GLUCURONIDE PRODRUG OF A NATURALLY DERIVED CYTOTOXIC PRODUCT

by

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A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Biochemistry
May 2019

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ACKNOWLEDGEMENTS

I would like to express my gratitude to my research advisor, Dr. Sean Kerwin, for the opportunity to work in his lab, his mentorship, and his unfailing patience and kindness towards me. I would like to thank my committee members, Dr. Wendi David and Dr. Liqin Du, for their support and guidance throughout my project. I would also like to thank everyone in the Kerwin research group, especially Zach Schwartz, Amanda Bohanon, and Mauricio Jemal, for their encouragement, collaboration, and willingness to assist and teach me concepts and techniques.

I would also like to thank the South Texas Doctoral Bridge Program Committee, Dr. Raquel Salinas, Dr. Ronald Walter, Dr. Nicquet Blake, and Dr. Babatunde Oyajobi for the opportunity to be a part of the program and the constant support and advice offered to me.

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ABSTRACT

African Potato (*Hypoxis hemerocallidea)* is utilized as a medicinal plant in South Africa to treat disorders such as cancer, prostate hyperplasia, and cardiac disease. The corm of *H. hemerocallidea* contains high levels of the bis-glycoside hypoxoside, which undergoes hydrolysis in the gut to produce the aglycone, rooperol. *In vitro* studies indicate rooperol is active against cancer cells, while human studies report rooperol is mostly metabolized to inactive phase II metabolites. It is important to note that a minor amount of the active compound bis-β-glucuronide is also generated during the metabolic process. Despite these findings, a phase I study of orally-administered hypoxoside in advanced lung cancer patients demonstrated significant tumor arrest and even complete remission. Because tumor tissues can have high levels of extracellular β-glucuronidase activity, we hypothesize that the clinical activity of hypoxoside is due to the hydrolysis of the rooperol bis-glucuronide to rooperol by β-glucuronidase present in tumor tissue.

The goal of this project is to chemically synthesize the bis-glucuronide of rooperol to study its use as a tumor-targeting prodrug. We have prepared a model 3,4-dihydroxy-substituted cinnamyl alcohol for use in the investigation of methods for the selective protection of the catechol and regioselective installation of a protected β-glucuronate group. Once conditions in the model system are established, we will apply this strategy to rooperol followed by global deprotection to generate rooperol bis-glucuronide. The final bis-β-glucuronide will be compared to the bis-β-glucuronate of

rooperol generated from *in vitro* metabolism studies.

I. INTRODUCTION

Plants have played a dominant role in the treatment of various ailments and diseases for centuries, with written records dating back as far as 5000 B.C.[1] These natural products offer a supply of remarkably diverse small organic molecules that are applicable to present day drug discovery.[2] The active compounds in these organic substances are produced by plant secondary metabolism.[3] These secondary metabolites often have cytotoxic properties that can lead to the advancement of promising chemotherapeutics.[1] In drug development, these compounds are often modified to increase their metabolic stability, cytotoxicity, and selectivity.[4] Plant-derived antitumor agents that have been commonly used as cancer chemotherapeutics include paclitaxel, camptothecin, and cytarabine.[4]

1.1 African Potato (*Hypoxis hemerocallidae*) is a Natural Source of Rooperol

African plants have played a role in drug development due to the diversity of species and a long-standing history of these plants in traditional medicine. *Hypoxis hemerocallidae*, commonly known as African potato, is well-known by native South Africans due to its use as a nutritional supplement for the treatment of a variety of ailments including cancer, immune dysfunction, diabetes, HIV, and prostate hyperplasia.[5-6] The portion of the African potato that is most commonly used for therapeutic purposes is the tuberous corm. Methanolic extracts of African Potato corm have been shown to have pharmacological properties such as anti-inflammatory and anti-diabetic effects in mice and rats.[7] Extracts from the corm contain norlignan glycosides, the most abundant of which is the glycoside hypoxoside. Hypoxoside is a diphenyl-1-en-4-yne-pentane biscatechol with a bis-β-D-glucoside covalently attached (Figure 1).[8] [9-10]

Hypoxoside is widely considered to be inactive, but β-glycosidases found in the gastrointestinal tract are believed to be responsible for hydrolysis of hypoxoside yielding the biologically active aglycone, rooperol.[11]

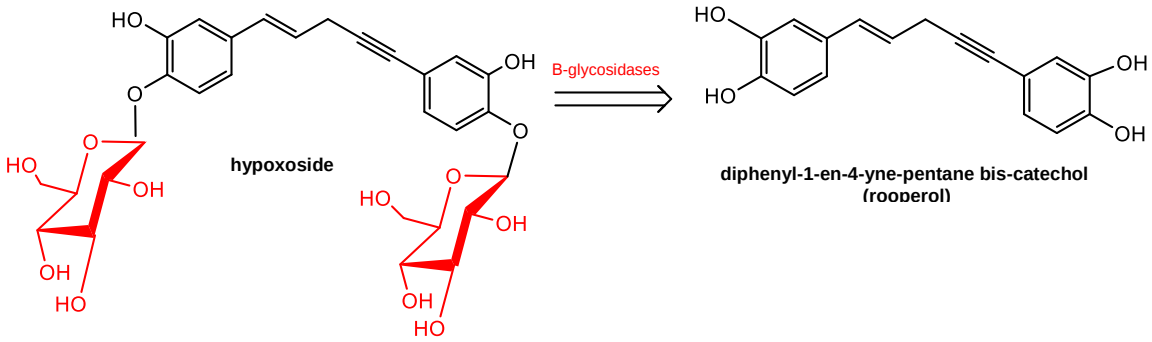


Figure 1: *Chemical structures of rooperol and hypoxoside found in the African Potato. The major glycoside hypoxoside (1,5-Bis[4-(beta-D-glucopyranosyloxy)-3-hydroxyphenyl]-1-pentene-4-yne). The hydrolosis of hypoxoside affords the biologically active rooperol*

1.2 The Anti-Cancer Properties of Rooperol

In vitro studies of hypoxoside indicate that it is not biologically active.[6, 8, 12] However, β-glycosidases in the gastrointestinal tract deconjugate the hypoxoside into the active aglycone, rooperol.[11] Studies have shown that rooperol immediately undergoes phase II metabolism in the liver, resulting in several inactive sulfate and glucuronide phase II metabolites (Figure 6).[13] Despite the rapid metabolism of rooperol, several studies have established that rooperol has anti-cancer activity and may function in this regard via a novel mechanism. [13-15].

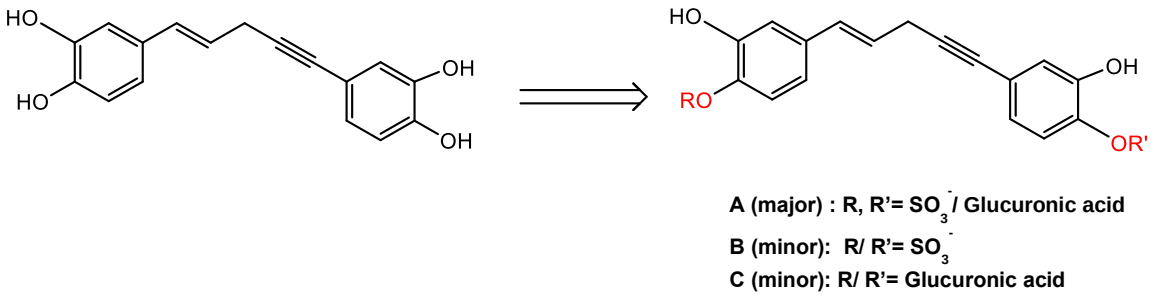


Figure 2: *Rooperol is quickly metabolized in the liver by the Phase II enzyme UDP-glucuronosyltransferase (UGT) to major and minor metabolites. The major metabolite is a mixed sulfate glucuronide and the minor metabolites are bis-glucuronides and bis-sulfates. The minor diglucuronide of rooperol is hypothesized to be converted back to active rooperol by β-glucuronidase in tumor tissue.*

Studies of the anticancer properties of rooperol indicate that it selectively inhibits the growth of cancer cell lines B-16, HeLa, HT-29, and MCF-7, but not non-tumorigenic transformed cell types such as MCF-10A.[12, 16-17] Furthermore, rooperol has been shown to preferentially target stem-cell-like cancer cells[14]. These cell types have the ability to extensively self-renew and are capable of unlimited proliferation. Targeting stem-cell like cancer cells could be advantageous since these cells can repopulate tumors after chemotherapy and lead to relapse.[18-19]

The biological mechanism by which rooperol inhibits cancer cell growth is not well defined. Studies in HeLa, MCF-7, and HT-29 have demonstrated that rooperol causes late G1 or early S phase arrest as a result of increased levels of p21, which causes cancer cells to become apoptotic.[15] Recently, a novel fluorescence-based assay identified rooperol as a p38α recruitment site ligand.[20] This is significant because rooperol is the first reported small molecule recruitment site inhibitor of p38α mitogen-activated protein kinase (MAPK).[20] MAPKs are involved in cancer cell survival and signaling, whereas

p38α specifically has been suggested to play a role part in tumor growth.[20-21] This further suggests that the cytotoxicity of rooperol may be due to interference with MAPK signaling.[20] In a preliminary study by Susan Mooberry of UTHSCSA using immunofluorescence microscopy, rooperol inhibited cancer cell proliferation by inducing microtubule depolymerization (unpublished). Together, these studies suggest rooperol may target several pathways in cancer cell proliferation.

Promising results demonstrating the effectiveness of rooperol as a chemotherapeutic agent has also been observed in humans. During phase I clinical trials, 24 patients with advanced-stage lung cancer were dosed daily with a hypoxoside-containing extract of African potato (800-1700mg) for 30 days to establish safety and pharmacokinetic parameters.[22-23] Efficacy was not studied in this trial; however, of the 24 patients, five experienced partial or complete remission including one patient who was cancer-free after five years.[23] No drug-induced toxicity was observed during treatment of the patients.[22] Blood analysis found no detectable hypoxoside or rooperol; however, a number of phase II metabolites were observed, including the minor bis-glucuronide metabolite.[13, 17, 22-23]. It has been hypothesized that the anti-tumor effects associated with ingesting hypoxoside is due to conversion of rooperol bis-glucuronide back to rooperol by β-glucuronidase found in tumor tissue. Tumors have been shown to have higher levels of β-glucuronidase than normal tissues, which may allow for tumor-selective delivery of rooperol.[24]

1.3 Drug Metabolism

Humans are exposed to a variety of potentially hazardous drugs and foreign compounds, referred to as xenobiotics. The resulting negative effects are due to the fact that most drugs are nonpolar, lipophilic molecules that can freely cross cellular membranes.[25] These xenobiotics, including rooperol, typically undergo enzymatic biotransformation, or drug metabolism, in the liver to allow for the clearance of the foreign compound from the body.[26],[27-28] A drug's pharmacologic activity and time of residence in the body can be affected by the chemical changes due to metabolizing enzymes.[27] The pathways of xenobiotic metabolism are categorized in two distinct phases: phase I and phase II (Figure 2).[29]

During phase I metabolism, polar functionalities are introduced by oxidation and hydrolysis reactions.[30] These reactions involve the modification or addition of functional groups that increase the polarity of the drug. [28] Phase I metabolism oxidation commonly involves membrane-bound monooxygenases, such as the cytochrome p450 which are localized in the endoplasmic reticulum of liver cells.[30],[28] While cytochrome p450 catalyzes the majority of phase I reactions, flavin-containing monooxygenase often oxidize drugs containing soft nucleophiles such as nitrogen[31]. Rooperol is a highly polar compound, therefore, phase I metabolism is does not play a major role in its metabolism. Instead, phase II metabolism is responsible for the major hepatic metabolic pathway of rooperol.

Many phase I metabolites are not eliminated rapidly and undergo subsequent phase II transformations. Phase II drug metabolism reactions serve as a detoxifying step for both exogenous and endogenous compounds. In phase II metabolism, parent compounds and phase I metabolites are commonly conjugated to an ionic hydrophilic

molecule such as sulfate, glycine, or glucuronic acids.[29] These conjugating reactions increase the hydrophilicity of the compound, allowing for their excretion in urine and bile.[29] Glucuronidation and sulfation are the major conjugation reactions for phenolic hydroxyl groups, including rooperol. These reactions are catalyzed by transferase enzymes, including UDP-gluronosyltransferases (UGTs), sulfotransferases, glutathione S-transferases, N-acetyltransferaces, and methyltransferases.[27] Transferase enzymes commonly convert pharmacologically active compounds to inactive forms by catalyzing reactions.[28]Glucuronidation is the major phase II reaction pathway that is applicable to this study.

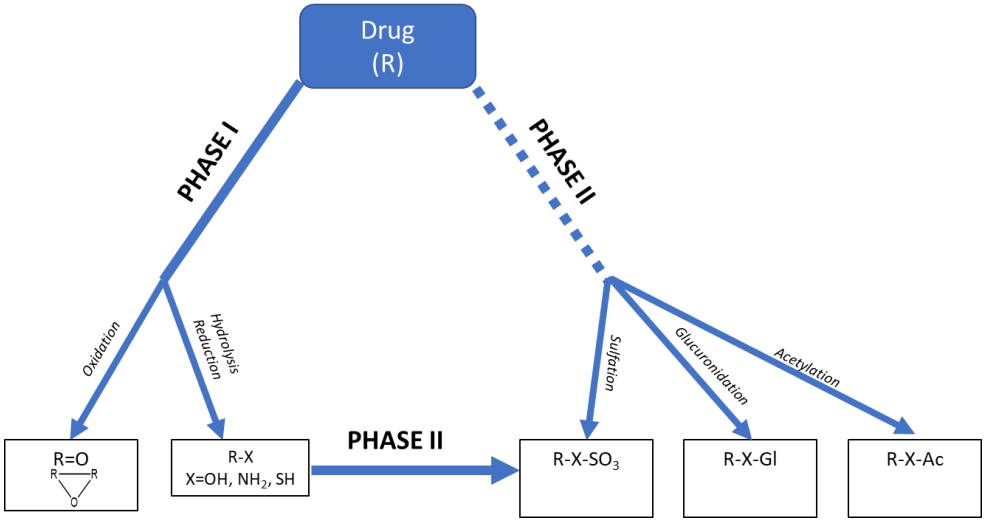


Figure 3: *Phase I and Phase II drug metabolism. Phase I reactions include oxidation and hydrolysis reduction. Phase II includes conjugation reactions of glucuronides, sulfates, and acetyl groups to increase the of a drug. Drugs may not always go through phase I metabolism. For example, rooperol proceeds directly to phase II metabolism. These reactions result in a substrate that is more hydrophilic than the original compound*

Glucuronidation is the most common phase II metabolic transformation, which is presumably due to an abundance of glucuronic acid found in the liver. Glucuronidation is the conjugation of phase I metabolites such as carboxylic acids, amines, phenols, and alcohols with a glucuronic acid moeity.[29] This is an S_N2 reaction where the nucleophilic group on the substrate attacks the electrophilic atom of UDP-glucuronic acid (UDPGA) forming a β-D-glucopyranosiduronic acid conjugate, or glucuronide (Figure 3).[28] For phenolic compounds, such as rooperol, glucuronidation reactions first undergo an acid base reaction where amino acid residues can donate and accept protons to facilitate the S_N2 reaction.[3, 32] This reaction is catalyzed by UDP-glucuronosyltransferase (UGT), one of the glycosyltransferases found in the endoplasmic reticulum.[33] The C-terminal of UGT is secured within the endoplasmic reticulum membrane. The active site faces the luminal side of the membrane, where the glucuronidation of lipophilic substrates take place.[31] The ability to efficiently synthesis these glucuronide metabolites is of great importance for drug development.

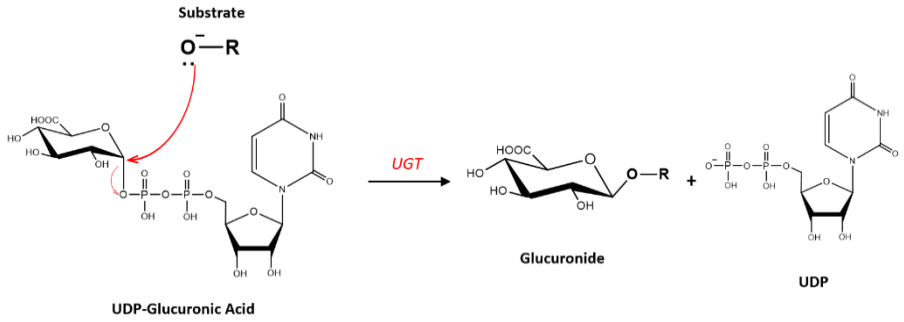


Figure 4: *Glucuronidation reaction of a phenol substrate. UDP acts as a leaving group while UGT deprotonates the oxygen so that the nucleophilic attack of the sugar can occur. The final products are the glucuronide and UDP.*

1.4 *O*-aryl Glucuronide Synthesis and Catalysis to Aglycone

Synthesis of glucuronide metabolites can be achieved through chemical or biological means. *O*-Glucuronides can be divided into three structural classifications: acyl, aryl, and alkyl[34]. O-aryl glucuronides are the most common class of glucuronides found in drug metabolites and can be chemically synthesized in acceptable yields with good stability by several methods. The Koenigs-Korr reaction is a widely used method where the aglycone is coupled with methyl peracetyl α-bromo-D-glucuronate (Figure 4) in the presence of silver salt catalysts like $AgCO_3$ and $Hg(CN)_2$ (Figure 8). [35-36] However, the reaction yields a mixture of the desired protected β-D-glucuronide (**1)** along with the α-anomer (**2)** and ortho ester (**3)** as side products (Figure 5).[37]If the aglycone has multiple sites of glucuronidation, this coupling can lead to a mixture of mono and polyglucuronides unless undesirable glucuronidation sites are selectively protected.[38] Besides the Koenigs-Korr method, alkylation of phenolates and halo sugar couplings are often used.[39-40] Direct alkylation of phenolates without the use of heavy metals is achievable with lithium salts in conjugation with phase-transfer catalysis, but gives low yields.[35] Halosugars have high donor abilities that are effective catalysts for the synthesis of β-D-glucuronide, however, one must be careful with the type and quantity of catalyst because it strongly influences β/α selectivity.[34, 38] Glucuronide synthesis is also prone to considerable problems in the deprotection step, such as dehydroglucuronide formation during ester hydrolysis.[34] Studies have often used Na_2CO_3 as a deacetylating agent because it is a strong enough base for hydrolysis to occur without the possibility of elimination as a side reaction.[38] One major problem with aryl glucuronides of acidic phenols is that the use of base hydrolysis of O-aryls can also lead to glycoside hydrolysis.

[34, 37] All of these considerations must be taken into account when chemically synthesizing glucuronides for attachment to an aglycone.

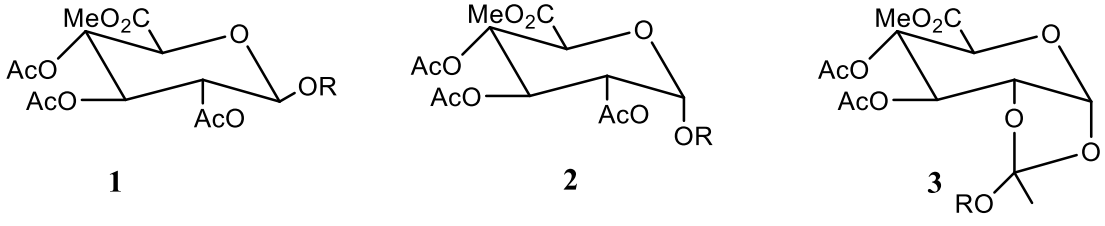


Figure 5: *Products of Koenigs-Knorr reaction before deprotection yielding the major product, β-D-glucuronide (1) and side products α-anomer (2) and ortho ester (3).*

1.5 Glucuronides as Prodrugs

Although chemotherapy is one of the most important treatments for cancer, this option lacks selectivity for tumor cells and results in dose-limiting side effects.[41] Prodrugs may be more selective, as they are bio-reversible derivatives of the parent compound that undergo an enzymatic transformation to release the active drug. Therefore, an important strategy to overcome selectivity and efficacy issues in chemotherapy is the use of enzyme-activated prodrugs. Because prodrugs can improve pharmacokinetics and physiochemical properties of pharmaceuticals, they are widely used in medicinal chemistry to overcome obstacles of poor metabolism, absorption, or solubility.

Various approaches can be employed for enzymatic prodrug therapy, including prodrug monotherapy utilizing enzymes that are naturally elevated in tumors, such as plasmin and β-glucuronidases.[41] Specifically, the enzymes used in prodrug monotherapy should differ from normal circulating enzymes present in normal tissues so that activation of the prodrug is restricted to the tumor site.[42]

One promising enzyme for tumor specific prodrug therapy is β-glucuronidase, a lysosomal enzyme that catalyzes the hydrolysis of β-D-glucuronic acid residues.[43] β-Glucuronidase, a building block of the basement membrane of tumor tissues, is found in greater concentrations in tumor cells and areas of inflammation. It is believed that overexpression of β-glucuronidase in cancer cells is part of the metastatic phenotype, allowing for cancer cells to leave the basement membrane and spread to other areas of the body. Because prodrugs with this enzyme contain hydrophilic glucuronic moieties, they do not diffuse across cell membranes, thereby reducing the prodrug toxicity in comparison to the parent compound.[44]

Because β-glucuronidase is found in greater concentrations in tumor tissues, glucuronic acid conjugates of anticancer drugs may allow for the selective delivery of the active drug to the tumor site.[45] β-Glucuronidase prodrugs usually have less systematic toxicity than the parent drug due to the hydrophilicity of the glucuronide. This prevents cellular uptake and further intracellular prodrug activation by lysosomal β-glucuronidase in non-malignant cells.[41] It is significant to note that as a result, prodrugs can be given at much higher doses with fewer side effects.

1.6 Research Aims

This motivation for this work is based on the hypothesis that the clinical activity of hypoxoside is due to the conversion of the minor rooperol bis-glucuronide metabolite back to rooperol by β-glucuronidase present in tumor tissue. If this hypothesis is correct, the development of an enzymatic prodrug of rooperol may lead to a compound that is site-selective for tumor tissue. The goal of this project is to chemically synthesize the bis-glucuronide of rooperol and later investigate its use as a promising tumor-targeting

prodrug. Initially, a model system for the regioselective installation of a glucuronate on a catechol will be utilized. We have prepared a model 3,4-dihydroxy-substituted cinnamyl alcohol bis-silyl ether in order to investigate the selective protection and deprotection of the 4-silyl ether followed by chemical glucuronidation (Figure 9). Once we have optimized these conditions in the model system, we will carry out selective deprotection of rooperol followed by chemical glucuronidation and global deprotection to afford the rooperol bis-glucuronide. The synthetic scheme of rooperol (Figure 7) followed by the attachment of the bis-glucuronide is described below (Figure 9). We believe that this glucuronide prodrug will display selective anti-tumor effects *in vivo*. All synthetic products will be characterized by ^{1}H- & ^{13}C-NMR and HPLC-MS.

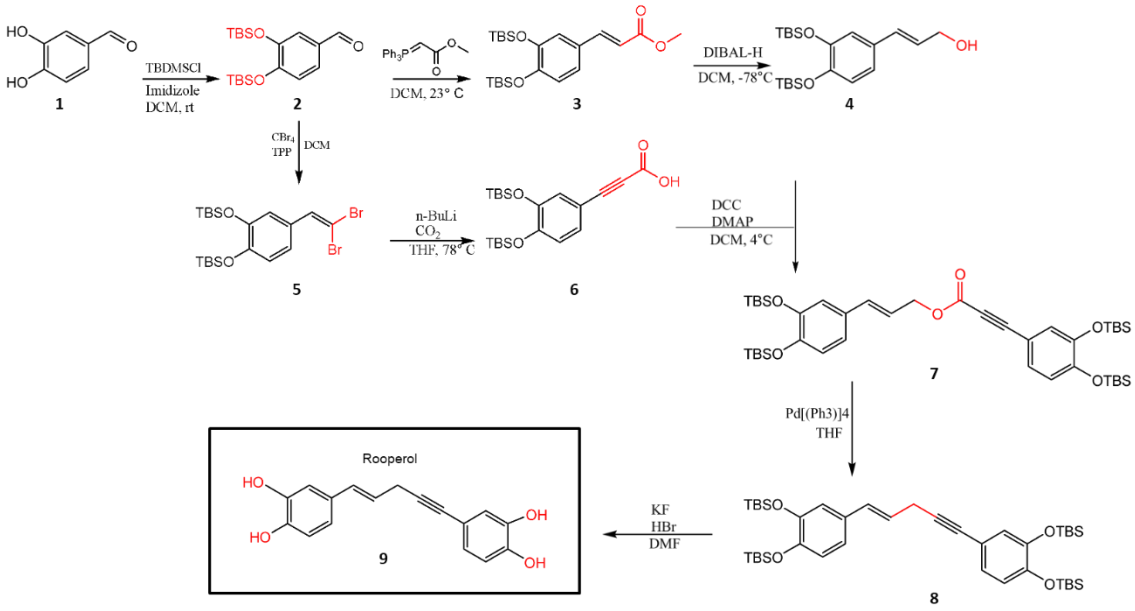


Figure 6: *Synthetic route to rooperol; selective deprotection methods will start from the benzaldehyde derivative (1).*

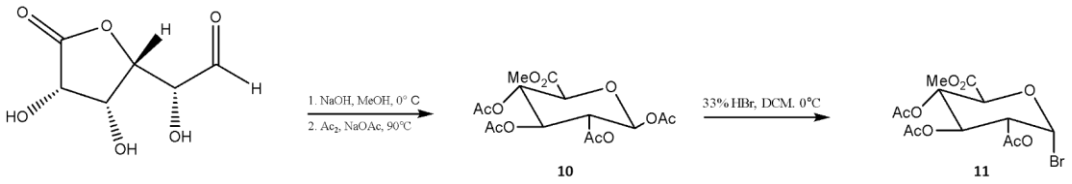


Figure 7: *Synthesis of the glucuronide. The acetal protected glucuronide (10) is brominated. The product (11) is then used in the Koenig's Knorr coupling to rooperol.*

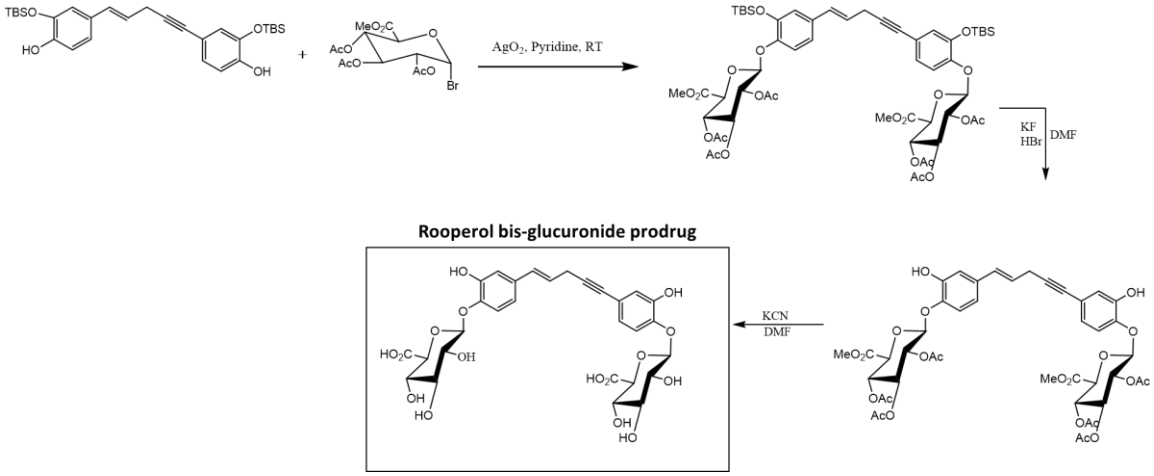
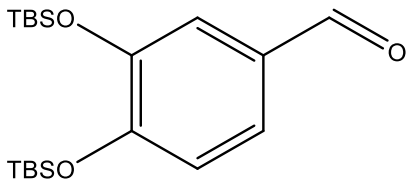


Figure 8: *Proposed synthetic scheme for the selectively protected rooperol coupled with the acetal protected glucuronide. This is followed by a global deprotection to afford the final product, a rooperol bis-glucuronide prodrug.*

II. EXPERIMENTAL

2.1 Rooperol Synthesis

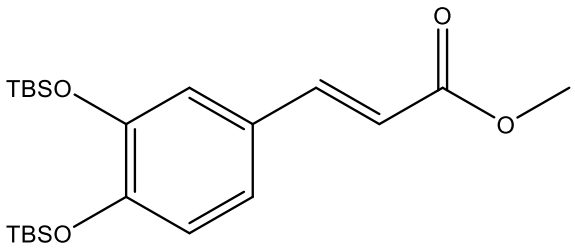
The synthesis of rooperol presented in this work is an efficient route developed by Sean Kerwin, both in terms of scalability and scope. There are a few issues with this modified synthesis in terms of yields for certain steps (Cory-Fuchs elimination/trapping and palladium decarboxylative coupling), and these are being further refined. The deprotection of the final enynes is also being carried out, so that the biological activity of these analogues of rooperol can be investigated. This synthesis is used as the template for the rooperol bis-glucuronide prodrug.



3,4-Bis-((ter-butyldimethyl-silyl)oxy)benzaldehyde (2): In a flask, 3,4-dihydroxybenzaldehyde (1) was placed in a reaction flask. The tert-butyldimethyl-silyl chloride (TBDMSCl, 2.4 equivalents) was added to a separate flask. Both flasks were purged with argon. The requisite amounts of dry DCM (4mL per mmol of aldehyde, 0.25 M/ 3.6mL per gram of TBDMSCl, 1.85 M) were added to each flask using an oven dried syringe. The reaction flask was placed in an ice water bath and allowed to cool. Imidazole (6 equivalents) was added in one portion. Once the imidazole was fully dissolved, the TBDMSCl solution was added dropwise to the reaction flask, the ice bath was removed, and the reaction was allowed to proceed for 2 hours. Progress was monitored by TLC. Ice-cold DI water was added to quench the reaction. The biphasic

mixture was then transferred to a separatory funnel and the DCM layer drained into a flask. The aqueous layer was extracted three times with 50 mL DCM and combined with the original DCM layer. Combined DCM layer extracts were returned to the separatory funnel and washed once with 30 mL ice cold 1N HCl, once with 50mL brine and then dried over anhydrous sodium sulfate. It was then filtered through fritted funnel and the solvent was evaporated and the resulting oil was purified with column chromatography. The product was a white flaky solid (95%).

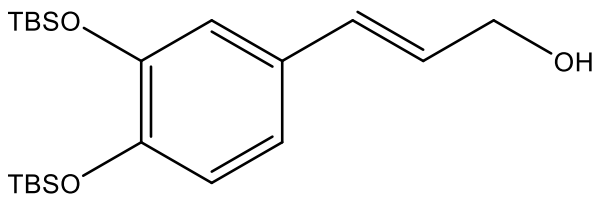
^{1}H NMR (400 MHz, $CDCl_3$) δ 0.21 (6H, s), 0.24 (6H, s), 0.966 (9H, s), 0.971 (9H, s), 6.92 (1H, d, J = 8.1 Hz), 7.33-7.35 (2H, m), 9.78 (1H, s).



Methyl (E)-3-(3,4-bis((tert-butyldimethylsilyl)oxy)phenyl)acrylate (3): Methyl (triphenylphosphoranylidene)acetate (Wittig reagent) (1.2 equivalents) was added to the reaction flask and purged with argon. The requisite amount of dry DCM (10 ml of DCM per mmol of aldehyde, 0.1 M) was added to the flask and Wittig reagent was allowed to fully dissolve. The protected aldehyde (2) was dissolved in a minimal amount of dry DCM and was added dropwise to the Wittig reagent solution. The solution was then allowed to react at room temperature for an hour and was monitored with TLC. The solvent was removed, 40 mL of hexanes were added, and the flask was swirled vigorously. The solids were filtered out and the frit was washed with an additional 40 mL of hexanes. The solvent was evaporated and the resulting oil was purified with column

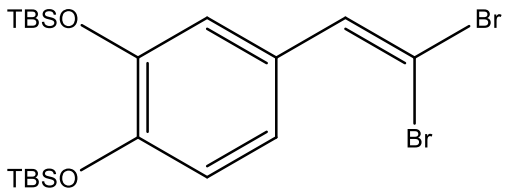
chromatography run at 0%, 2.5%, 5%. The resulting product was a white fluffy solid (82%)

^{1}H NMR (400 MHz, $CDCl_3$) 7.61 (d, 1H, J=16.1 Hz), 7.26 (d, 2, *J* = 2.1), 6.26(t, 1, *J* = 2.1), 5.98 (d, 1, *J* = 15.9), 3.81 (s, 3), 1.01 (s, 18), 0.23 (s, 12).

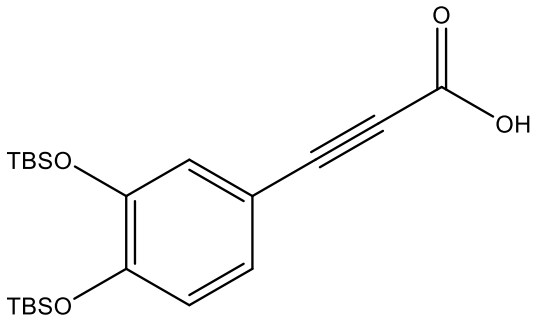


(E)-3(3,4-Bis((tert-butyldimethylsulyl)oxy)phenyl)prop-2-en-1-ol (4): The ester (3) was added to the reaction flask and purged with argon. The requisite amount of DCM (10 mL of DCM per mmol of aldehyde, 0.1 M) was added and the reaction flask was cooled in a saturated dry ice/acetone bath. DIBAL-H (2.5 equivalents) was added to the reaction flask dropwise, using a gastight syringe. The ice bath was removed and the reaction mixture was allowed to warm to room temperature over 2 hours. Progress was monitored with TLC and when complete, the reaction mixture was added in small portions to a stirred mix of 100 mL 2N HCl and ice. The biphasic mixture was transferred to a separatory funnel and the DCM layer was drained into a flask. The aqueous layer was then extracted 3 times with DCM and combined with the original DCM layer. The combined extracts were dried over anhydrous magnesium sulfate and filtered through a celite pad and concentrated. Column chromatography was not necessary. Product was colorless oil or white, waxy solid (85%).

^{1}H NMR (400 MHz, $CDCl_3$) 6.53 (d, 2, J = 1.8), 6.48 (d, 1, J = 15.4), 6.28 (dt, 1, J = 15.4, 5.5), 6.25 (t, 1, J =1.8), 4.31 (d, 2, J = 5.5), 1.02 (s, 18), 0.23 (s, 12).

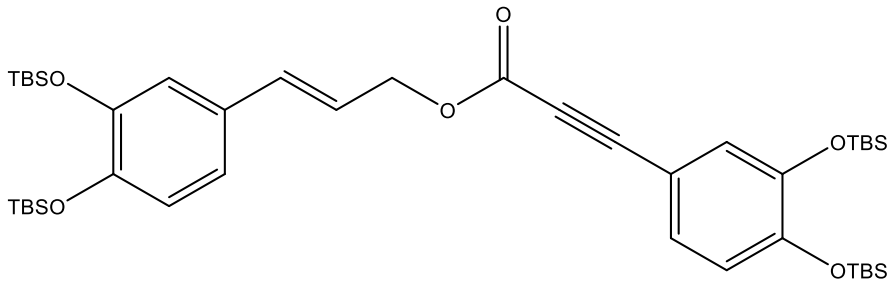


4-(2,2-Dibromovinyl)-1,2-phenylene)bis(oxy))bis(tert-butyldimethylsilane (5): The CBr_4 (2 equivalents) was added to the oven dried flask and purge with argon. The first portion of DCM (2 mL per mmol of aldehyde) was added with a syringe and placed in an ice bath. Once the CBr_4 was dissolved, triphenylphosphine (4 equivalents) was added in portions. A color change was observed. Once the triphenylphosphine had fully dissolved, the protected aldehyde (2) (1 equivalent) was dissolved in a separate flask using a second portion of DCM (1 mL of DCM per mmol of aldehyde). The solution of aldehyde was added to the reaction flask dropwise using a syringe. The ice bath was removed and the reaction mixture was stirred at room temperature for 30 minutes. The progress was monitored with TLC. When completed, saturated $NaHCO_3$ was added solution to the reaction mixture and the pH of the aqueous phase was checked periodically until it was neutral. The biphasic mixture was transferred to a separatory funnel and the DCM layer was drained into a flask. The aqueous layer was extracted three times with DCM. The aqueous layer was discarded, and the organic layer was returned to the separatory funnel which was then washed once with brine then dried over Na_2SO_4. The product was purified with column chromatography. The product was a light yellow oil (81%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.41 (2H, d, J=8.8 Hz), 7.32 (1H, s), 6.89 (2H, d, J=8.8 Hz), 1.01 (9H, s,), 0.25 (6H, s).



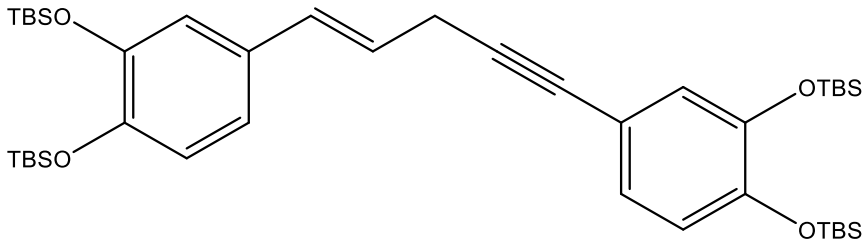
3-(3,4-Bis((tert-butyldimethylsilyl)oxy)phenyl)propiolic acid (6): The vinyl dibromide (5) (1 equivalent) was added to an oven dried flask and purged with argon. Anhydrous THF (2 mL per mmol of vinyl dibromide) was added to the reaction flask with a syringe. And cooled in a saturated dry ice/acetone bath. Once the flask was cooled, the n-BiLi (2.2 equivalents) solution was added dropwise using a gas tight syringe and allowed to react for 1 hour in the dry ice bath. The dry ice bath was removed, and the solution was allowed react for an additional hour. An excess of dry ice was added directly to the reaction flask. The aqueous layer was transferred to a separatory funnel and extracted with DCM. An emulsion formed, so 1N HCl was added to the separatory funnel using a pipette until the emulsion was broken. The aqueous layer was extracted twice more with DCM and the DCM extracts were combined and returned to the separatory funnel. Wash with 1N HCl followed by brine and dried over sodium sulfate and concentrate. It was purified by column chromatography 0:0/ 2.5:0/ 5:1% ethyl acetate: acetic acid in hexanes. The product was a white fluffy solid (79%).

^{1}H NMR (400 MHz, $CDCl_3$) d 7.16 (1H, dd, J = 8.30, 2.06), 7.01 (1H, d, J = 2.05), 6.85 (1H, d, J = 8.27), 1.01 (18H, q, J = 2.73), 0.25 (12H, m).

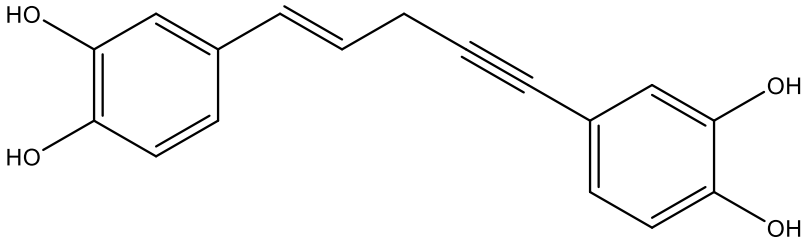


(E)-3-(3,4-Bis((tert-butyldimethylsulyl)oxy)phenyl)allyl-3-(3,4-bis((tert-butyldimethylsilyl)oxy)phenyl)propiolate (7): The propiolic acid (6) (1.3 equivalent), alcohol (3)(1 equivalent), and DMAP (0.2 equivalents) were added to the reaction flask. DCC (1.25 equivalents) was added to the pear-shaped flask. Both flasks were purged with argon and the requisite amounts of DCM (10 mL of DCM per mmol of alcohol and minimal amount to prepare DCC solution) were added to both flasks. The reaction flask was cooled in an ice water bath and the DCC solution was added to the reaction mixture dropwise using a syringe. The ice bath was removed after addition was complete and the reaction mixture was allowed to warm to room temperature over 2 hours. The reaction was filtered through a plug of silica and washed with 30% ethyl acetate:hexanes. After solvent was evaporated, the resulting oil was purified with column chromatography. The resulting product was light yellow solid (87%).

^{1}H NMR (400 MHz, $CDCl_3$) d 7.53 (2H, m), 6.59-6.73 (2H, m), 6.39-6.32 (2H, m), 6.60 (1H, d, J = 15.76), 6.15 (1H, dt, J = 15.76, 6.79), 4.91 (2H, dd, J = 6.76, 0.83), 1.57(36H, m), 0.26 (24H, m)



1,5-Bis(3',4'-di(tert-butyldimethylsilyl)oxyphenyl)pent-4-en-1-yne (8): The ester (7) (1 equivalent) was added to a pear shaped flask and purge with argon. Dry THF (10 mL per mmol of ester) was added and stirred until the ester was dissolved. TPP (0.085 equivalents) was added to a pressure tube in a glove box and sealed to ensure the area was free of oxygene. The ester solution was taken up in a gas tight syringe and placed in glove box with the pressure tube. The ester solution was added to the pressure tube and resealed. The reaction tube was heated in an oil bath to 80°C and stirred for 4 hours. It was removed from the oil bath and allowed to cool to room temperature. Once cooled, it was filtered through a plug of silica and Purified using column chromatography (16%). ^{1}H NMR (400 MHz, $CDCl_3$) d 7.23 (1H, m), 6.94 (4H, m), 6.93 (2H, m), 6.88(1H, d, J = 15.68), 6.07 (1H, m), 4.15 (2H, dd, J = 5.73, 1.58), 1.01(36H, m), 0.22 (24H, m)

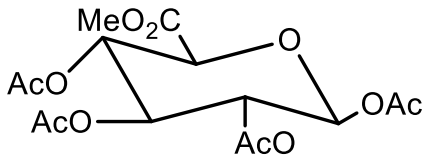


1,5-Bis(3',4'-dihydroxyphenyl)pent-4-en-1-yne (9): The silyl ester (8) was placed in a flask and then purged with argon. The ester was dissolved in anhydrous THF and then placed in an ice bath for 10 minutes. TBAF (6 equivalents) was added dropwise to the ester solution and then allowed to warm to room temperature for about 1 hour. The

progress was monitored by TLC. Once the reaction was completed, the reaction was quenched with water and extracted three times with ethyl acetate. The combined organic layers were then washed with brine and dried over Na_2SO_4. The solvent was then evaporated off and the resulting oil was purified using column chromatography.

2.2 Glucuronide Synthesis

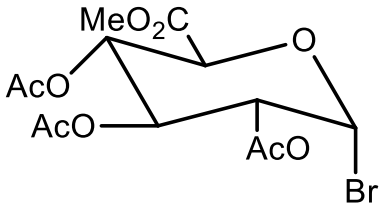
This brominated glucuronide synthesis was successful and chosen for the future glucuronidation of cytotoxic products. It was an efficient three step synthesis; however, one issue was poor yields. The products were confirmed both by NMR spectroscopy and melting point temperatures.



Methyl 1,2,3,4-tetra-O-acetyl-β-D-glucopyranuronate (10): Methanol was added to a two necked flask and NaOH (0.02 equivalents) was added and stirred until NaOH was completely dissolved. The round bottom was then cooled in an ice water bath and the D-glucuronolactone (1 equivalent) was added portion-wise over 20 minutes. The solution was stirred for 30 minutes. The solvent was removed by rotovap and then dried under vacuum for 2 hours and a pale-yellow foam formed. Acetic anhydride (19 equivalents) and anhydrous NaOAc (4.1 equivalents) were added. A reflux condenser was attached, the reaction was placed under argon, and the mixture was heated to 90℃ for 1.5 hours. The mixture was no longer stirred and then cooled and left standing over night. It was diluted with ethyl acetate and solids are broken up. It was stirred for 5 minutes and then

the solids were filtered with a fritted funnel. Sodium bicarbonate (38 equivalents) was placed in a large beaker and distilled water was added. It was stirred until the majority of the sodium bicarbonate was dissolved. The reaction filtrate collected previously was added slowly to the sodium bicarbonate solution until the effervescence had ceased. This was then transferred to a separatory funnel. The aqueous layer was extracted once with ethyl acetate and the organics were washed with water, and then dried over $MgSO_4$. The solvent was removed by rotovap and a sticky brown oil remained. The product was purified using recrystallization and was an off-white, flaky solid (30%).

MP: 176°C ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.04 (s, 3H), 2.05 (s, 6H), 2.13 (s, 3H), 3.75 (s, 3H), 4.19 (d, J = 9.4, 1H), 5.15 (t, J = 8.3, 1H), 5.26 (t, J = 9.3, 1H) 5.32 (t, J=9.1, 1H), 5.78 (d, J=7.8, 1H) ppm.

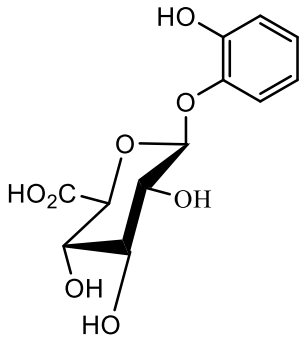


Methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranosiduronate (11): Add (10) (1 equivalent) to a flask and purge with argon. After it had completely dissolved, it was cooled in an ice water bath. Acetic anhydride and 33% HBr in acetic acid were added via syringe. The reaction was stirred for 20 minutes and then quenched with 200 mL of ice-cold water. The aqueous layer was extracted three times with DCM and the combined organics were extracted with ice-cold water twice, cold $NaHCO_3$ twice, and then brine. The combined organics were dried over $MgSO_4$ and then concentrated on the rotovap. The resulting brown syrup was dissolved in DCM and filtered through a silica plug. The

product was purified by recrystallization in hexanes, resulting in a tan crystal (40%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.67 (d,1H), 5.64 (t, 1H), 5.27 (dd, 1H), 4.89 (dd, 1H), 4.60 (d, 1H), 3.79 (s, 3H), 2.13 (s, 3H), 2.09 (s, 3H), 2.09 (s,3H) ppm.

2.3 Koenig's Knorr

The pyrocatechol was chosen as a model system for the coupling of the glucuronide to a catechol. This glucuronidation of the pyrocatechol was unsuccessful using this present synthesis. Examination of the NMR product reveled both the pyrocatechol and glucuronide were present, however, mass spectra data concluded they were not coupled together. The reason for this may be attributed to the fact that pyrocatechol undergoes oxidation rapidly and may have been too unstable for these purposes.

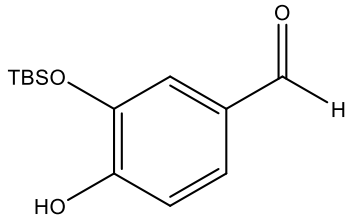


2-hydroxyphenyl β-D-glucopyranosiduronic acid (12): The catechol (1.3 equivalents) was dissolved along with the brominated glucuronide (11) in dry acetonitrile (to give 0.1 M of sugar). Ag_2O (2.5 equivalents), and the reaction was stirred vigorously overnight at room temperature in the dark. The reaction was filtered through a plug of silica and concentrated on a rotovap. The resulting syrup was then dissolved in ethyl acetate and

washed with $NaHCO_3$ three times, water twice, and brine twice. The collected organic phases were dried over $MgSO_4$. The compound was not successful most likely because pyrolcatechol is easily oxidized.

2.4 Other Protection Reactions

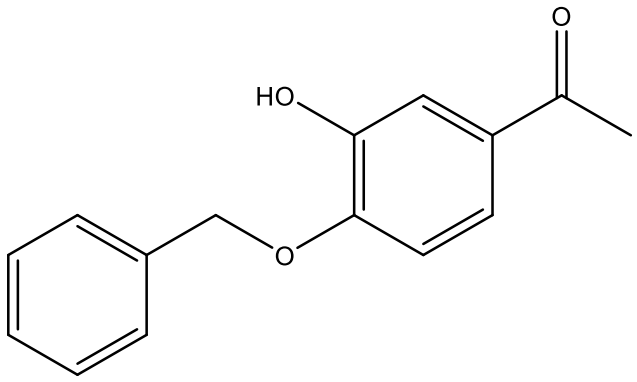
To accomplish the synthesis of the rooperol glucuronide prodrug, a method to selectively protect the 4-hydroxyl group of 3,4-dihydroxybenzaldehyde was explored. The need for a protecting group that can be removed from target molecules under mild conditions was also taken into consideration, because the final protected rooperol bis-glucuronide is both acid and base labile. Another method described here is protecting the rooperol alcohol (4) with triphenyl methyl chloride in an attempt to selectively deprotect the TBS protecting groups on the catechol.



3-((tert-butyldimethyl-silyl)oxy)-4-hydroxy-benzaldehyde (13): In a flask, 3,4-dihydroxybenzaldehyde (1equivalent) was added. The tert-butyldimethyl-silyl chloride (1 equivalents) was added to a separate flask. Both flasks were purged with argon and the requisite amounts of dry DMF (4mL per mmol of aldehyde, 0.25 M/ 3.6mL per gram of TBDMSCl, 1.85 M) were added to each flask using an oven dried syringe. The reaction flask was placed in an ice water bath and allowed to cool. Imidazole (2 equivalents) was added in one portion and once the imidazole was fully dissolved, TBDMSCl solution was

added dropwise to the reaction flask. The ice bath was removed after addition and the mixture was allowed to react for 2 hours. The progress was monitored by TLC. Ice-cold DI water was added to quench the reaction. The biphasic mixture was then transferred to a separatory funnel and the DCM layer drained into a flask. The aqueous layer was extracted three times with 50 mL DCM and combined with the original DCM layer. Combined DCM layer extracts were returned to the separatory funnel and washed once with 30 mL ice cold 1N HCl, once with 50mL brine and then dried over anhydrous sodium sulfate. Filter through fritted funnel and remove solvent using rotovap and purified with column chromatography. Product was a tan solid (39%).

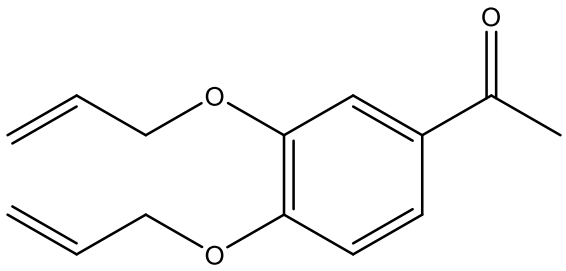
^{1}H NMR (400 MHz, $CDCl_3$) δ 0.10 (6H, s), 0.32 (6H, s), 0.92 (9H, s), 6.95 (1H, d, J = 8.1 Hz), 7.33-7.46 (2H, m), 9.82 (1H, s).



3-hydroxy-4-benzyloxy-benzaldehyde (14): In a reaction flask, 3,4-dihydroxybenzaldehyde (1equivalent) was added and purged with argon. Acetonitrile was added and stirred until dissolved. Then, KI (0.1 equivalent) and $NaHCO_3$ (1.5 equivalents) were added. Benzyl bromide (1.5 equivalents) was added dropwise to the reactions flask and then stirred for 2-3 days at 40°C and monitored by TLC. The mixture

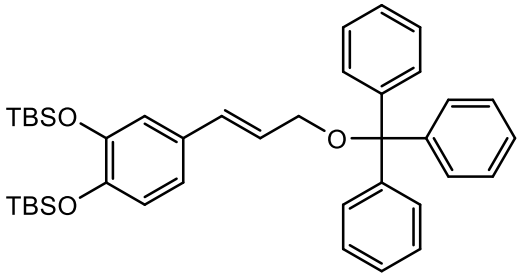
was cooled to room temperature. 0.1 M HCl was added to quench the reaction and then the aqueous layer was extracted three times with toluene. The combined organics were washed once with brine and dried over $MgSO_4$. It was then concentrated and purified through recrystallization. The product was a yellow oil (55%).

MP: 113 ℃; ^{1}H NMR (400 MHz, $CDCl_3$) 9.87 (s, 1H), 7.49 (d, 1H, 1.7 Hz), 7.26 (m, 6H), 7.07 (d, 1H, J = 8.1 Hz), 5.79 (s, 1H), 5.23 (s, 2H).



3,4-Allyloxyhydroxybenzaldehyde (15): In a flask, 3,4-dihydroxybenzaldehyde (1 equivalents) was added and purged with argon. It was dissolved in anhydrous DMF and sodium hydride suspension in mineral oil (2 equivalents) was added. The mixture was then cooled to 0℃. The solution was stirred for 30 minutes and allylbromide (1 equivalent) was added dropwise over a period of 10 minutes. The ice bath remained on the reaction and was stirred for an additional hour and monitored with TLC. Once the reaction was complete, 1M HCl was added to quench the reaction, which was transferred to a separatory funnel. The organic layer was extracted three times with ethyl acetate and the combined organics were washed with water three times and dried over sodium sulfate. The solvent was removed and the resulting oil was purified by column chromatography. The product was a tan solid (15%).

MP 54℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.91 (d, J = 5.6 Hz, 2 H), 6.84 (m, 2 H), 4.73 (s, 1 H), 5.48 (m, 1 H), 7.28 (d, J = 8.4 Hz, 1 H), 7.46 (m, 2 H), 9.86 (s, 1 H).



(E)-3(3,4-Bis((tert-butyldimethylsilyl)oxy)phenyl)triphenylmethyl (16): In a flask, the alcohol (4) (1 equivalent) was purged with argon and dissolved in pyridine. The flask was placed on an ice bath and cooled to 0°C. Trityl methylchloride (2 equivalents) were added quickly to the flask. The reaction was allowed to react for 2 hours and monitored by TLC. Once complete, the reaction was quenched with water and transferred to a separatory funnel. 1M HCl was added and the organic layer was drained. The aqueous layer was extracted three times with DCM. The combined organics were washed with water twice and brine once and then dried over sodium sulfate. The solvent was then evaporated and the resulting oil was purified by column chromatography. The product was a yellow oil (45%).

III. DISSCUSSION

4.1 Summary and Conclusion

The brominated glucuronide was successfully synthesized in a 40% yield which can be coupled to a cytotoxic natural product, thereby creating a prodrug. For the purpose of this study, we studied rooperol which is a biscatechol system. Because the brominated glucuronide must be selectively coupled on the 4' position of rooperol, many selective protection methods were attempted. These selective protection methods helped aid in the future synthesis of the glucuronide prodrug and gave insight to the best protection methods for our product.

The challenge of this rooperol bis-glucuronide synthesis is the fact that the desired product is the glucuronide attached to only the 4' position of the catechol. It has been hypothesized that the glucuronide is most accessible for cleavage by β-glucuronidase when conjugated to the 4' position. Since the two 4' catecholic hydroxyl groups in product (**9**) are preferred to for glucuronide conjugation, we searched for a method to selectively protect a catechol. Once the catechol moiety is selectively protected, it can then be glucuronidated using silver coupling. Another aspect of the synthesis to consider is that the product is both base and acid sensitive. Because many of the widely-used catechol protecting groups, such as acetals and cyclic sulfates, require harsh conditions for their removal, they would likely not be useful for this present synthesis. Examples of selective protection of 3,4-dihydroxybenzaldehyde are presented in this work.

The first protection attempted used the methyl protecting group for the protection of the 4-hydroxyl group and a benzyloxy protecting group in the 3-hydroxyl group of 3,4-dihydroxybenzaldehyde (13). We had success in this protection, with an overall yield of

~55%. However, it was concluded this protecting group was inappropriate for our purpose because of the harsh conditions needed for the removal of the methyl group.

Table 1: *The various conditions attempted for TBDMSCl selective protection. The most successful of which is D. Using NaH as a base was unsuccessful and only starting material was found on NMR.*

TBDMSCl	Base (eq)	Temperature	Solvent	Yield (monoprotected)
A	DIPEA (2)	0°C	DMF	*32%*
B	NaH (2)	-78°C	DMF	*Unsucessful*
C	Imidazole (2)	0°C	DMF	*26%*
D	Imidazole (1)	-78°C	DMF	*40%*
E	DIPEA (1)	-78°C	DCM	*12%*

The protection of 3,4-dihydroxybenzaldehyde was accomplished in ~ 40% yield using the TBDMSCl protecting groups (14) using varying equivalents of base. However, ^{1}H NMR suggested that a 2/1 ratio mixture of the 4-monoprotcted and the 3-monoprotected isomers were present. Changing the base to a weaker DIPEA to deprotonate only the 4-hydroxyl group was unsuccessful in reducing the ratio of isomers in the mixture. The various conditions attempted are shown in Table 1 and all were done on about 200-500 mg scales. Methods as described in the above experimental section were used for all experiments. Method D is suggested to be used in further experiments, possibly taking the mixture of the 3' and 4' monoprotected and carrying out the rooperol synthesis. This mixture can be coupled with the brominated glucuronide and then globally deprotected.

Table 2: *The various conditions attempted for benzyl bromide selective protection. The most successful of which is A. Using MeOH as a solvent was unsuccessful and only starting material was found on NMR.*

Benzyl Bromide	Salt	Temperature	Solvent	Yield
A	KI	40°C	Acetonitrile	*55%*
B	KI	60°C	MeOH	*Unsuccessful*
C	K_2CO_3	40°C	Acetone	*21%*
D	K_2CO_3	60°C	DMF	*34%*
E	$NaHCO_3$	60°C	Acetonitrile	*43%*

Having no success with silyl protecting groups or methylation deprotection, more reactive alkyl halides were examined. Benzyl bromide was used under a varying set of conditions as shown in Table 2, with the conditions of A presented in this work having the most positive effect on the yield. The monoprotected aldehyde was isolated in ~55% yield. We were able to determine that it was selectively protected based on the ^{1}H NMR chemical shift of the 4' protected versus the 3' protected. However, it was determined that the removal of the benzyl bromide would require too harsh of conditions for this synthesis. This may be a possible starting material for a rooperol analog to test if the cytotoxicity remains the same. If the cytotoxicity remains, replacing the hydroxy group of the catechol may also reduce the phase II metabolism of the system, therefore increasing half- life of the molecule.

The regioselective protection of 3,4-dihydroxybenzaldehyde using allyl bromide (15) was attempted as well. In this case, we were able to obtain the deprotected product, but in very poor yields of ~15%. This may be a route worthy of further investigation in order to increase the yield and also the regioselectivity of the product. We have successfully selectively protected the 4' and 3' hydroxyl groups of 3,4-dihydroxybenzaldehyde using various methods. However, a selective protection method

that is suitable for our present synthesis has not been identified. Future work includes searching for a suffic ient protecting group that maintains a high yielding product and is labile under correct conditions.

Another protection method that was tested was the trityl chloride protection of the alcohol (4). This was successfully synthesized in ~45% yields.

4.2 Future Directions

Selective protection reactions on catechol moieties as presented in this work have possible future implications for the synthesis of rooperol analogues and the synthesis of the glucuronide prodrug. These reactions can be helpful in experimental design for varying protection methods of rooperol analogues. However, more experiments will need to be done to find a method of protection that produces a high-yielding product that will withstand the total synthesis of rooperol. The successful synthesis of the brominated glucuronide can be a valuable tool for the glucuronidation of varying cytotoxic products.

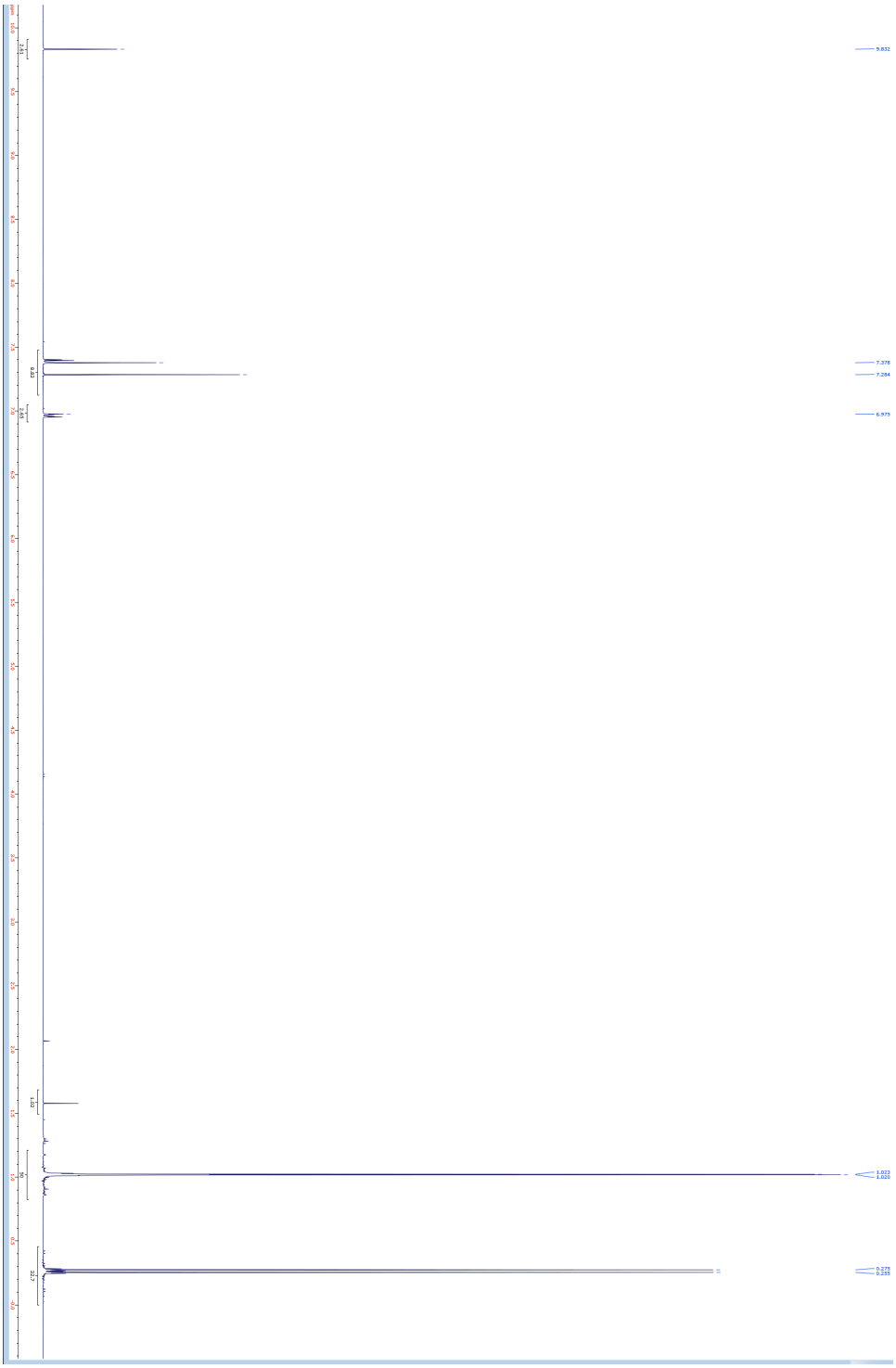
The Koing's Knorr method attempted on a catechol can be employed for the glucuronidation of rooperol as well. Another approach to obtain the desired bis-glucuronide rooperol is by coupling the glucuronide to the final product of rooperol without selective protection. We may be able to separate out the desired product by HPLC and then characterize the metabolic stability of the product in an *in vitro* metabolism assay. If the metabolic activity is increased, further studies can be conducted to assess the cytotoxicity of the compound.

These selected protected aldehydes synthesized in this work may also be used as possible candidates for rooperol analogs. For example, the 3-hydroxy 4-benzyloxybenzaldehyde may be a viable starting material for a possible rooperol analog.

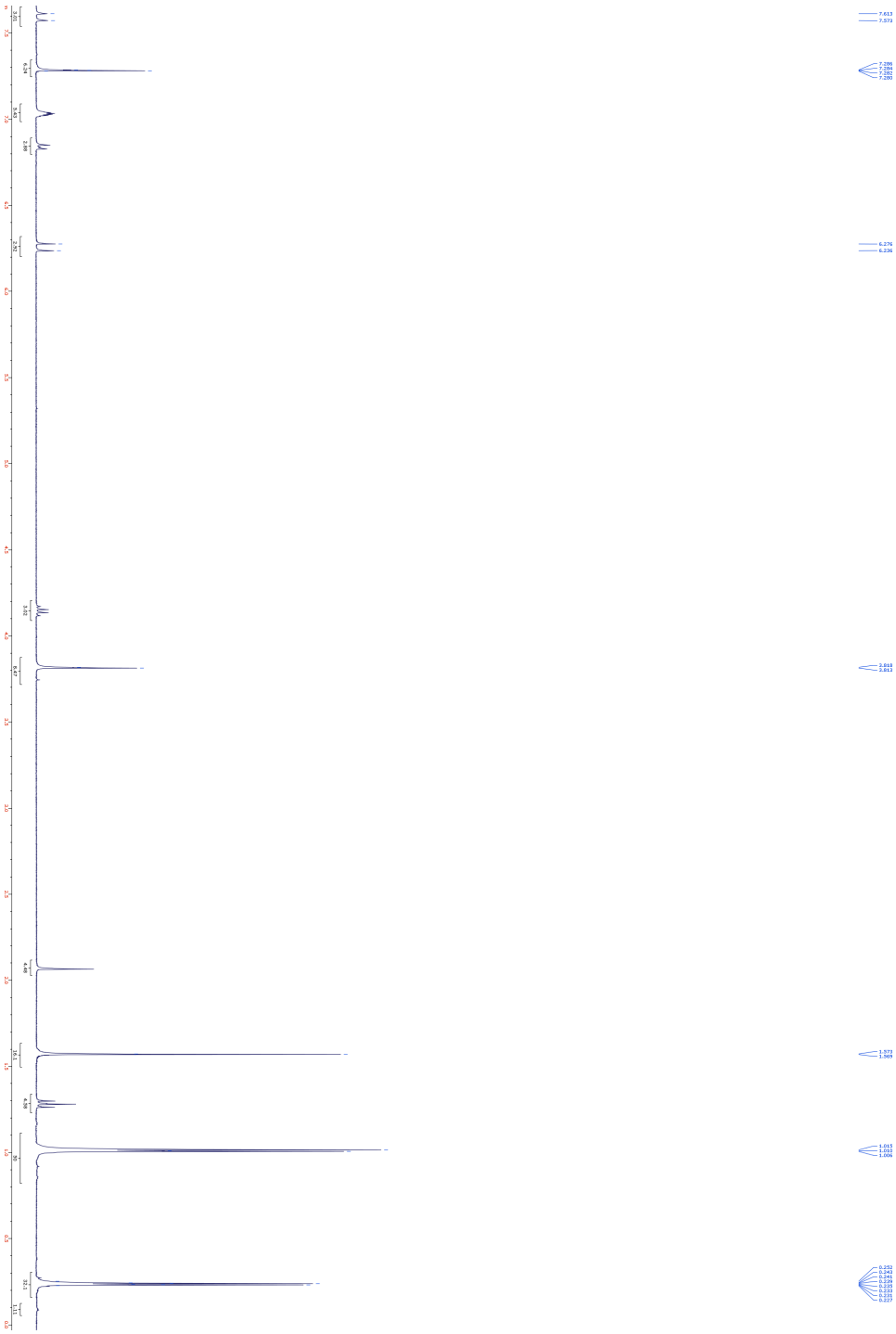
Another route that may be explored is the biological activity of the rooperol backbone though the synthesis of various backbones and determine if cytotoxicity is still present. All of these future directions can potentially give insight to the mechanism of action of rooperol as well as the major pharmacophore of the structure.

APPENDIX SECTION

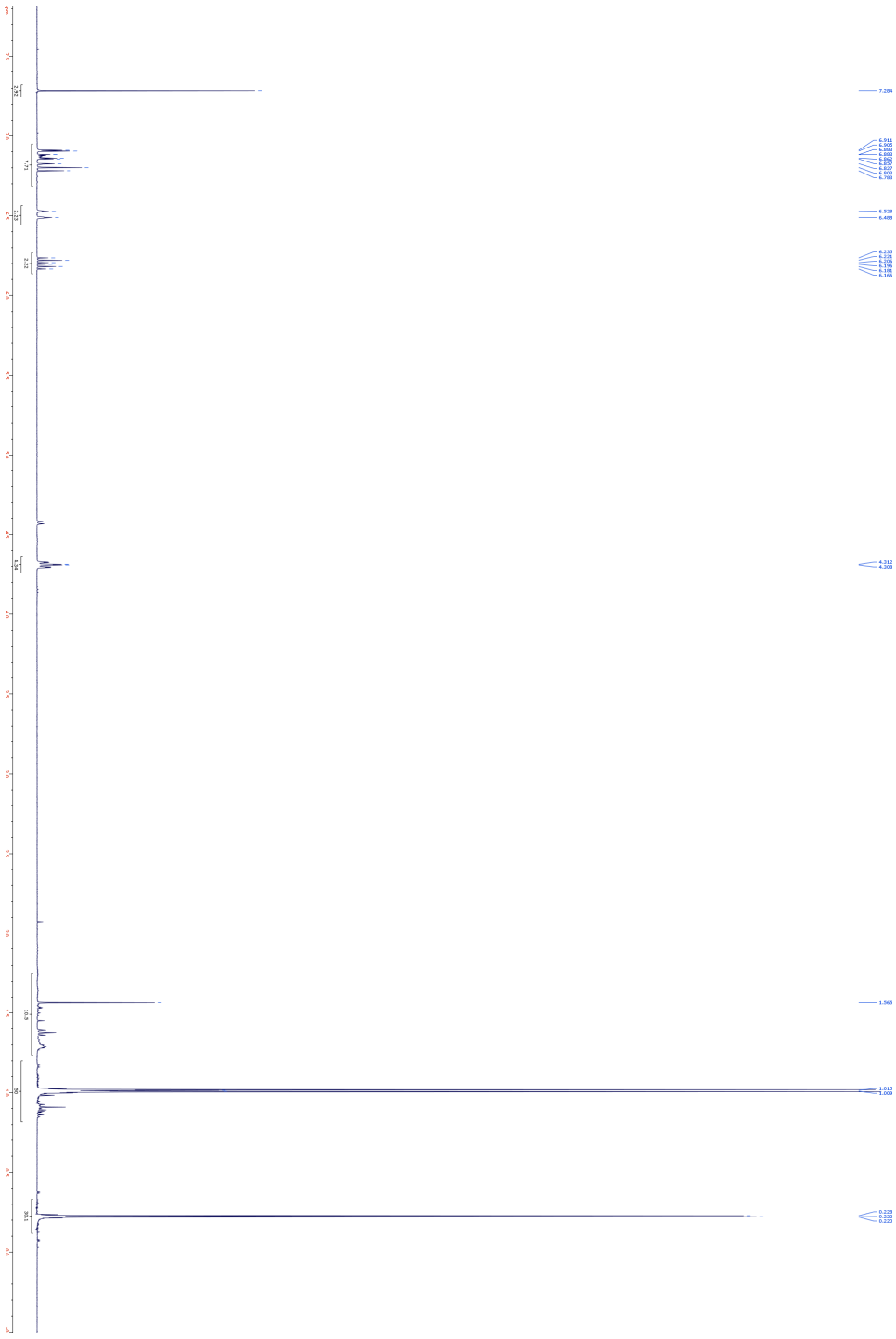
2 ^{1}H NMR in $CDCl_3$



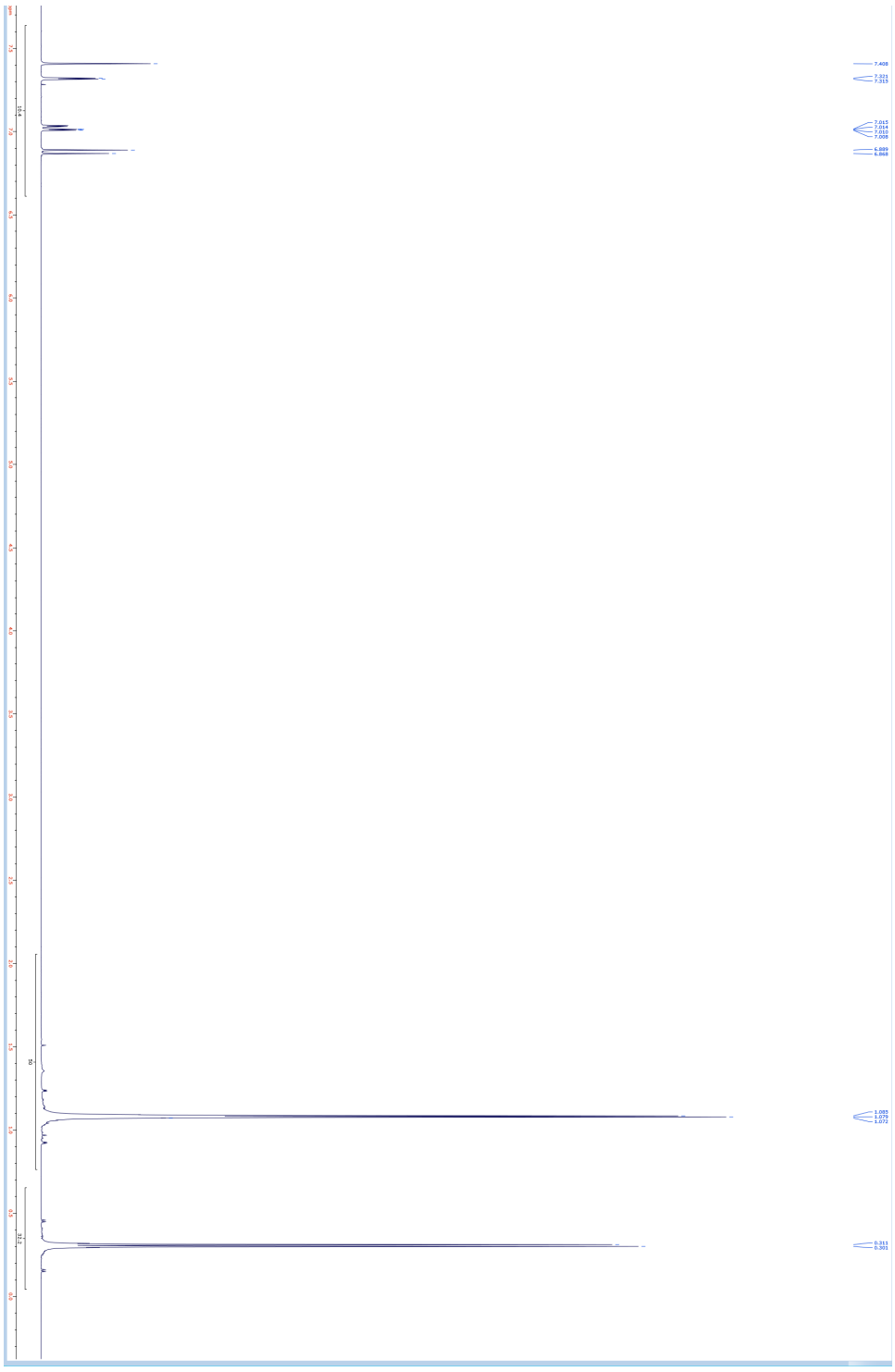
3 ^{1}H NMR in $CDCl_3$



4 ^{1}H NMR in $CDCl_3$

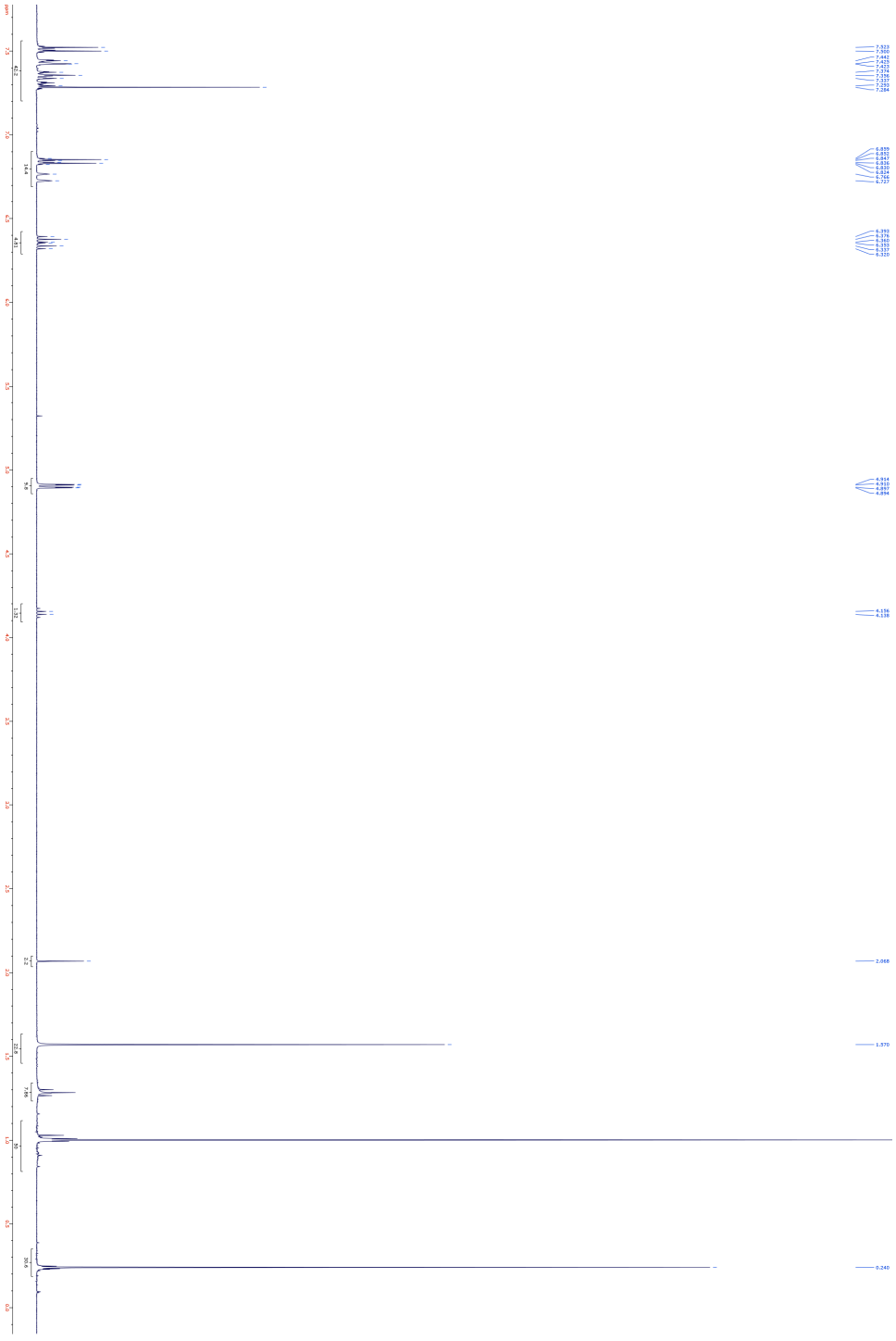


5 ^{1}H NMR in $CDCl_3$

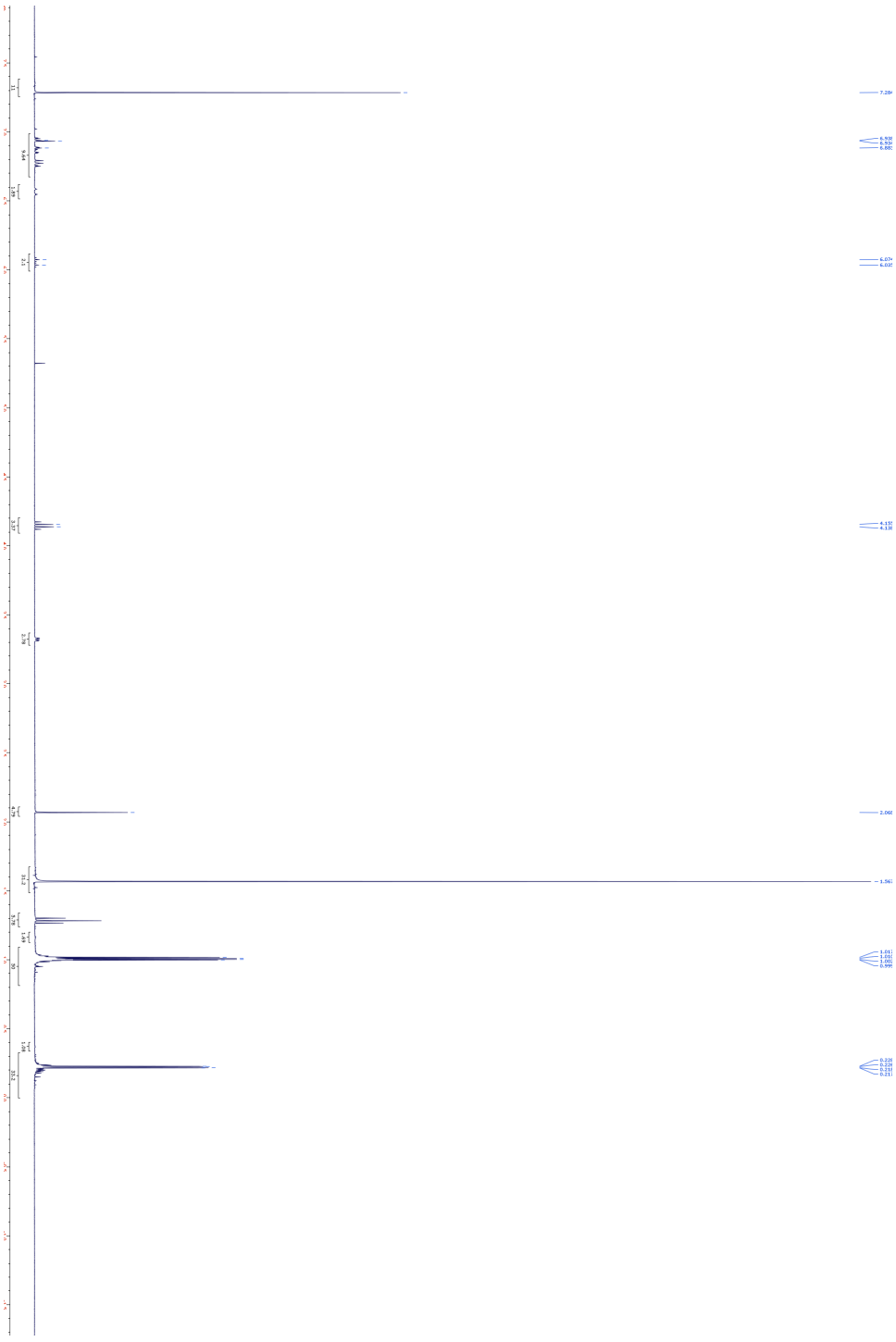


6 ^{1}H NMR in $CDCl_3$

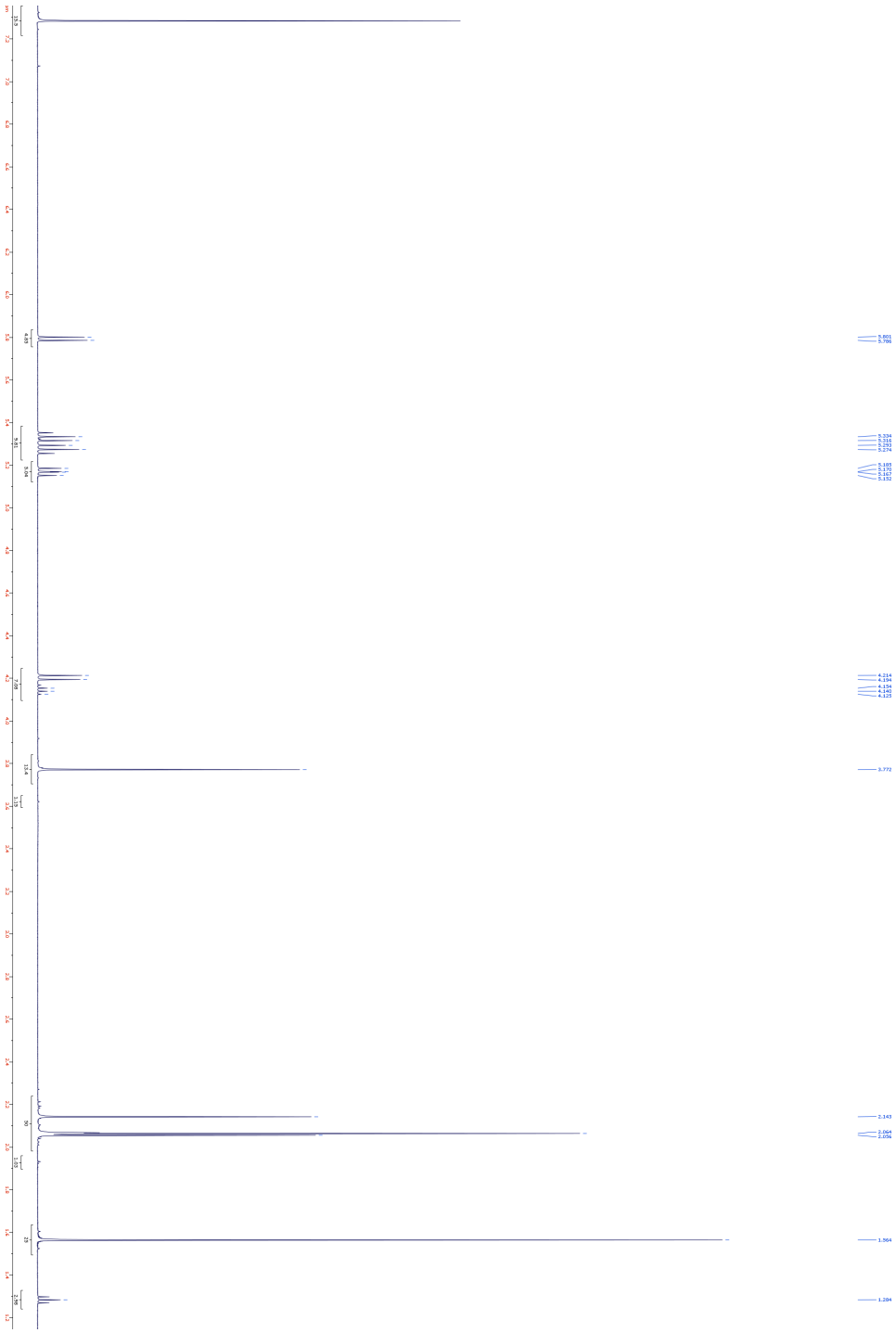
7 ^{1}H NMR in $CDCl_3$



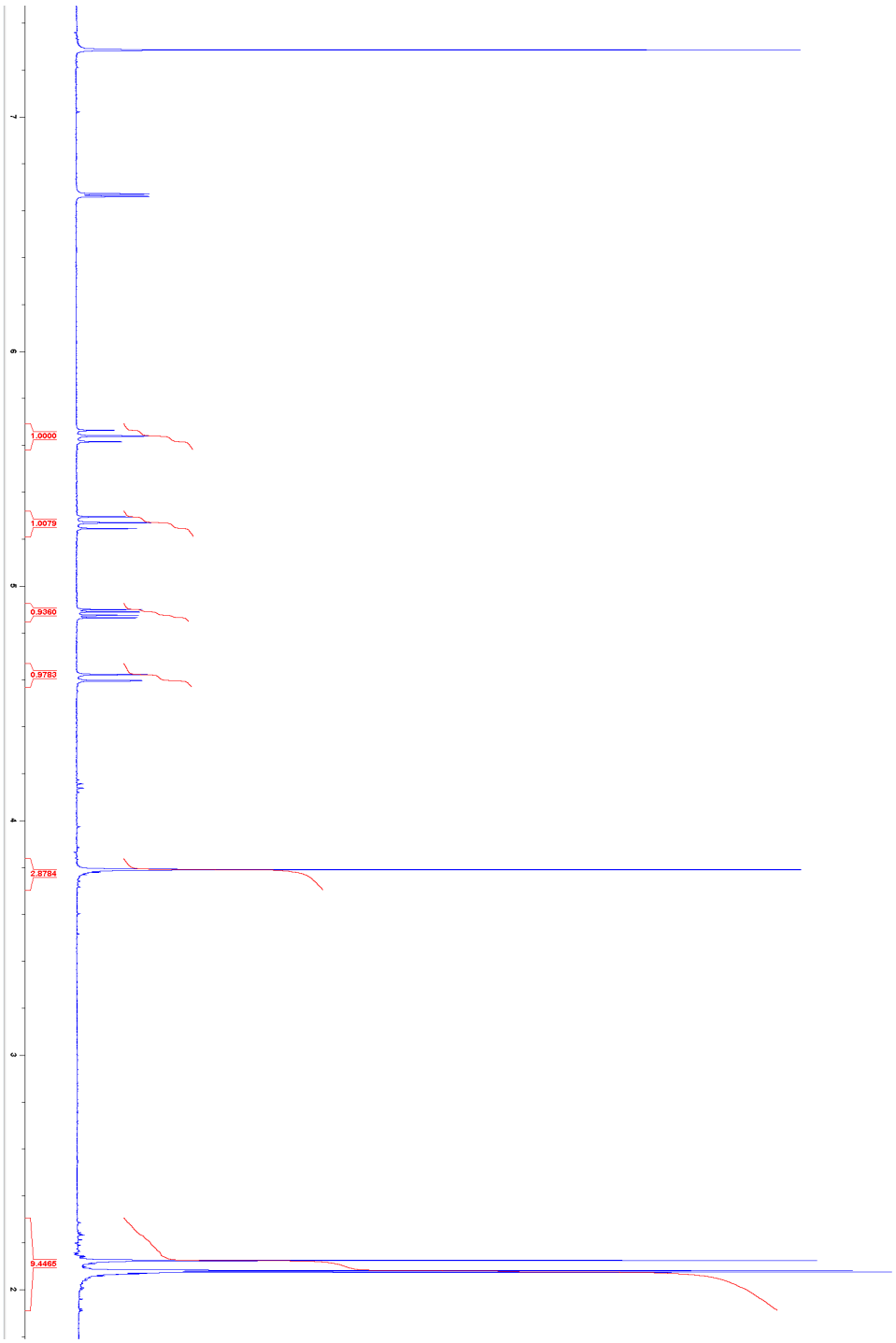
8 ^{1}H NMR in $CDCl_3$



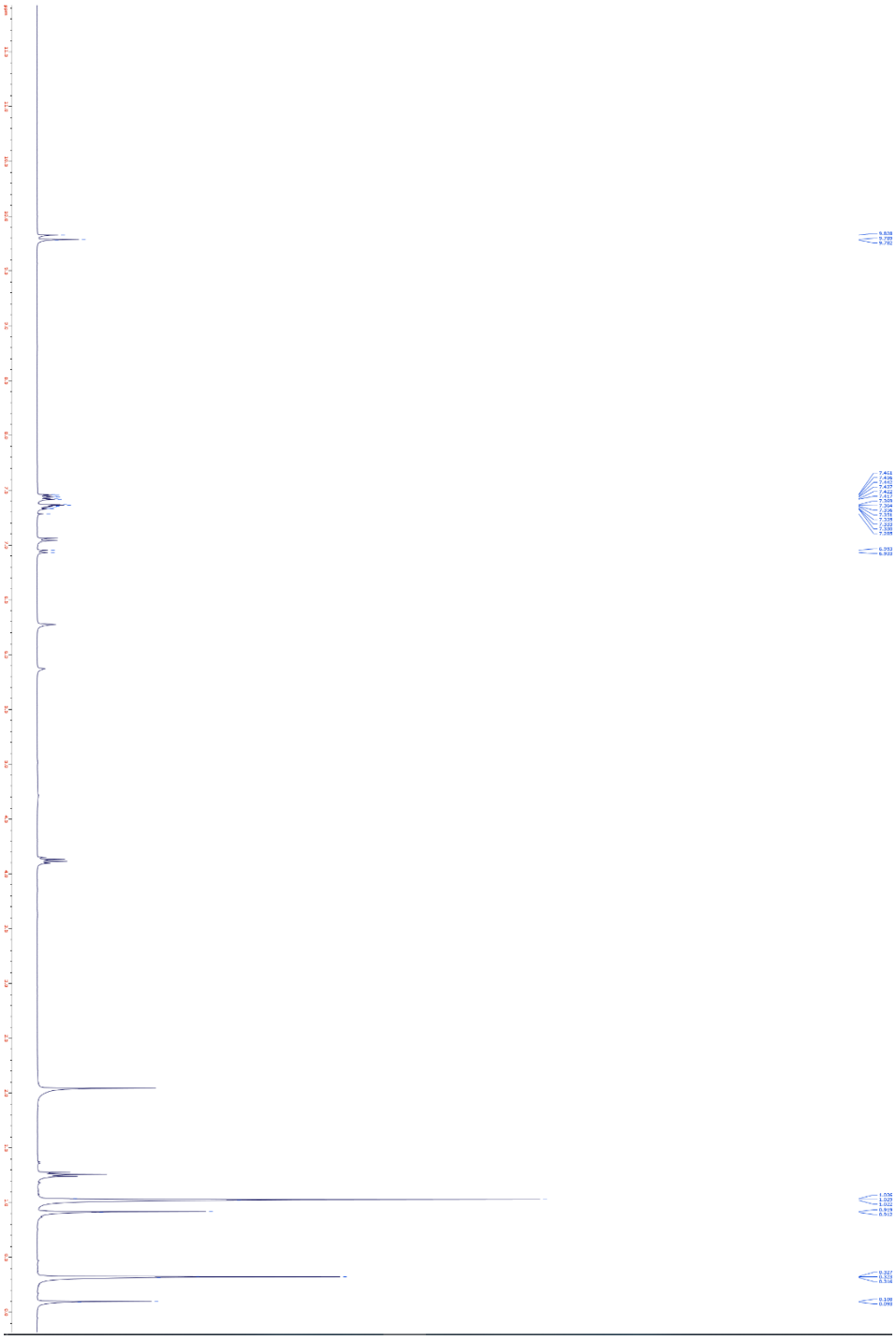
10 ^{1}H NMR in $CDCl_3$



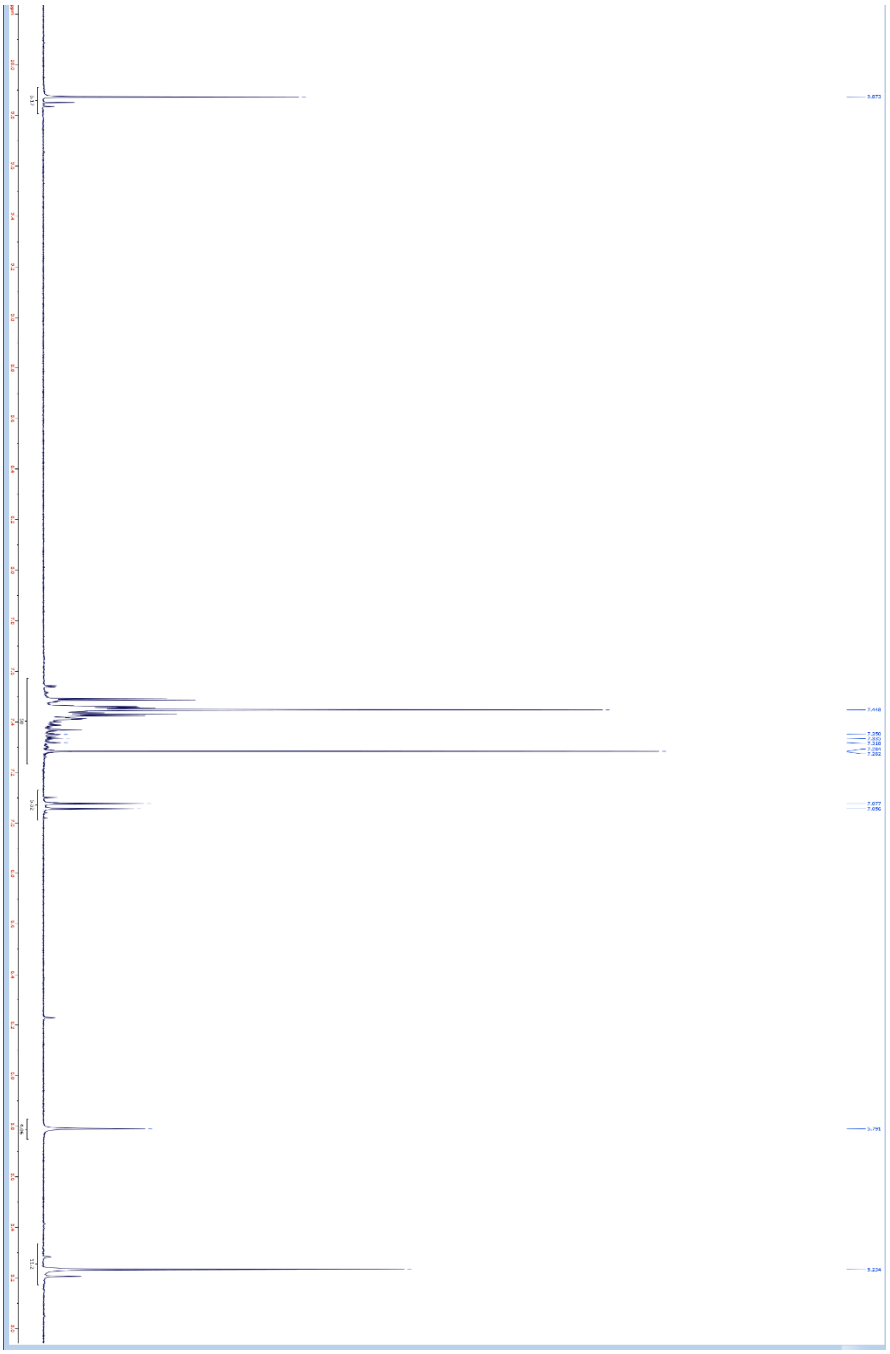
11 ^{1}H NMR in $CDCl_3$



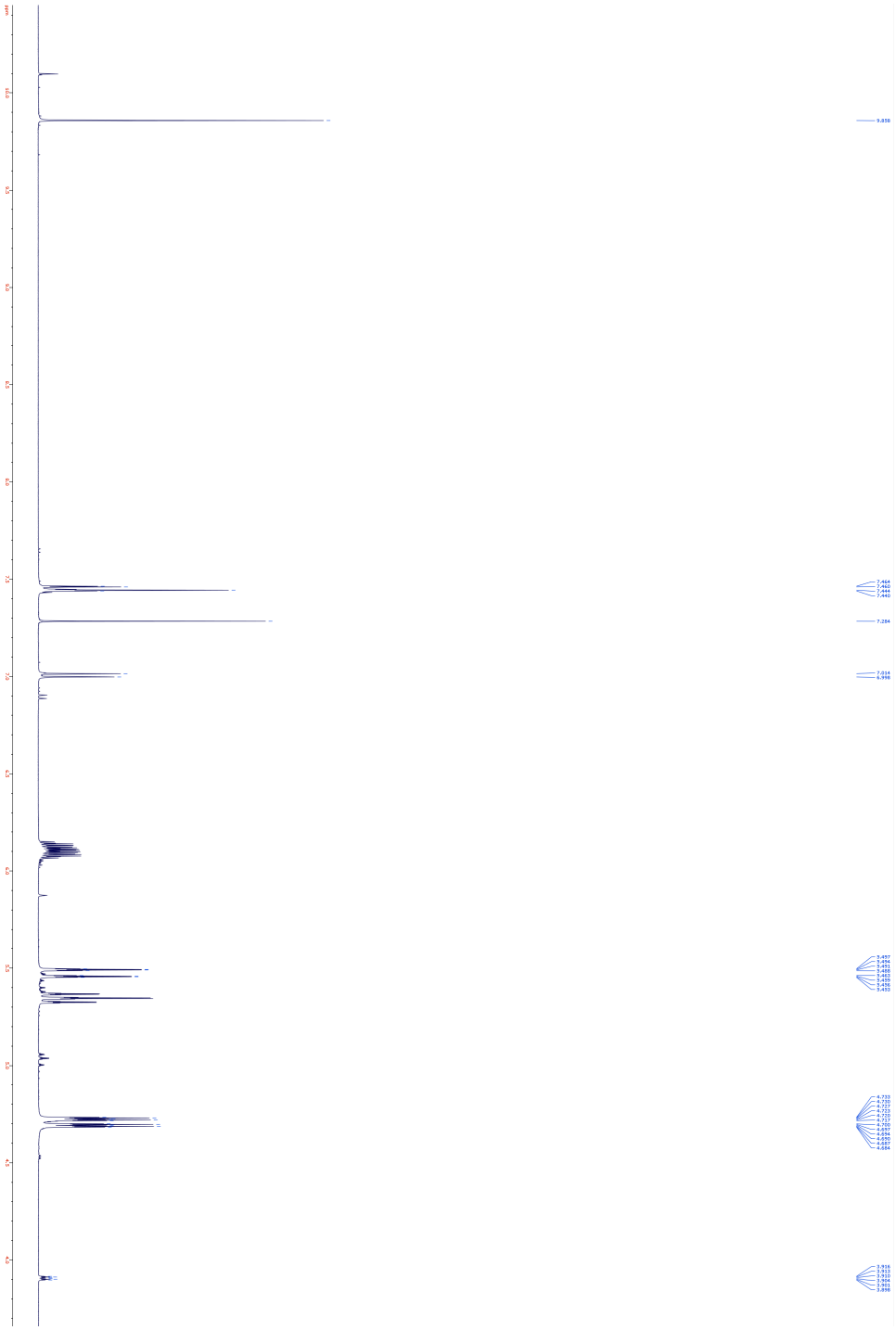
13 ^{1}H NMR in $CDCl_3$



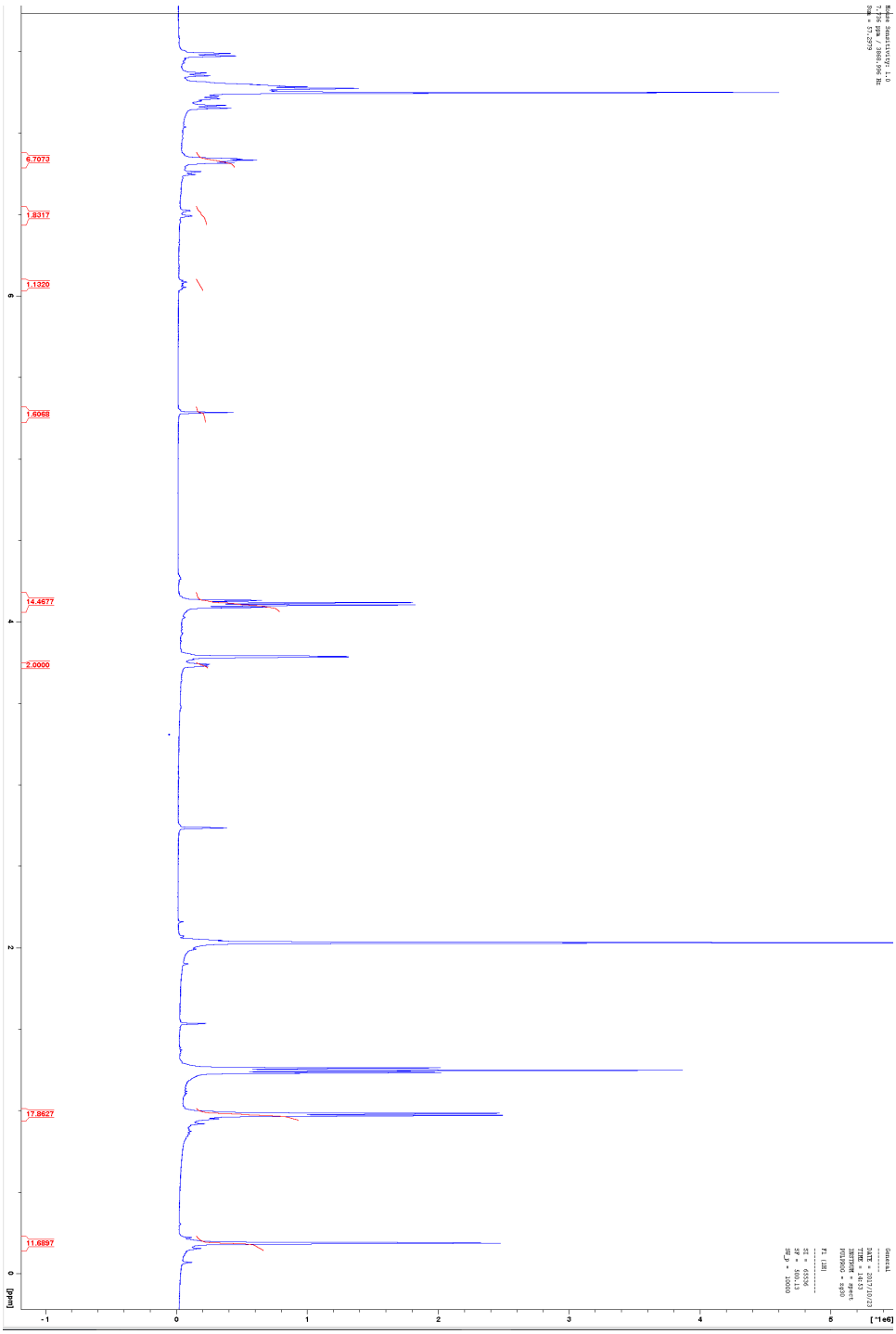
14 ^{1}H NMR in $CDCl_3$



15 ^{1}H NMR in $CDCl_3$



16 ^{1}H NMR in $CDCl_3$



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