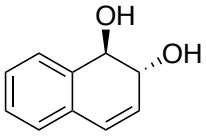
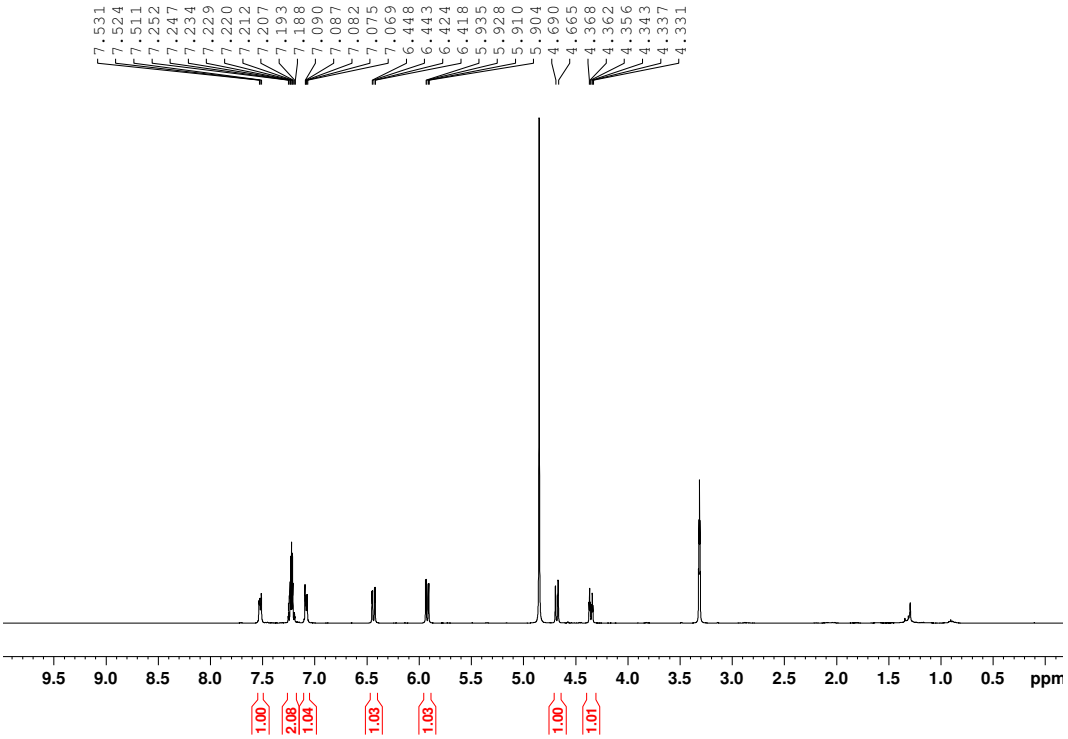
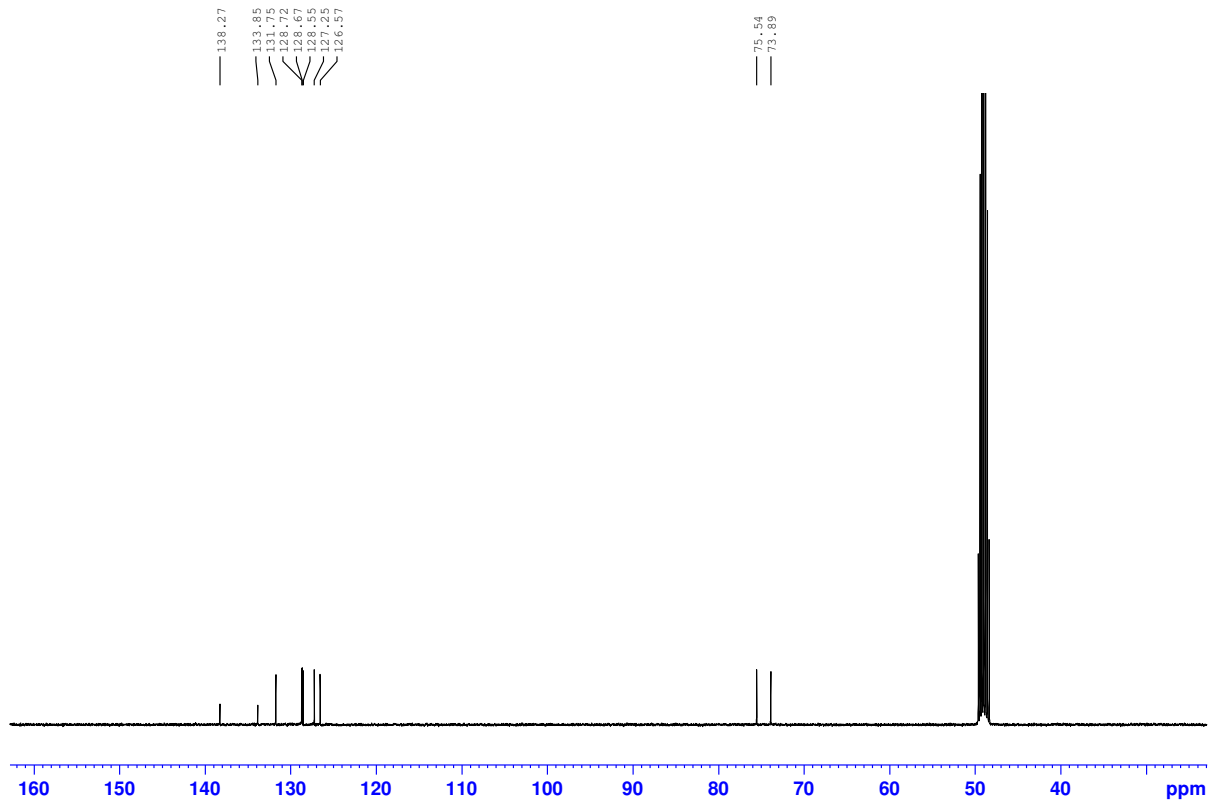
Appendix

^{1}H-NMR and ^{13}C-NMR

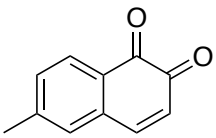
trans-1,2-Dihydronaphthalene-1,2-diol (13±)

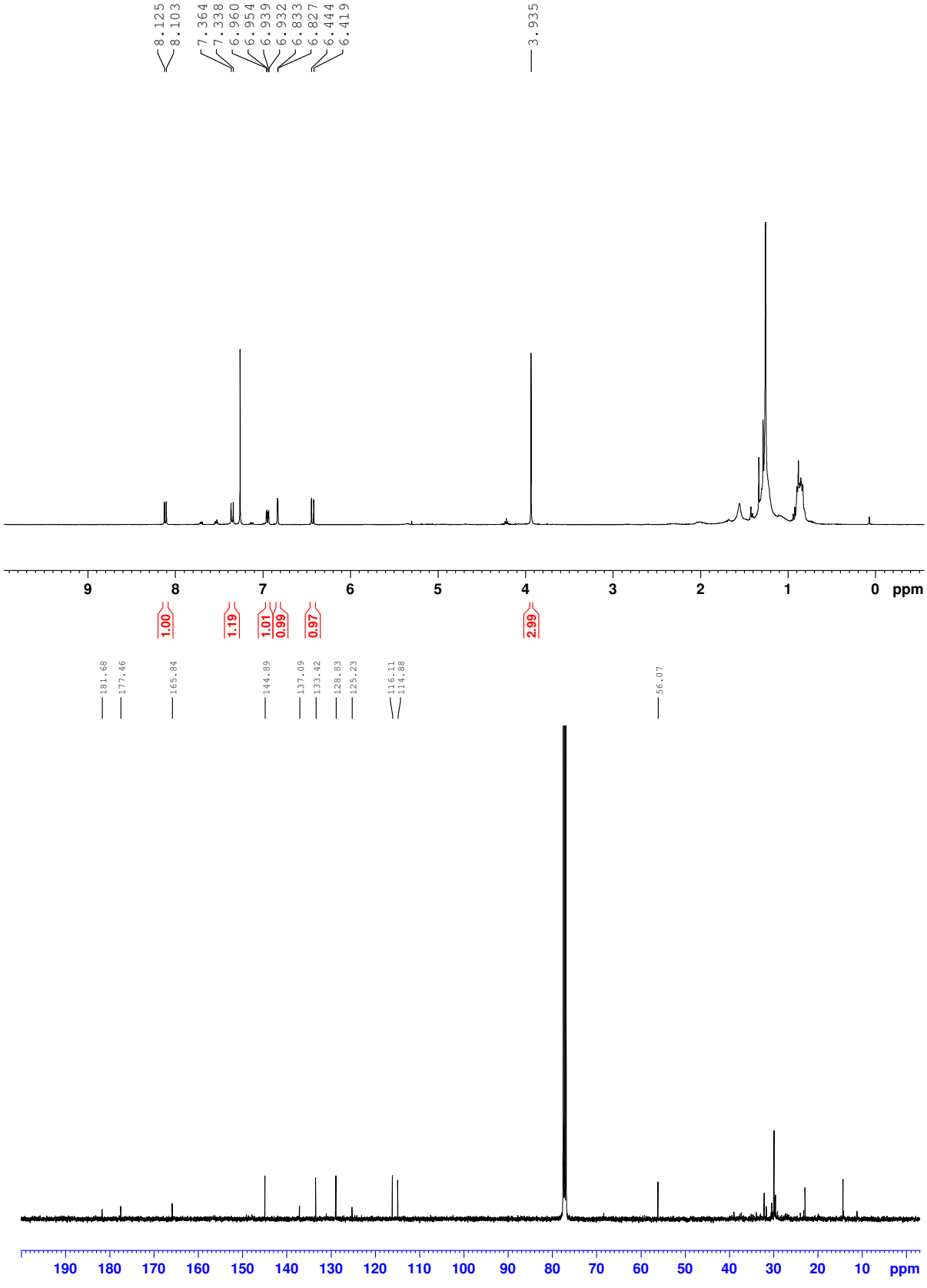




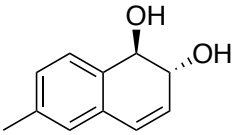


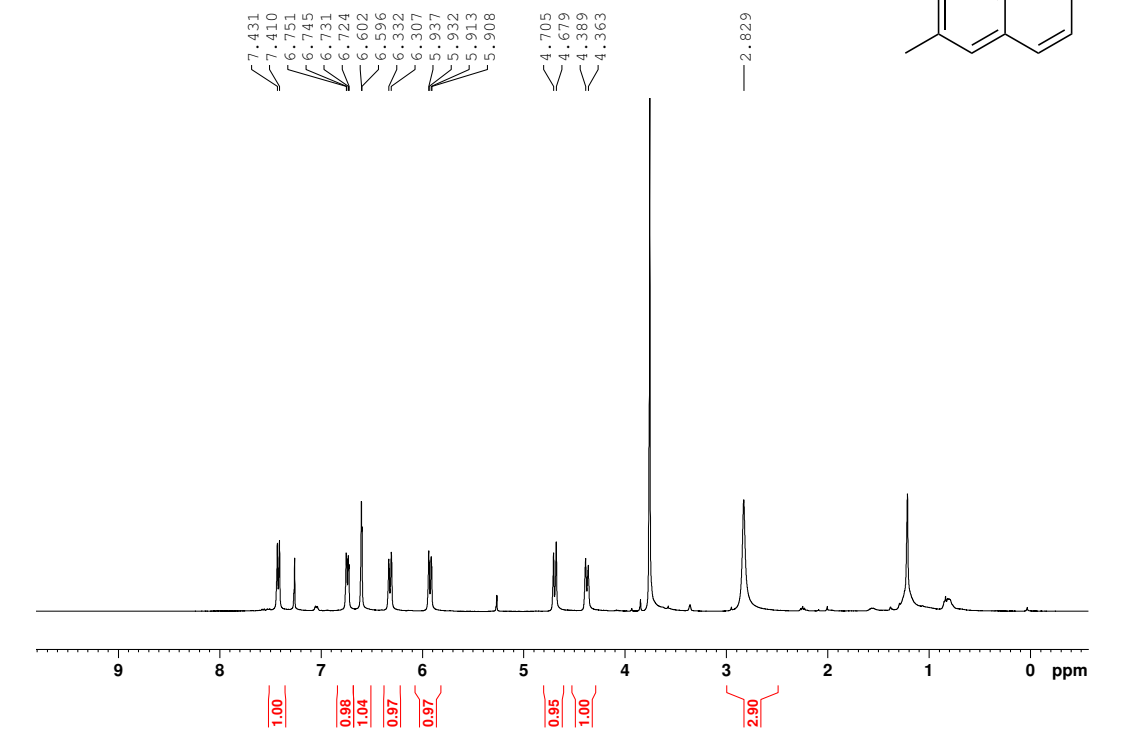
6-Methylnaphthalene-1,2-dione (43)

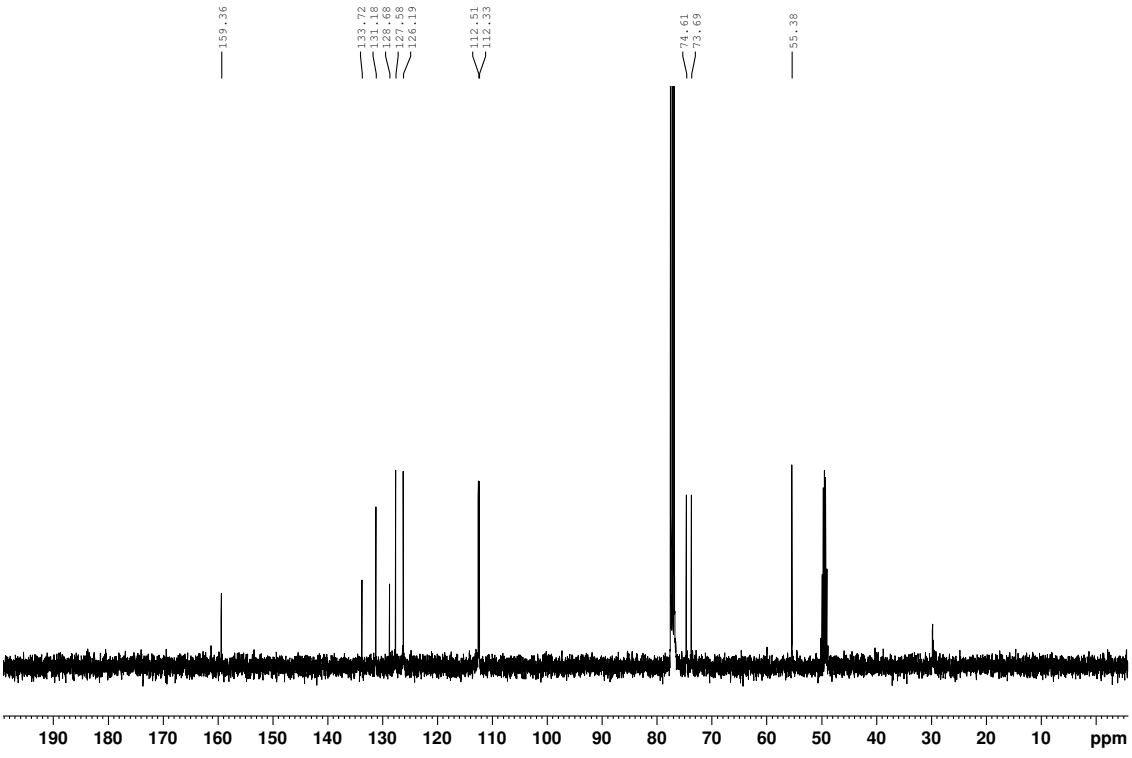




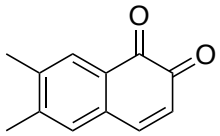
***trans*-6-Methyl-1,2-dihydronaphthalene-1,2-diol (19±)**

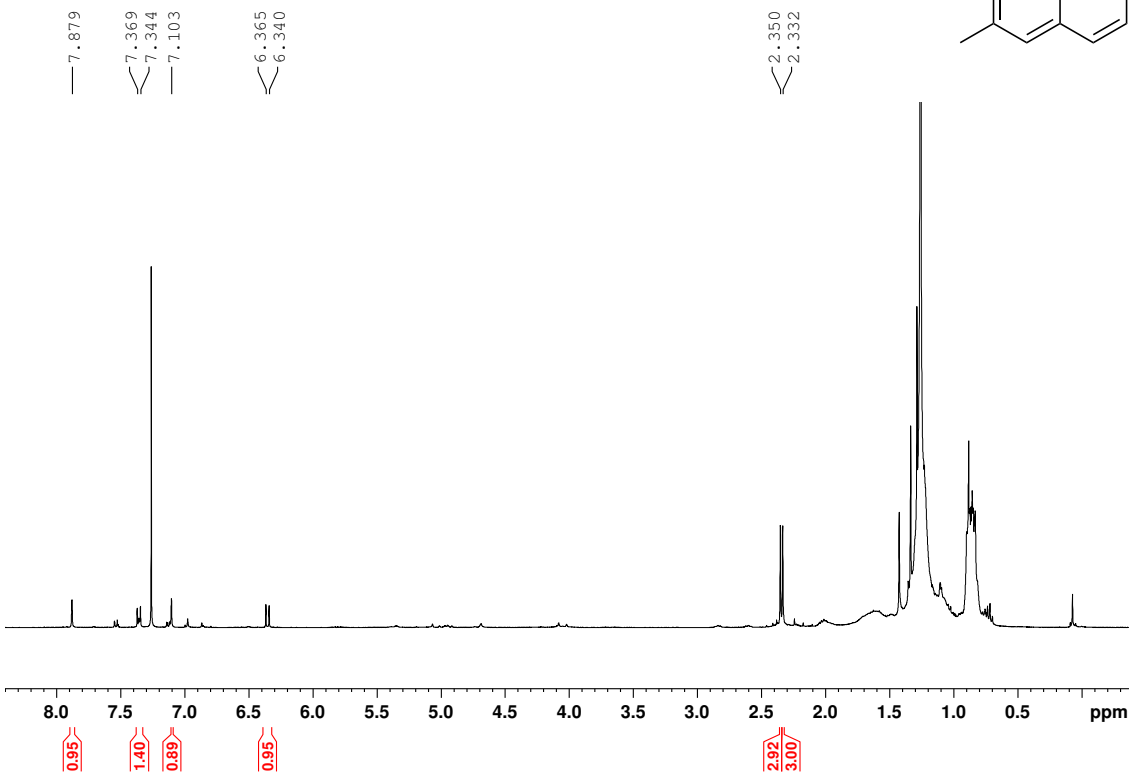


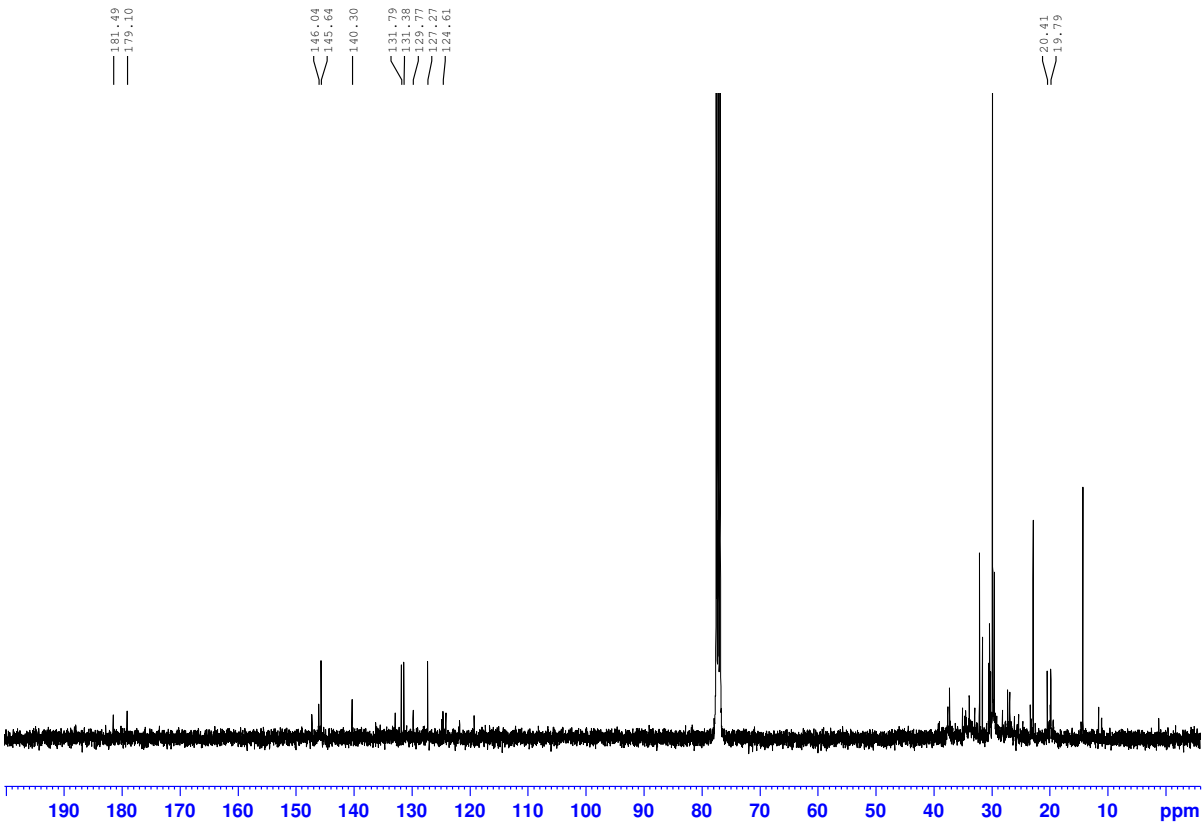




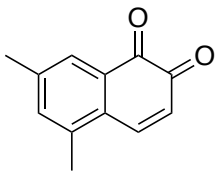
6,7-Dimethylnaphthalene-1,2-dione (28)

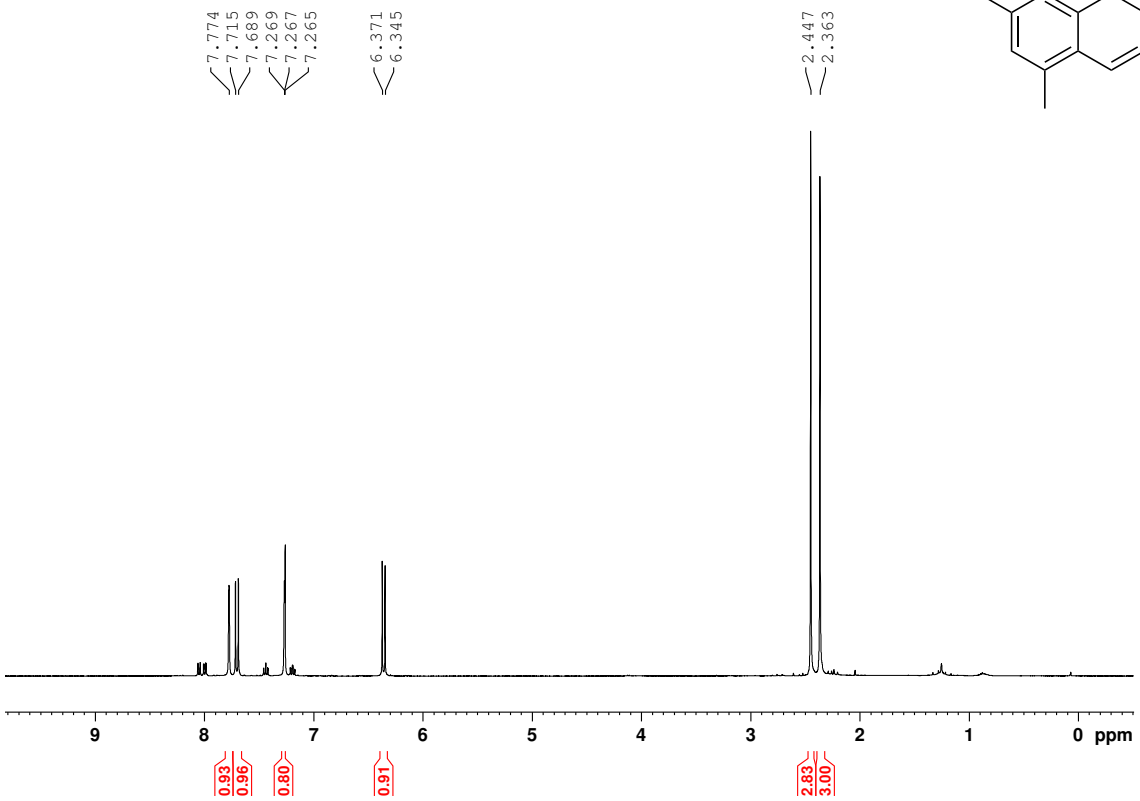


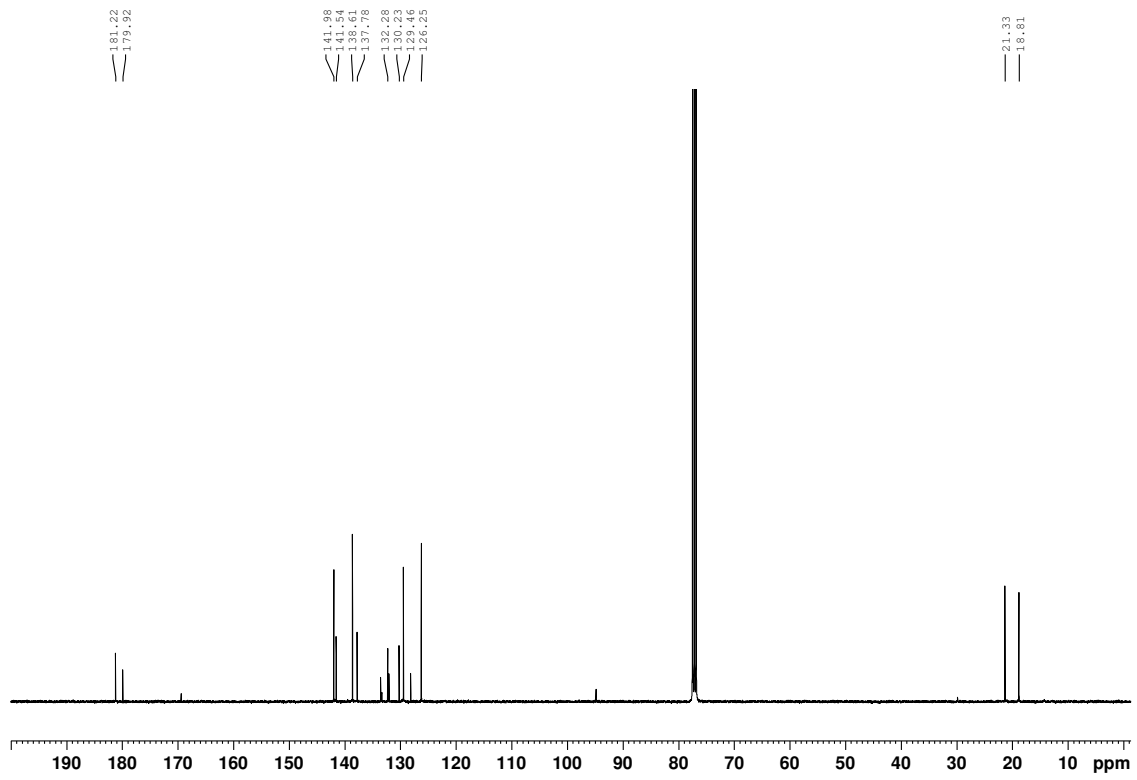




5,7-Dimethylnaphthalene-1,2-dione (44)







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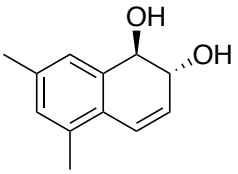
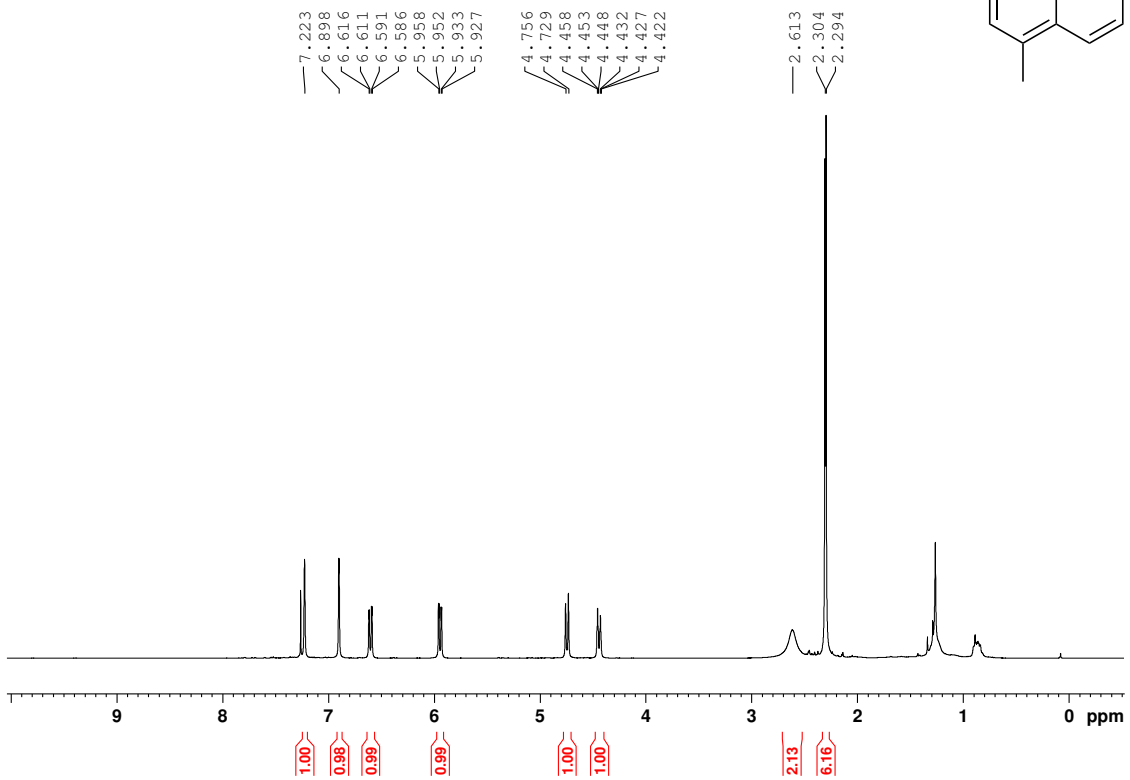
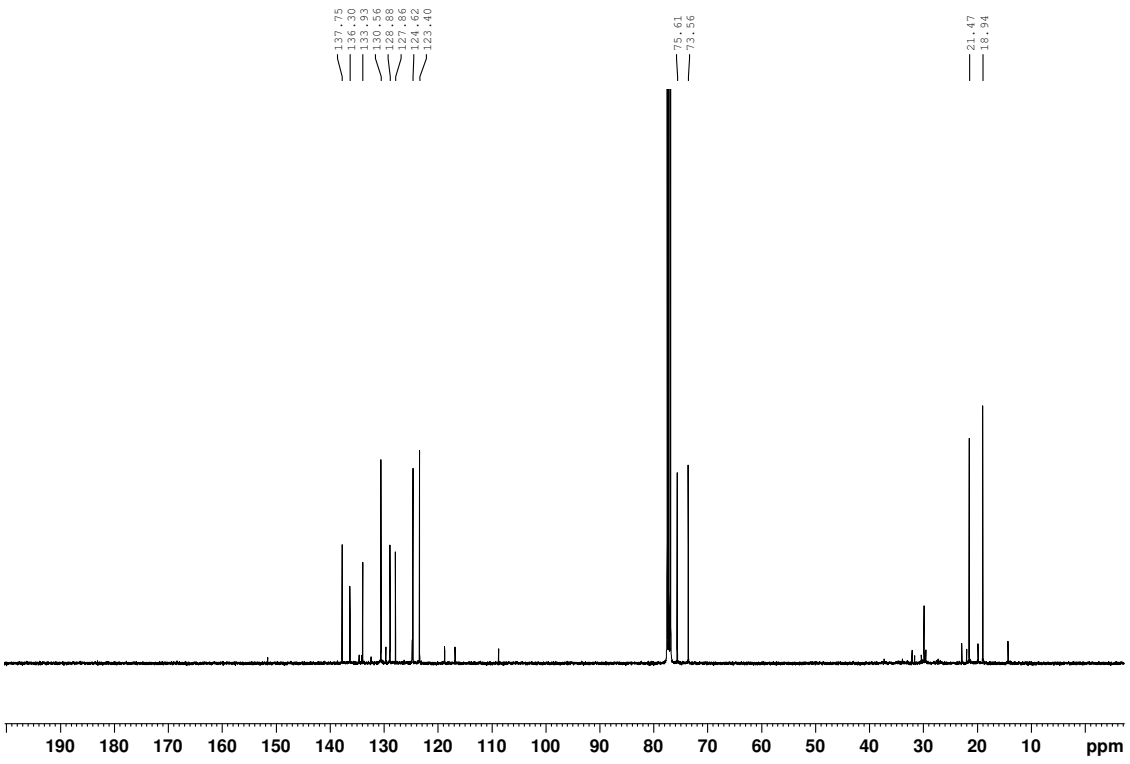
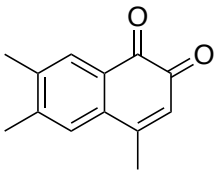
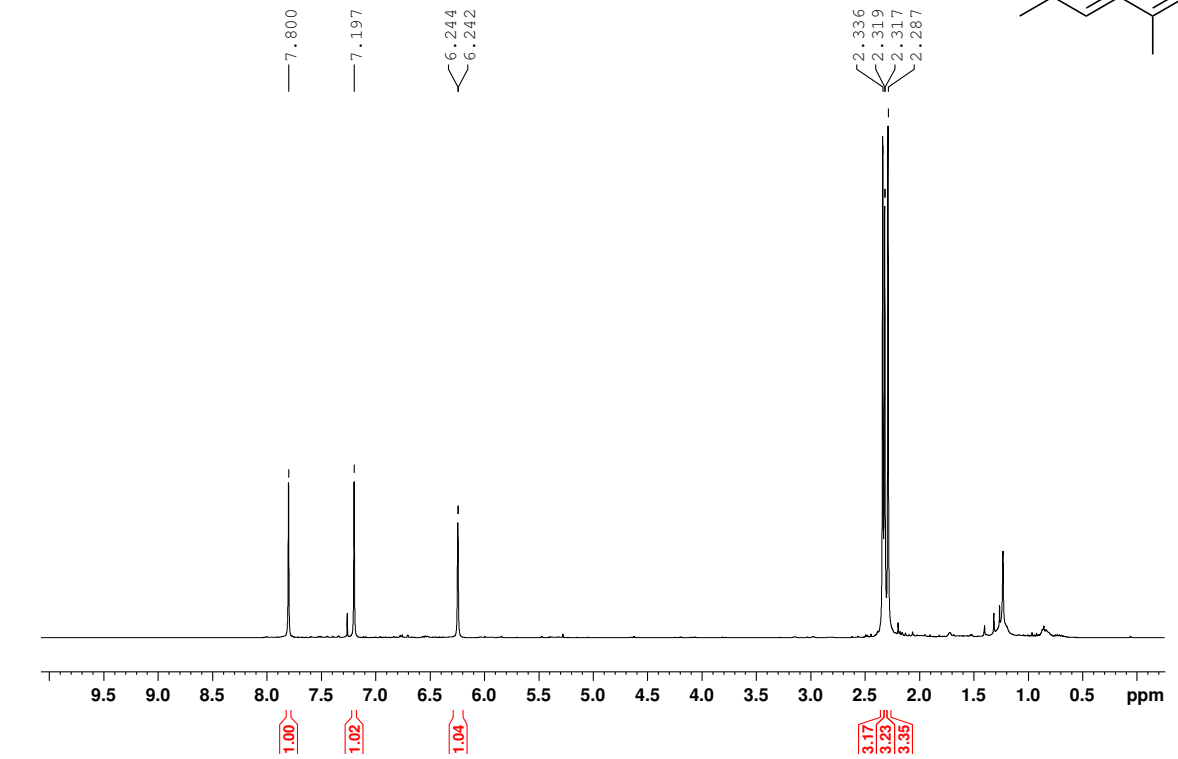


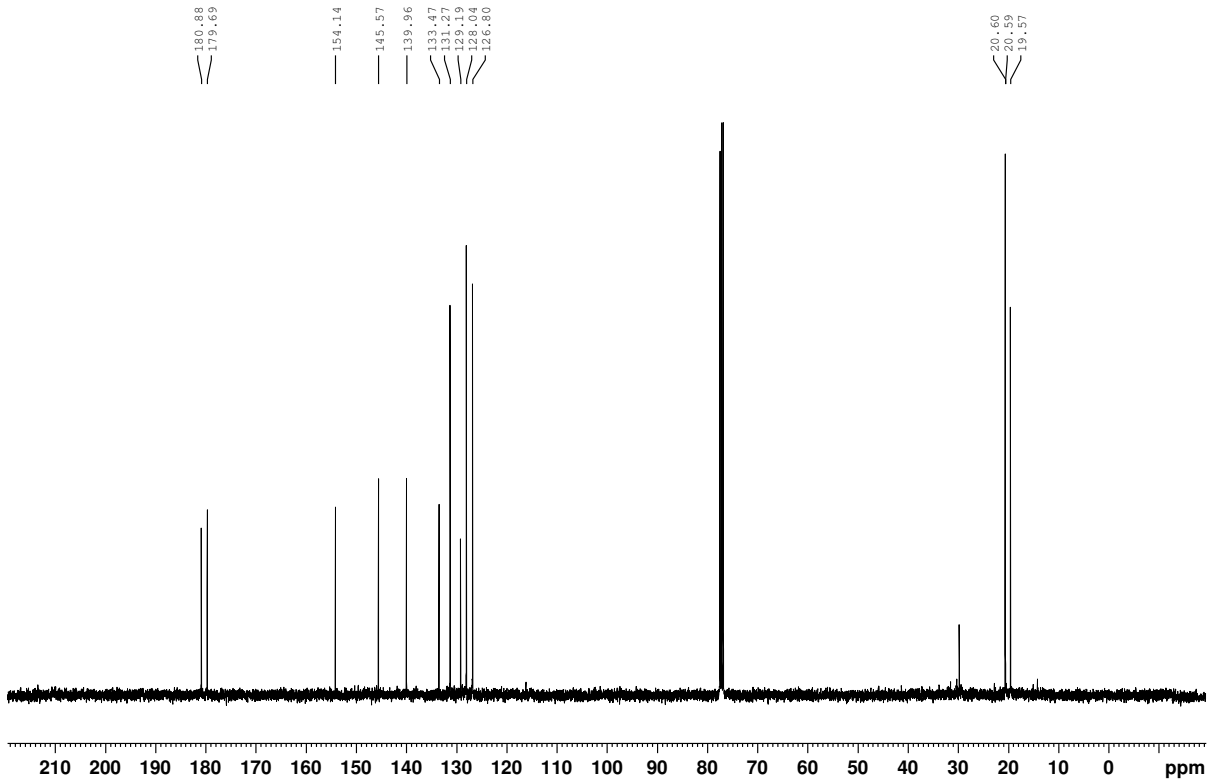




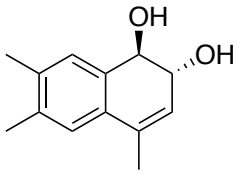
4,6,7-Trimethylnaphthalene-1,2-dione (39)

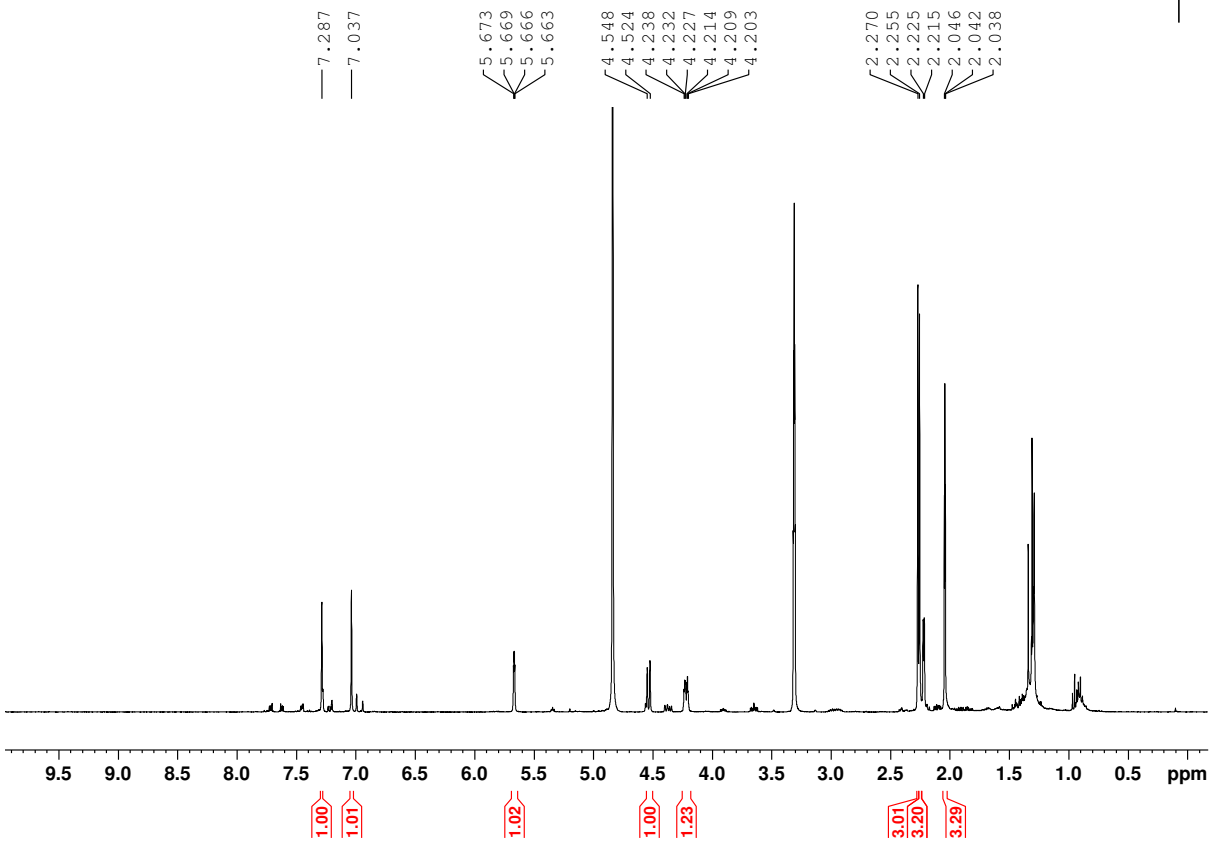


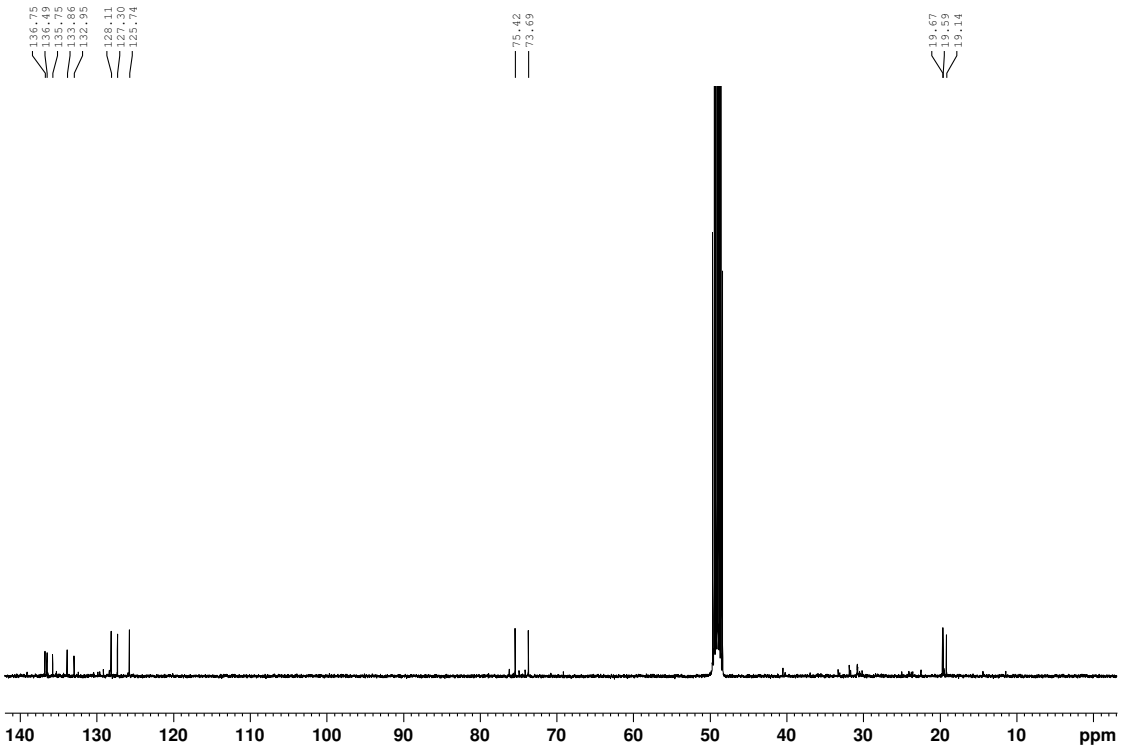




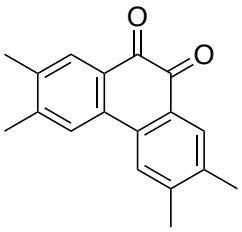
trans-4,6,7-Trimethyl-1,2-dihydronaphthalene-1,2-diol (21±)

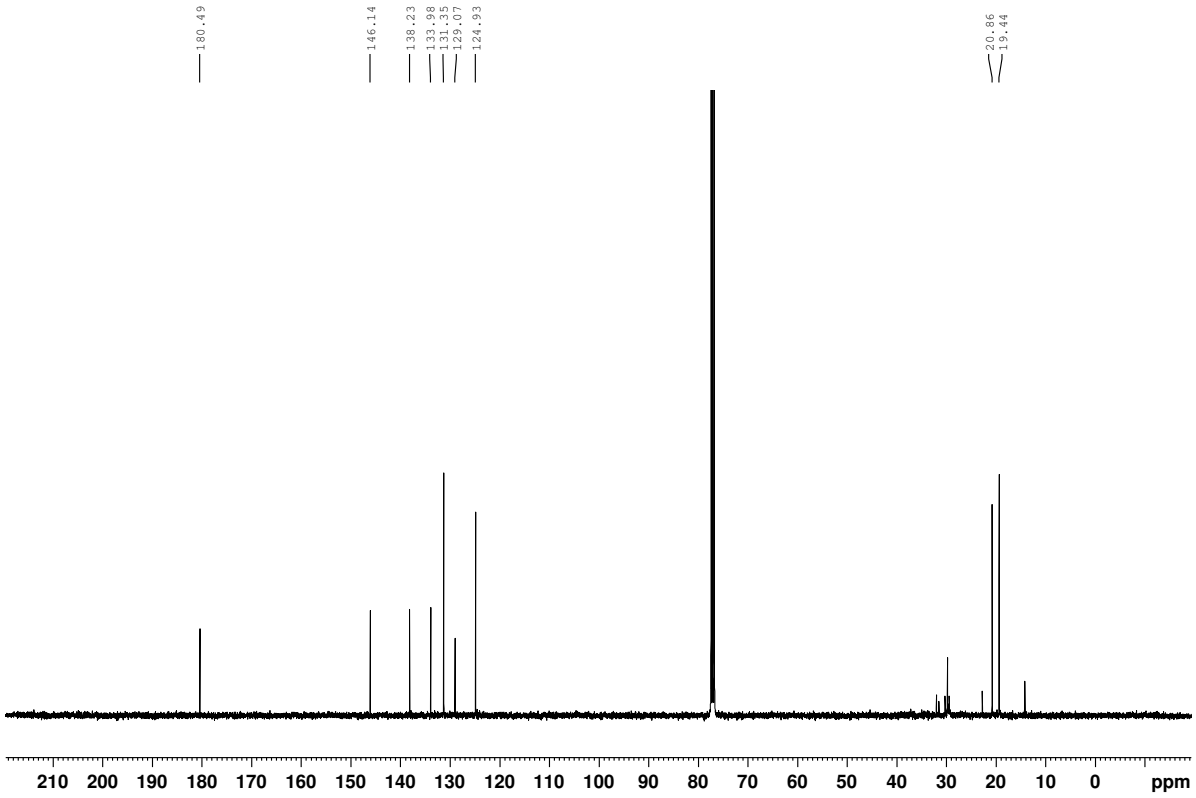




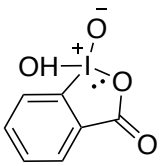


2,3,6,7-Tetramethylphenanthrene-9,10-dione (40)





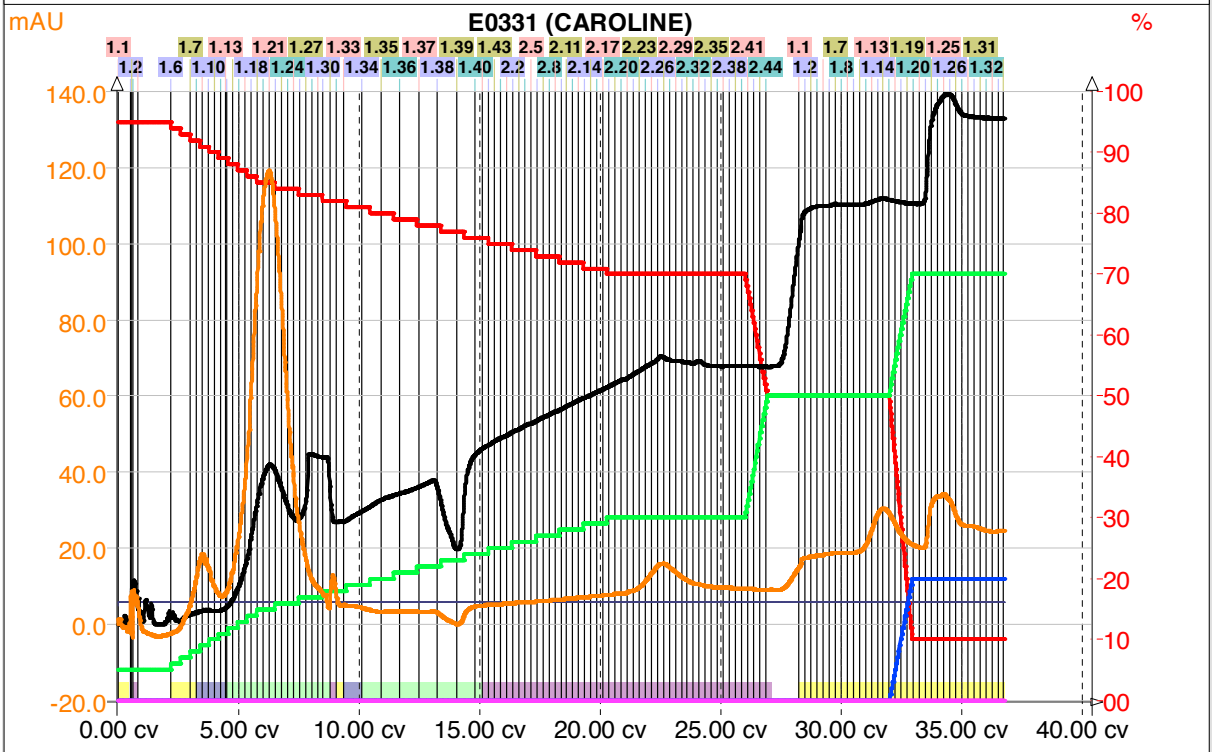
2-Iodoxybenzoic acid (31)



Interchim PuriFlash® 215 chromatography reports

6-Methylnaphthalene-1,2-dione (43)

Title : CAROLINE	Author :
Sample : E0331	Column : PURIFLASH COLUMN 50 STD - 25.0 g (15 bar)
Solvent A : petroleum ether	Solvent C : methanol
Solvent B : ethyl acetate	Solvent D : dcm
Channel 1 : UV600:SCAN	Channel 2 : UV600:SIG2
Channel 3 : UV600:SIG1	
Equil+Inject Mode : Dry Load+Auto	Stop Mode : Pause
RUN Time : 8/24/2018 10:19:14 AM	



Peak Tracking

	25 mL				25 mL			
	088	067	066	045	044	023	022	001
	087	068	065	046	043	024	021	002
	086	069	064	047	042	025	020	003
	085	070	063	048	041	026	019	004
	084	071	062	049	040	027	018	005
	083	072	061	050	039	028	017	006
	082	073	060	051	038	029	016	007
	081	074	059	052	037	030	015	008
	080	075	058	053	036	031	014	009
	079	076	057	054	035	032	013	010
	078	077	056	055	034	033	012	011

<< RackSet #1 >>

	25 mL				25 mL			
						111	110	089
						112	109	090
						113	108	091
						114	107	092
						115	106	093
						116	105	094
						117	104	095
						118	103	096
						119	102	097
					123	120	101	098
					122	121	100	099

<< RackSet #2 >>

Page 1/2

Data Time : 8/24/2018 10:27:02 AM

180824-1019-E0331

Printing Time : 8/24/2018 12:21:41 PM

Collection Table

Tube	Peak	Rack	Pos.	RSet	Coll.	Volume	Surface	%Surface	Start Time	End Time	Begin	End
001	001	1	1	1	1	14.5	0.3	0.0 %	00:00:01	00:00:59	0.01	0.46
002	002	1	2	1	1	0.3	0.0	0.0 %	00:01:07	00:01:08	0.52	0.53
003	003	1	3	1	1	0.3	0.1	0.0 %	00:01:09	00:01:10	0.54	0.55
004/005	004	1	4/5	1	1	6.5	2.3	0.1 %	00:01:19	00:01:45	0.62	0.82
006/007	005	1	6/7	1	1	33.0	2.6	0.1 %	00:04:40	00:06:52	2.19	3.22
008/013	006	1	8/13	1	1	41.3	6.1	0.2 %	00:06:52	00:09:37	3.22	4.51
014/030	007	1	14/30	1	1	136.8	167.8	6.0 %	00:09:37	00:18:44	4.51	8.78
031	008	1	31	1	1	8.0	9.3	0.3 %	00:18:44	00:19:16	8.78	9.03
032	009	1	32	1	1	9.5	10.3	0.4 %	00:19:16	00:19:54	9.03	9.33
033	010	1	33	1	1	25.0	28.3	1.0 %	00:19:54	00:21:34	9.33	10.11
034/040	011	1	34/40	1	1	160.0	215.2	7.7 %	00:21:34	00:32:14	10.11	15.11
041/044	012	1	41/44	1	1	32.0	61.5	2.2 %	00:32:14	00:34:22	15.11	16.11
045/088	012	2	1/44	1	1	351.8	890.0	31.6 %	00:34:22	00:57:49	16.11	27.10
041/088	012	-	-	-	-	383.8	951.5	33.8 %	00:32:14	00:57:49	15.11	27.10
089/123	013	1	1/35	2	1	272.5	1300.9	46.3 %	01:00:15	01:18:25	28.24	36.76
-	-	-	-	-	-	85.0	117.7	4.2 %	00:00:00	00:00:00	0.00	0.00

Elution Steps

N°	CV	Flow Rate	%A	%B	%C	%D
01	0.00	15.0	95	05	00	00
02	1.99	15.0	95	05	00	00
03	5.99	15.0	85	15	00	00
04	20.97	15.0	70	30	00	00
05	25.97	15.0	70	30	00	00
06	26.97	15.0	50	50	00	00
07	31.97	15.0	50	50	00	00
08	32.97	15.0	10	70	20	00
09	37.97	15.0	10	70	20	00

Detection Steps

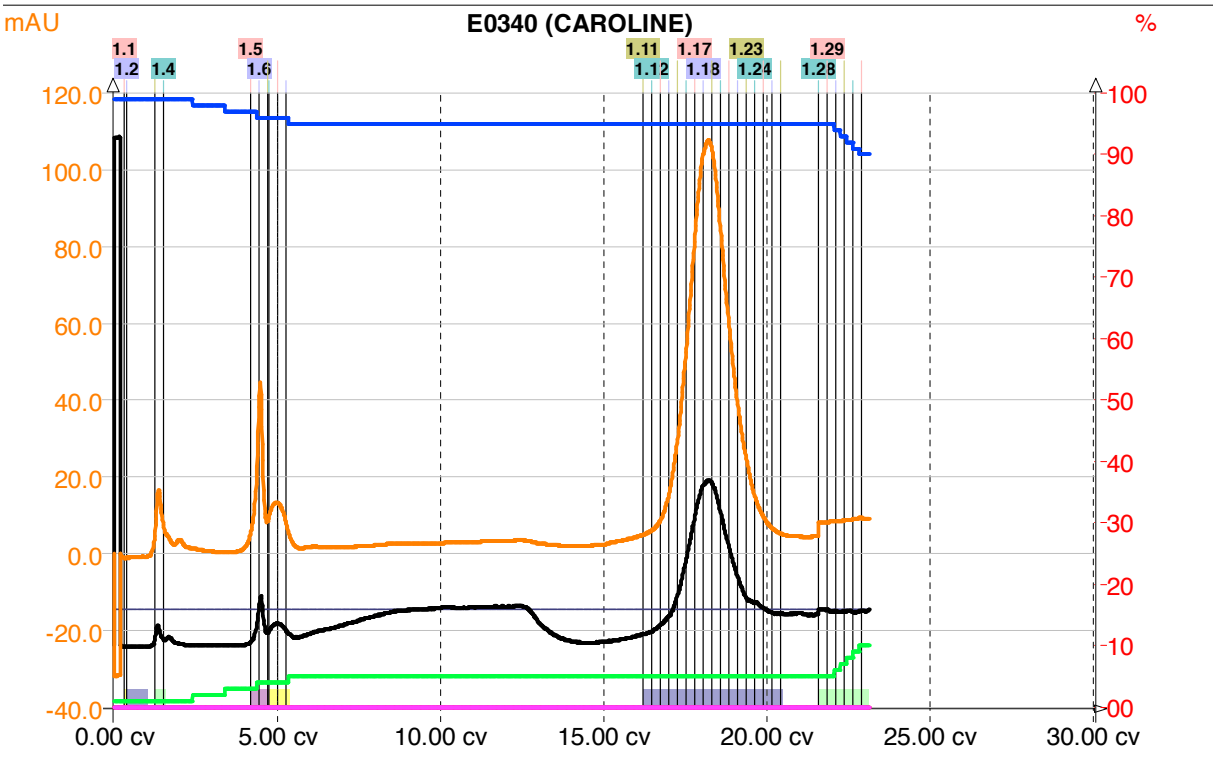
N°	CV	WL (nm)	>> Scan	Gain	XIC <<	>> XIC	Collect	Threshold	F1
01	0.00	200	599				No		1
		254					Yes	15	1
		250					No		1

Collection Steps

N°	CV	Local	Volume	Mode	Action
01	0.00	Yes	6.0	All	None
02	0.23	Yes	6.0	All	None
03	0.45	Yes	6.0	Threshold	None
04	2.18	Yes	8.0	All	None
05	24.42	Yes	8.0	Threshold	None

trans-6-Methyl-1,2-dihydronaphthalene-1,2-diol (19±)

Title : CAROLINE		Author :
Sample : E0340	Column : PURIFLASH COLUMN 30 SILICA HP - 12.0 g (15 bar)	
Solvent A : petroleum ether	Solvent C : chloroform	
Solvent B : ethyl acetate	Solvent D : methanol	
Channel 1 : UV600:SCAN	Channel 2 : UV600:SIG2	
Channel 3 : UV600:SIG1		
Equil+Inject Mode : Dry Load+Auto	Stop Mode : Pause	
RUN Time : 8/20/2018 12:04:39 PM		



Peak Tracking

	25 mL	25 mL
		023 022 001 024 021 002 025 020 003 026 019 004 027 018 005 028 017 006 029 016 007 030 015 008 031 014 009 032 013 010 033 012 011

<< RackSet #1 >>

<< RackSet #2 >>

180820-1204-E0340

Collection Table

Tube	Peak	Rack	Pos.	RSet	Coll.	Volume	Surface	%Surface	Start Time	End Time	Begin	End
001	001	1	1	1	1	1.0	-0.0	-0.0 %	00:00:24	00:00:28	0.32	0.37
002	002	1	2	1	1	12.3	-0.0	-0.0 %	00:00:29	00:01:18	0.38	1.03
003/004	003	1	3/4	1	1	6.5	0.5	0.5 %	00:01:35	00:02:01	1.25	1.59
005/007	004	1	5/7	1	1	10.5	1.5	1.6 %	00:05:17	00:05:59	4.17	4.72
008/010	005	1	8/10	1	1	12.3	1.5	1.6 %	00:05:59	00:06:48	4.72	5.37
011/027	006	1	11/27	1	1	81.5	37.5	39.4 %	00:20:32	00:25:58	16.21	20.50
028/033	007	1	28/33	1	1	29.3	6.7	7.0 %	00:27:20	00:29:17	21.58	23.12
-	-	-	-	-	-	286.0	47.5	49.8 %	00:00:00	00:00:00	0.00	0.00

Elution Steps

N°	CV	Flow Rate	%A	%B	%C	%D
01	0.00	15.0	00	01	99	00
02	1.98	15.0	00	01	99	00
03	5.98	15.0	00	05	95	00
04	20.97	15.0	00	05	95	00
05	21.97	15.0	00	05	95	00
06	22.97	15.0	00	10	90	00
07	27.97	15.0	00	10	90	00

Detection Steps

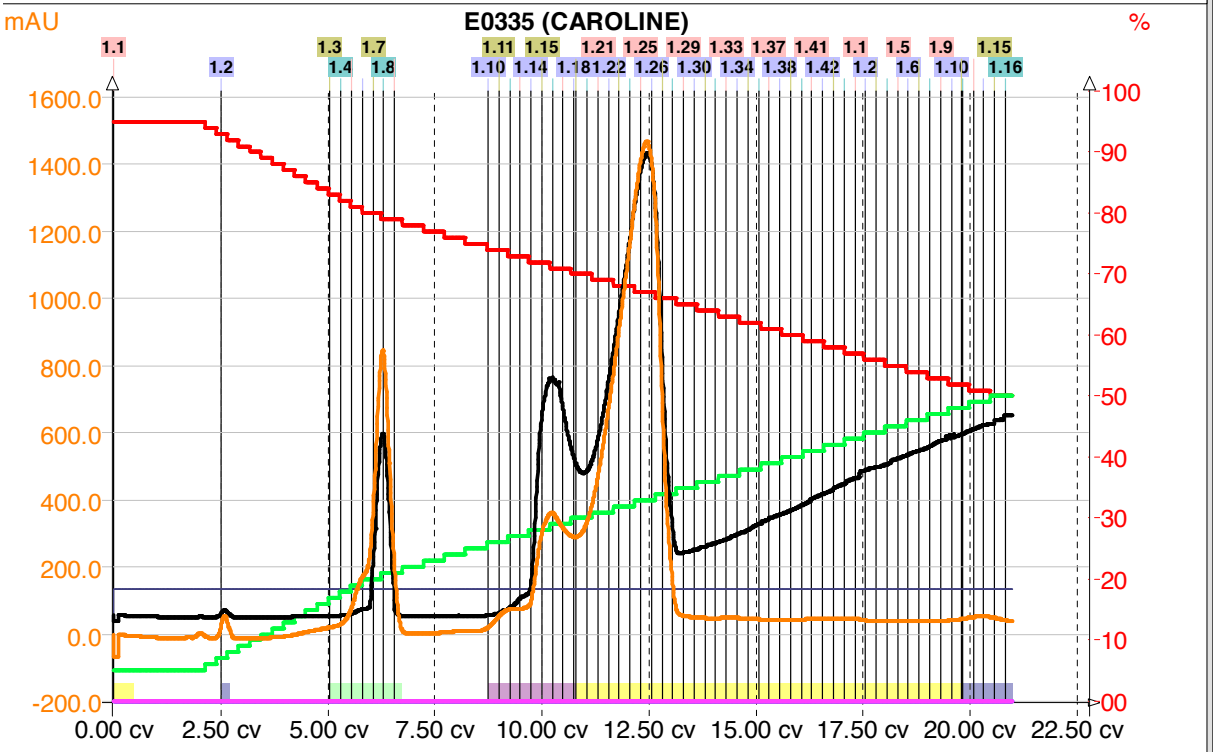
N°	CV	WL (nm)	>> Scan	Gain	XIC <<	>> XIC	Collect	Threshold	F1
01	0.00	200	599				No		1
		254					Yes	5	1
		250					No		1

Collection Steps

N°	CV	Local	Volume	Mode	Action
01	0.00	Yes	5.0	All	None
02	0.28	Yes	5.0	Threshold	None
03	0.29	Yes	5.0	Threshold	None
04	0.29	Yes	5.0	All	None
05	0.36	Yes	5.0	Threshold	None
06	0.36	Yes	5.0	All	None
07	0.38	Yes	5.0	All	None
08	1.01	Yes	5.0	Threshold	None

5,7-Dimethylnaphthalene-1,2-dione (44)

Title : CAROLINE		Author :
Sample : E0335	Column : PURIFLASH COLUMN 50 STD - 25.0 g (15 bar)	
Solvent A : petroleum ether	Solvent C : methanol	
Solvent B : ethyl acetate	Solvent D : dcm	
Channel 1 : UV600:SCAN	Channel 2 : UV600:SIG2	
Channel 3 : UV600:SIG1		
Equil+Inject Mode : Dry Load+Auto	Stop Mode : Pause	
RUN Time : 8/14/2018 9:47:42 AM		



Peak Tracking

		25 mL			
		044	023	022	001
		043	024	021	002
		042	025	020	003
		041	026	019	004
		040	027	018	005
		039	028	017	006
		038	029	016	007
		037	030	015	008
		036	031	014	009
		035	032	013	010
		034	033	012	011

<< RackSet #1 >>

		25 mL	
			045
			046
			047
			048
			049
			050
		060	051
		059	052
		058	053
		057	054
		056	055

<< RackSet #2 >>

Data Time : 8/14/2018 9:55:10 AM

180814-0947-E0335

Printing Time : 8/14/2018 11:36:44 AM

Collection Table

Tube	Peak	Rack	Pos.	RSet	Coll.	Volume	Surface	%Surface	Start Time	End Time	Begin	End
001	001	1	1	1	1	14.8	-0.2	-0.0 %	00:00:01	00:01:00	0.01	0.47
002	002	1	2	1	1	6.3	0.2	0.0 %	00:05:20	00:05:45	2.50	2.70
003/009	003	1	3/9	1	1	53.0	19.2	3.4 %	00:10:47	00:14:19	5.05	6.71
010/018	004	1	10/18	1	1	66.0	61.7	10.8 %	00:18:39	00:23:03	8.74	10.80
019/044	005	1	19/44	1	1	208.0	307.6	53.7 %	00:23:03	00:36:55	10.80	17.30
045/055	005	1	1/11	2	1	80.5	120.7	21.1 %	00:36:55	00:42:17	17.30	19.82
019/055	005	-	-	-	-	288.5	428.4	74.8 %	00:23:03	00:42:17	10.80	19.82
056/060	006	1	12/16	2	1	37.0	65.9	11.5 %	00:42:17	00:44:45	19.82	20.98
-	-	-	-	-	-	205.8	-2.4	-0.4 %	00:00:00	00:00:00	0.00	0.00

Elution Steps

N°	CV	Flow Rate	%A	%B	%C	%D
01	0.00	15.0	95	05	00	00
02	1.99	15.0	95	05	00	00
03	5.99	15.0	80	20	00	00
04	20.97	15.0	50	50	00	00

Detection Steps

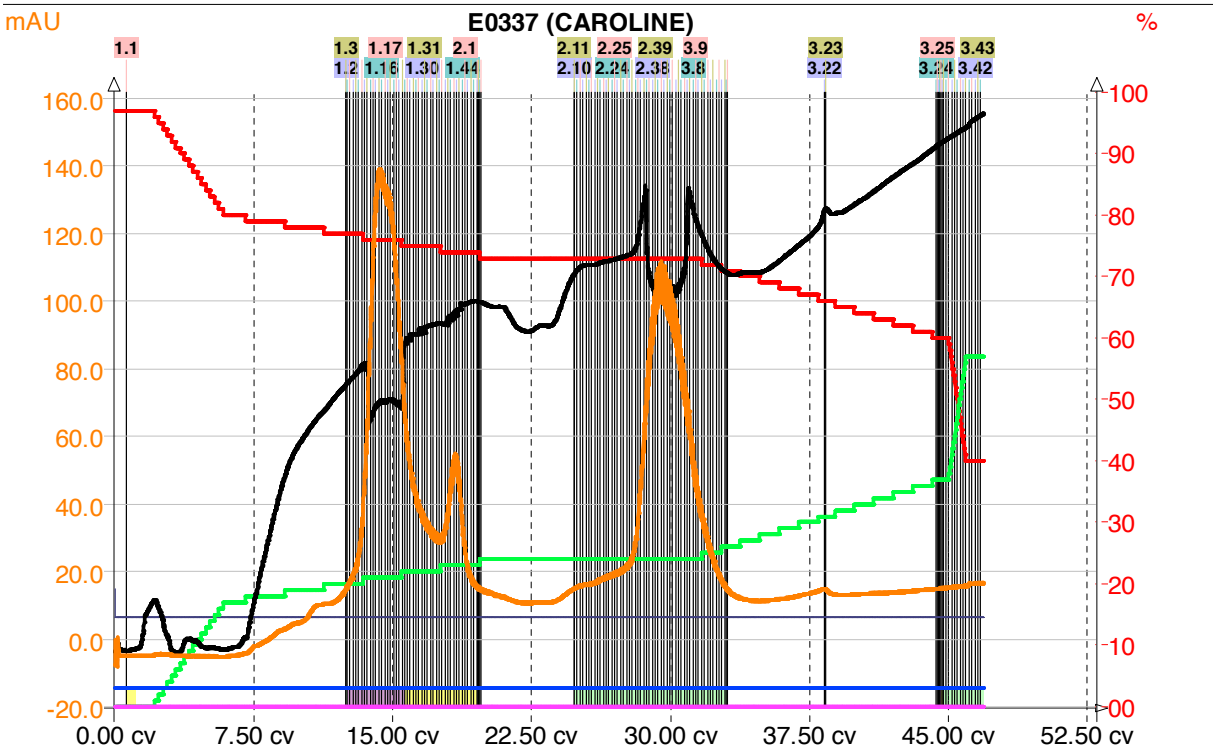
N°	CV	WL (nm)	>> Scan	Gain	XIC <<	>> XIC	Collect	Threshold	F1
01	0.00	200	599				No		1
		254					Yes	20	1
		250					No		1

Collection Steps

N°	CV	Local	Volume	Mode	Action
01	0.00	Yes	8.0	All	None
02	0.46	Yes	8.0	Threshold	None

trans-5,7-Dimethyl-1,2-dihydronaphthalene-1,2-diol (20±)

Title : CAROLINE		Author :
Sample : E0337	Column : PURIFLASH COLUMN 30 SILICA HP - 25.0 g (15 bar)	
Solvent A : petroleum ether	Solvent C : chloroform	
Solvent B : ethyl acetate	Solvent D : methanol	
Channel 1 : UV600:SCAN	Channel 2 : UV600:SIG2	
Channel 3 : UV600:SIG1		
Equil+Inject Mode : Dry Load+Auto	Stop Mode : Pause	
RUN Time : 8/17/2018 10:30:07 AM		



Peak Tracking

25 mL				25 mL				25 mL			
132	111	110	089	088	067	066	045	044	023	022	001
131	112	109	090	087	068	065	046	043	024	021	002
130	113	108	091	086	069	064	047	042	025	020	003
129	114	107	092	085	070	063	048	041	026	019	004
128	115	106	093	084	071	062	049	040	027	018	005
127	116	105	094	083	072	061	050	039	028	017	006
126	117	104	095	082	073	060	051	038	029	016	007
125	118	103	096	081	074	059	052	037	030	015	008
124	119	102	097	080	075	058	053	036	031	014	009
123	120	101	098	079	076	057	054	035	032	013	010
122	121	100	099	078	077	056	055	034	033	012	011

<< RackSet #1 >>

<< RackSet #2 >>

Data Time : 8/17/2018 10:43:53 AM

180817-1030-E0337

Printing Time : 8/17/2018 1:06:28 PM

Collection Table

Tube	Peak	Rack	Pos.	RSet	Coll.	Volume	Surface	%Surface	Start Time	End Time	Begin	End
001	001	1	1	1	1	16.0	0.1	0.0 %	00:01:19	00:02:23	0.62	1.12
002	002	1	2	1	1	0.3	0.5	0.0 %	00:26:33	00:26:34	12.45	12.45
003	003	1	3	1	1	1.3	2.4	0.1 %	00:26:37	00:26:42	12.48	12.52
004/023	004	1	4/23	1	1	99.0	184.9	5.6 %	00:26:43	00:33:19	12.52	15.62
024/044	005	1	24/44	1	1	105.0	247.3	7.5 %	00:33:19	00:40:19	15.62	18.90
045/049	005	2	1/5	1	1	21.5	54.1	1.6 %	00:40:19	00:41:45	18.90	19.57
024/049	005	-	-	-	-	126.5	301.4	9.1 %	00:33:19	00:41:45	15.62	19.57
050	006	2	6	1	1	1.0	2.5	0.1 %	00:41:47	00:41:51	19.59	19.62
051	007	2	7	1	1	1.3	3.1	0.1 %	00:41:53	00:41:58	19.63	19.67
052	008	2	8	1	1	1.0	2.5	0.1 %	00:42:00	00:42:04	19.69	19.72
053	009	2	9	1	1	0.3	0.6	0.0 %	00:42:07	00:42:08	19.74	19.75
054	010	2	10	1	1	0.5	1.4	0.0 %	00:52:50	00:52:52	24.77	24.78
055/088	011	2	11/44	1	1	170.0	475.5	14.3 %	00:52:54	01:04:14	24.80	30.11
089/107	011	3	1/19	1	1	92.3	265.6	8.0 %	01:04:14	01:10:23	30.11	32.99
055/107	011	-	-	-	-	262.3	741.0	22.4 %	00:52:54	01:10:23	24.80	32.99
108	012	3	20	1	1	0.5	1.4	0.0 %	01:10:26	01:10:28	33.02	33.03
109	013	3	21	1	1	0.5	1.4	0.0 %	01:10:33	01:10:35	33.07	33.09
110	014	3	22	1	1	0.3	0.8	0.0 %	01:21:44	01:21:45	38.31	38.32
111	015	3	23	1	1	0.3	0.8	0.0 %	01:21:50	01:21:51	38.36	38.37
112	016	3	24	1	1	0.3	0.9	0.0 %	01:34:39	01:34:40	44.37	44.38
113	017	3	25	1	1	0.3	0.9	0.0 %	01:34:46	01:34:47	44.42	44.43
114	018	3	26	1	1	0.5	1.8	0.1 %	01:34:52	01:34:54	44.47	44.48
115	019	3	27	1	1	0.8	2.7	0.1 %	01:34:58	01:35:01	44.52	44.54
116	020	3	28	1	1	0.8	2.7	0.1 %	01:35:05	01:35:08	44.57	44.59
117	021	3	29	1	1	1.0	3.7	0.1 %	01:35:11	01:35:15	44.62	44.65
118	022	3	30	1	1	1.0	3.7	0.1 %	01:35:18	01:35:22	44.67	44.70
119/132	023	3	31/44	1	1	70.0	263.8	8.0 %	01:35:24	01:40:04	44.72	46.91
-	-	-	-	-	-	915.8	1789.7	54.0 %	00:00:00	00:00:00	0.00	0.00

Elution Steps

N°	CV	Flow Rate	%A	%B	%C	%D
01	0.00	15.0	97	00	03	00
02	1.99	15.0	97	00	03	00
03	5.99	15.0	80	17	03	00
04	20.97	15.0	73	24	03	00
05	25.97	15.0	73	24	03	00
06	30.97	15.0	73	24	03	00
07	31.13	15.0	73	24	03	00
08	45.00	15.0	60	37	03	00
09	46.00	15.0	40	57	03	00
10	51.00	15.0	40	57	03	00

Detection Steps

N°	CV	WL (nm)	>> Scan	Gain	XIC <<	>> XIC	Collect	Threshold	F1
01	0.00	200	599				No		1
		254					Yes	15	1
		250					No		1

Collection Steps

N°	CV	Local	Volume	Mode	Action
01	0.00	Yes	5.0	All	None
02	0.61	Yes	5.0	All	None
03	1.11	Yes	5.0	Threshold	None