

The Synthesis of Selective Activators of Adenylyl Cyclase Isoform 6 for the Treatment of Persistent Pulmonary Hypertension of the Newborn

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A Thesis submitted to the Faculty of Graduate Studies
of The University of Manitoba in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Abstract

In this piece of work, Persistent Pulmonary Hypertension of the New-born (PPHN), a condition that reduces the dilation of blood vessels, preventing a normal transition to independent breathing, will be discussed. PPHN leads to hypoxia and, if untreated, can be fatal.

Under these conditions, an enzyme known as adenylyl cyclase (AC) has decreased activity; AC isoform 6 is associated with vasodilation and, if activated, could produce a vasodilation effect. This would allow blood vessels to relax and breathing to return to normal.

Forskolin, a natural product from plants, can activate all nine transmembrane isoforms of AC; if forskolin was given as a treatment for PPHN, it could activate AC6, causing vasodilation, returning breathing and blood oxygen levels to normal.

Unfortunately, forskolin does not activate AC6 specifically, and activating all isoforms of AC would cause side effects throughout the body and may worsen the condition.

Previously forskolin has been successfully derivatized to activate different isoforms of AC, AC2 and AC5 selectively; similar studies have not been completed for AC6. However, this previous work suggests that increasing isoform selectivity is possible.

With selective activation of AC6, a forskolin derivative could be an effective treatment for PPHN.

Acknowledgements

During my graduate studies, I received support from friends, family and colleagues, too many to name here. I want to thank Dr Sorensen and the Sorensen Group; John has always supported my ideas and given me the freedom to carry them out. I want to thank Harjasmin and my collaborators, Shyamala and her research team, particularly Vikram, Martha and most recently, Saeid. The project was more successful than I could have hoped. I'm excited to see what happens next, and I will follow the work of our collaborators in the future.

I'm grateful to all the undergraduate and graduate students I have mentored. Teaching them helped me become a more compassionate and better communicator, especially Andrew and Scott. I would also like to thank April, Harman and Randi for keeping things interesting.

Volunteering during Science Rendezvous allowed me to grow and gain many science communication-related skills while engaging young people with science. I want to thank Seema for pushing me to try something new and participate in a fantastic event. I'd also like to thank Sari, Harjasmin and Scott.

I want to thank my parents for always encouraging me to take chances and opportunities presented to me, and for their continued emotional and financial support. Also, I would not have gotten here without Lyric and Ryan's support.

Finally, I thank the department and support staff, especially Emy, Terry and Dave, for putting up with my questions and always being friendly faces and my committee for their comments, feedback and discussions.

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Chapter 1 – An Introduction to Persistent Pulmonary Hypertension of the Newborn, Adenylyl Cyclase and Forskolin

1.1 Persistent Pulmonary Hypertension of the Newborn

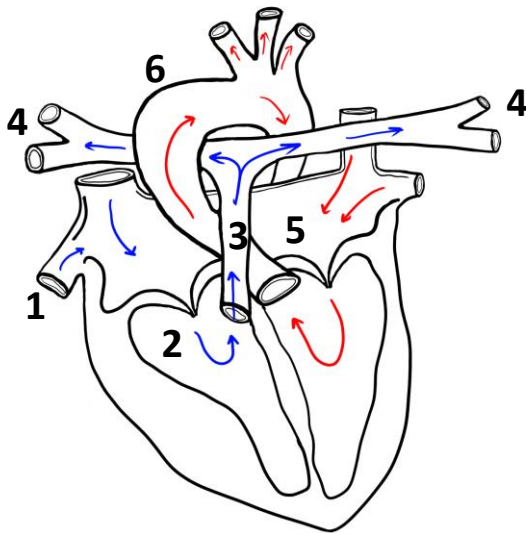
Persistent pulmonary hypertension of the newborn (PPHN) is a condition that affects the pulmonary vasculature. The typical pulmonary vascular adaptation that occurs at birth fails, characterized by constricted pulmonary arteries and vascular remodelling^{1, 2}. Before birth, the foetus receives oxygen from the mother via the placenta. Blood bypasses the lungs at this time, so reoxygenation will not occur. To maintain this state, pulmonary vascular resistance (PVR) is elevated, due to low oxygen levels³, while the pulmonary vascular flow is low². This will cause oxygenated blood from the mother to flow to the fetal brain, heart and other essential areas through a process known as right-to-left shunting⁴, while avoiding the lungs.

In utero, the organ of oxygen exchange is the placenta, not the lungs. To accommodate for this, there are two additional connections in the fetal heart that are not present in the adult heart. The patent foramen ovale (PFO) connects the right and left atriums, allowing blood to pass between them. The ductus arteriosus allows blood to bypass the lungs; instead, blood passes from the pulmonary artery to the aorta⁵.

When deoxygenated blood enters the heart, instead of passing through the right ventricle to the pulmonary artery, it crosses from the right ventricle to the left ventricle, allowing the blood to return to the body. This occurs because of constricted blood vessels between the heart and lungs, as the blood will follow the path of least resistance away from the lungs⁶.

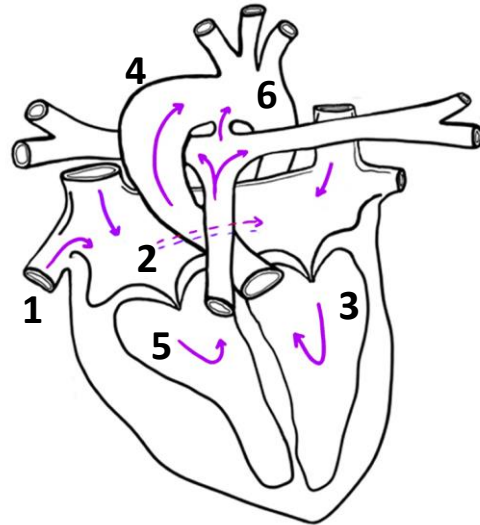
Before birth, this is a healthy physiological state and beneficial as it reduces the volume of blood required for oxygenation and allows deoxygenated blood to bypass the lungs while providing oxygen to essential areas.

Adult Heart Circulation



1. Deoxygenated blood enters heart from body
2. Blood enters the right atrium, to the right ventricle
3. From the right ventricle to the pulmonary artery, to the lungs
4. Reoxygenation of blood in lungs
5. Blood returns to heart from lungs
6. Through left atrium, left ventricle into the aorta to rest of body

Fetal Heart Circulation



1. Oxygenated blood enters heart from umbilical cord, deoxygenated from body
2. Enters right atrium, passes through the PFO to the left atrium (right to left shunting) or to the right ventricle
3. Blood flows from left atrium to left ventricle
4. Blood travels through the aorta, oxygenated blood goes to brain first
5. Simultaneously partially oxygenated blood returns to the right atrium, and towards the lungs
6. To by-pass the lungs blood will pass through the ductus arteriosus to the aorta, to the lower half of the body and to return to the placenta

Figure 1. Diagram depicting healthy adult heart circulation and healthy fetal heart circulation. Red arrows represent oxygen rich blood, purple medium, while blue arrows represent oxygen low blood.

After birth, PVR decreases rapidly, and pulmonary vascular flow increases 8-10 fold⁷; this is in response to an increase in oxygen tension, removal of fluid from and expansion of the lungs⁸ and a decrease in carbon dioxide in the blood, increasing pH⁹. Blood vessels connecting to the lungs relax, reducing the right to left shunting as it is no longer the path of least resistance, supported by the PFO beginning to close. Blood can then travel throughout the body and enter the lungs once the remaining fluid has been expelled/reabsorbed^{8, 10}. Blood will then be oxygenated, return to the heart, enter by the left atrium, left ventricle, and through the aorta to return to the body. This process is undergone for a healthy transition to independent breathing.

Before birth, various enzymes maintain elevated PVR, including endothelin-1 and thromboxane¹¹. Thromboxane is present in high concentrations in the neonatal circulation at birth, in the closure of the umbilical vessels¹². Acidosis and other mediators of inflammation can also contribute to raised PVR^{4, 11}.

To initiate the transition process before birth, nitric oxide (NO) production is increased due to an increase in the expression of endothelial nitric oxide synthase (eNOS)² and soluble guanylate cyclase (sGC). There is also preparation for increased activation of adenylyl cyclase (AC), as cyclooxygenase is upregulated, increasing production of prostacyclin¹³, which acts as an activator of AC via a G-protein coupled receptor (GPCR) pathway¹⁰. NO acts as a vasodilator by activating sGC¹⁴.

The levels of phosphodiesterase-5 (PDE5), an enzyme that terminates a vasodilatory response, are also decreased⁹, increasing the duration of a vasodilation response. For a healthy new-born, these changes happen rapidly, with full pulmonary perfusion occurring within the first few minutes. PVR decreases more steadily, usually stabilising within three weeks¹⁵.

PPHN is characterized by a sustained high PVR and continued right-to-left shunting. A significant percentage of blood returning to the heart from the body will cross the ductus arteriosus, passing from the right to the left ventricle and bypassing the lungs, as observed in fetal circulation (**Figure 1**). By passing the lungs, oxygenation of the blood is prevented; the blood

recirculates through the body. This state is then maintained as the blood vessels cannot relax, and functional breathing cannot commence¹⁵.

Due to increased blood pressure, there is also an increase in pulmonary arterial fibrosis and damage to or scarring of the arteries surrounding the heart. Remodelling of pulmonary vasculature leads to an increase in PVR, as a small decrease in artery diameter can significantly affect vascular resistance. This is due to the proliferation or growth of the predominant cell type area, endothelial and smooth muscle cells¹⁶.

Treatment for PPHN primarily involves selective pulmonary vasodilation to reduce PVR¹⁷. PPHN causes decreasing blood oxygen levels, difficulty breathing, and a blue tinge to the skin; if untreated, it can be fatal. PPHN occurs in approximately 0.4-6.8 per 1000 live births in the UK and 2 per 1000 in the USA (released 2015)¹⁸ and is associated with a mortality rate ranging from 5-35%¹⁹. However, this has decreased significantly in recent years²⁰.

1.1.1 PPHN Categorisation

Persistently high pulmonary vascular resistance and vascular remodelling are characteristic of PPHN, but PPHN has several different causes and presentations so PPHN diagnosis can be separated into three distinct categories²¹.

1.1.2 Irritation or Obstruction of Airways

The first type involves the blood vessels remaining abnormally constricted, but there appears to be little or no development of structural damage. This can be caused by meconium aspiration syndrome (MAS), damage to or inflammation of the lungs by other causes. MAS can cause irritation, obstruction of the airways and deactivation of surfactants in the lungs, affecting alveoli surface tension and contributing to breathing difficulties^{11,22}. MAS accounts for approximately 40% of PPHN cases, while sepsis accounts for 25%. Patients suffering from inflammation or sepsis have the worst response to vasodilators²³.

1.1.3 Idiopathic PPHN

The second type, idiopathic PPHN, includes antenatal remodelling, thickening of the blood vessel walls, and an increase in smooth muscle formation. This could be caused by

constriction of the fetal ductus arteriosus, a blood vessel in the neonatal heart; it contributes to deoxygenated blood bypassing the lungs in utero and is essential for maintaining oxygenation before birth²⁴. Premature constriction of this blood vessel is associated with pulmonary vascular remodelling and increases in arterial pressure; this has increased risk in the presence of nonsteroidal anti-inflammatory drugs (NSAIDs)^{11,25}. The connection between constriction of the ductus arteriosus and PPHN is well established²⁶. Idiopathic PPHN is associated with loss of activity from both sGC and AC vasodilation pathways; eNOS is less effective under PPHN conditions, with decreased expression and activity²⁷, producing less nitric oxide (NO). Abnormally high levels of endothelin-1 (a vasoconstrictor) are found in neonates with PPHN²⁸; idiopathic PPHN potentially involves multiple interlinked pathways combined with remodelling of the vasculature.

1.1.4 PPHN Due to Developmental Issues

The third category of PPHN involves developmental issues within the lung or pulmonary vasculature, the most discussed condition in the literature being congenital diaphragmatic hernias (CDH)²⁹. The diaphragm sits between the thorax and abdomen as a barrier to keep them separate; in cases of CDH, the diaphragm fails to form completely. This allows organs from the abdomen to migrate into the chest, preventing full lung development. Repairing the diaphragm surgically is possible, and combined with treatments for the associated pulmonary hypertension (PH), has reduced the mortality rate to between 10 and 20%²⁹. Surgery is no longer considered essential as the mortality arises in association with PH/PPHN as opposed to the misplaced organs in cases where the lungs are properly formed¹¹.

Another developmental condition is alveolar capillary dysplasia (ACD); patients have improperly formed air sacs in the lungs combined with or without misaligned pulmonary vasculature, which is rare but often fatal³⁰. This condition could be an option for investigation if a patient suffers from PPHN-like symptoms and does not respond to standard treatment (vasodilators, ECMO)³¹.

1.1.5 Persistent Pulmonary Hypertension of the Newborn Causes

PPHN appears to be a secondary condition in most cases, an undetermined condition is causing the maintenance of high PVR, the cause and appropriate treatment for PPHN should therefore be determined on a case-by-case basis, guided by the three categories discussed.

1.1.6 Contributing factors of PPHN

PPHN is associated with several risk factors, such as premature birth, maternal asthma³², and caesarean section³³. The risk of PPHN was especially high when the caesarean was performed before 39 weeks, suggesting the risks are especially high in cases where the neonate's pulmonary system is undeveloped³³. Caesarean section does not trigger the usual enzymatic response supporting the transition to post-birth breathing⁸.

Emergency caesarean sections were conducted in PPHN cases approximately 50% of the time due to maternal or foetal complications, compared to 20% in the control population, suggesting the caesarean section may have been conducted in response to an existing condition³².

Dysregulation of Thromboxane Prostanoid and NO Receptors

Two main pathways control pulmonary vascular resistance, the nitric oxide-endothelin and the prostacyclin-thromboxane system³⁴. Described below are several of the mechanisms that cause an increase in vasoconstriction; these mechanisms are less effective under hypoxic conditions, contributing to a positive feedback loop, and raising PVR.

Prostacyclin-Thromboxane

Thromboxane A₂ (TxA₂) is an inflammatory prostanoid with vasoconstrictive properties, activated in the presence of cell damage or bleeding. It contracts pulmonary arterial myocytes through a G α_q -coupled thromboxane receptor, which in turn causes an increase in Ca²⁺ concentration, causing a contractile response³⁵. Phosphorylation is the primary regulator of vascular prostanoid receptors²³.

TxA₂ is present in high concentrations in the neonatal circulation at birth, as it is required to close umbilical vessels upon birth. Hypoxic PPHN increases the ratio of TxA₂ to prostacyclin²³

(ratio of vasoconstrictor to vasodilator), and elevated thromboxane levels have been associated with PPHN³⁶. A study of rat lungs under hypoxic conditions showed an increased vasoconstriction response to a thromboxane mimetic¹². Inhibition of thromboxane receptors results in a decrease in pulmonary hypertension caused by hypoxia³⁶; during treatment with ECMO, thromboxane levels decrease³⁷.

In normoxic neonatal pulmonary arteries, Thromboxane Prostanoid (TP) receptors are phosphorylated on a serine residue, prostacyclin receptor activity induces this phosphorylation, decreasing thromboxane receptor-ligand affinity and, therefore, thromboxane receptor activity. TP receptor sensitization in hypoxic myocytes is also increased due to prostacyclin (IP) receptor inactivation; this sensitization may be reversible using a cAMP-specific phosphodiesterase (PDE3) inhibitor Milrinone²³.

Prostacyclin mediates vasodilatory and anti-inflammatory actions via activation of the IP receptor, which is coupled to adenylyl cyclase via G α s. Postnatal prostacyclin production is high in healthy infants, but hypoxia decreases the concentration of prostacyclin (PGI₂), by reducing production³⁸.

Hypoxia has a priming effect on pulmonary arterial myocytes, increasing the production of inositol 1,4,5-triphosphate (IP₃) in the presence of vasoconstrictors³⁹. IP₃ mobilises intracellular calcium ion stores, increasing calcium ion sensitivity; this causes an increase in smooth muscle proliferation (pulmonary remodelling) and vasoconstriction⁴⁰.

Nitric Oxide-Endothelin

The nitric oxide-endothelin mediated vasodilatory system induces vasodilation by nitric oxide binding to soluble guanylate cyclase (sGC), which catalyses the conversion of guanylyl triphosphate (GTP) to cyclic guanylyl monophosphate (cGMP). cGMP activates calcium-gated potassium channels that induce relaxation of the smooth muscle tissue⁴¹.

The counterpart to NO is endothelin, a peptide that induces vasoconstriction by binding with a GPCR, which results in an increase in intracellular Ca²⁺ concentration, causing vasoconstriction.

Increased levels of vasoconstrictors such as endothelin-1 or thromboxane decrease the vasodilatory response produced by NO; vasoconstrictors remaining present in the system could prevent vasoactive mediators from contributing to a healthy transition and reduce how effective treatments are.

Both in infants born before⁴² and after 37¹⁴ weeks increase in oxygenation levels when iNO is given is only observed in 50% of cases. This effect is also observed in adults but is not permanent⁴³. In a study of hypoxic porcine cells, there is evidence that hypoxia directly inhibits the activity of AC in both in vivo and in vitro models⁴⁴.

Selective Serotonin Reuptake Inhibitors

PPHN has also been associated with using SSRIs, such as fluoxetine, in the later stages of pregnancy. Although the mechanism for this is currently unknown, there have been several suggestions for possible routes, such as the accumulation of SSRIs in the newborn lungs and increasing serotonin levels. Serotonin is known to amplify a vasoconstriction response⁴⁵, increasing the effects of present vasoconstrictors and potentially contributing to the maintenance of high PVR via the increased production of pulmonary smooth muscle cells (PSMCs)⁴⁶. A more recent meta-analysis suggested that while there was an increase in absolute risk, it was a small one⁴⁷, but in these cases, SSRIs may exacerbate an already existing issue.

1.1.7 PPHN Treatment

When an infant experiences respiratory distress, a standard evaluation would involve an x-ray of the chest and a measure of arterial blood gas. At the same time, attempts are made to stabilise breathing/oxygenation. If symptoms of difficult breathing are disproportionate to the appearance of any disease in the x-ray results, it would suggest idiopathic PPHN. This can be confirmed by measuring blood oxygen levels in various areas of the body and comparing them. A difference of 5% or more suggests PPHN⁴⁸.

Suppose PPHN is suspected, the following diagnostic step would be the completion of an echocardiograph. This is an ideal diagnostic tool, as right to left shunting at the ductus arteriosus and elevated arterial blood pressure⁴⁸ can be observed.

The priority when treating PPHN is selective pulmonary vasodilation; treatments will attempt to deliver oxygen or improve cardiac function. Lung expansion must be maintained at an appropriate level and carefully controlled as too much or little expansion can increase pulmonary vascular resistance in patients, worsening the condition⁹. An increase in systematic arterial pressure can cause a reduction in right-to-left shunting, improving oxygenation.

Treatment should include monitoring or measures to counteract several physiological imbalances, such as hypoglycaemia, acidosis and hypocalcaemia⁴⁸.

Surfactants

Surfactants are a mix of phospholipids and proteins in the lungs that support alveoli function, allowing them to expand by decreasing surface tension without the risk of collapsing. They also inhibit the growth of pathogens and support the elimination of larger particles from the lungs⁴⁹. Introducing surfactant therapy increased oxygenation while decreasing the need for ECMO and improving iNO responses⁵⁰.

The use of surfactants as a treatment was not supported in cases of CDH⁵⁰, unless there is evidence specifically of surfactant inactivation. However, in cases of PPHN associated with MAS and pneumonia, there was a decrease in the length of hospital stays and an improvement in general condition⁵¹.

Inhaled Nitric Oxide

Nitric oxide is used as a treatment for PPHN as it acts as a vasodilator; a patient inhaling NO (iNO) will experience a decrease in PVR, while a reduced response to NO can contribute to the failure of postnatal transition⁵². Response to iNO as a treatment varies widely between individual patients, approximately 50% of patients respond when iNO is given, and it is ineffective for treating patients with a congenital diaphragmatic hernia¹⁴. Another study states that 30% of PPHN patients treated with iNO do not see a sustained increase in blood oxygen levels⁵³. Sufficient lung recruitment is required for iNO to be an effective treatment; low lung recruitment may indicate poor treatment outcomes, which may need to be combined with surfactants or high-frequency ventilation⁴.

iNO has been shown to reduce the impact of arterial remodelling in rats exposed to chronic hypoxia⁵⁴. While this provided a temporary reduction in pulmonary artery pressure, once NO administration was halted, artery pressure increased rapidly, this effect occurred with increasing vasoconstriction. As there was a decrease in pulmonary remodelling in these cases, it was suggested that it might be due to the anti-proliferation effects of NO decreasing proliferation of aortic muscle cells⁵⁵. If a patient responds poorly to iNO ventilation, other strategies such as mechanical ventilation or PDE inhibitors will be used⁵¹.

Prostacyclin

Prostacyclin can be administered as a medication for PPHN⁵⁶; in humans, prostacyclin is produced in endothelial cells from arachidonic acid, increasing Gas production and, in turn, causing vasodilation. A cyclooxygenase (COX) enzyme supports prostacyclin production and is the rate-limiting enzyme in prostacyclin production. COX-1 is upregulated during the development and formation of the neonatal lung, increasing prostacyclin production around the time of birth. This treatment will be used alongside others to improve lung function. There is a risk in using prostacyclin to stimulate AC activity in a clinical setting due to the rapid desensitisation of IP-mediated vasodilatory response⁵⁷. While improving patient condition in the short term, decreased vasodilation may occur due to inhibition of the AC pathway.

Inhaled Oxygen

Introduction of excess oxygen can cause a rapid reduction in PVR at birth⁴⁸ initially, non-invasive methods are used, but progression to invasive methods such as ECMO may be required if initial therapy is unsuccessful²⁰.

Ventilation with O₂ causes a significant decrease in PVR, especially when 100% O₂ is given, which has previously been recommended as a treatment for newborns⁵⁸. However, this treatment appears to cause delayed issues; despite the rapid fall in PVR lambs treated with 100% O₂, iNO/sGC responsiveness is significantly decreased in lambs treated with O₂. Reduced responsiveness occurs even if the oxygen exposure is brief. These effects were found to be reduced if less than 100% O₂ was given⁵⁹. This could be for several reasons; oxidant stress may increase the expression of cGMP-specific PDEs, and increased oxygen content can also react

with NO to form peroxynitriles (ONOO⁻). Peroxynitriles can cause lung injury, dysregulate vasocontrolling signalling pathways and causes an increase in NO, increasing nitrosylation signalling. The decrease in NO concentration will also affect the vasodilatory response, as it can no longer activate the appropriate enzyme⁶⁰. Reactive oxygen species also have a role in rebound pulmonary hypertension, following NO withdrawal, by reducing NO production⁶¹.

Phosphodiesterase Inhibitors (PDEs)

Phosphodiesterase (PDEs) are a family of enzymes that catalyse the breaking of phosphodiester bonds. Two examples of PDEs, PDE3 and PDE5, catalyse the conversion of cAMP to AMP and cGMP to GMP respectively (**Figure 2**)⁶². cAMP and cGMP cause vasodilation, while their non-cyclic counterparts are unable to activate the appropriate enzyme, terminating the vasodilation response.

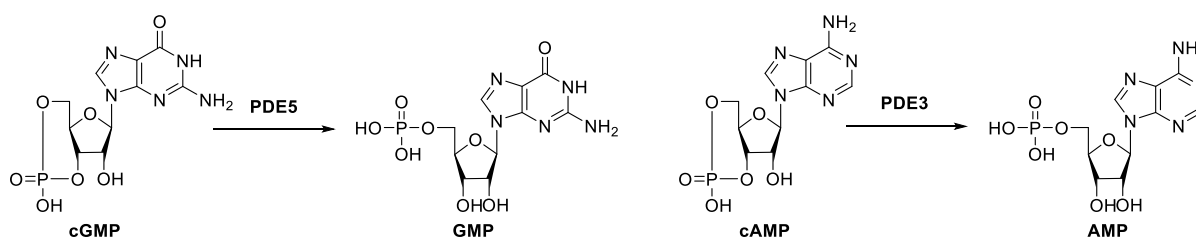


Figure 2. Cyclic GMP and Cyclic AMP are converted to non-cyclic counterpart by phosphodiesterase enzymes. This reaction can be prevented with appropriate phosphodiesterase inhibitors. cGMP and cAMP are responsible for a vasodilatory response, by inhibiting their breakdown the vasodilatory response continues.

PDE3 and PDE5 inhibitors reduce response termination, allowing cAMP/cGMP to continue causing a vasodilatory effect. Sildenafil is the only PDE inhibitor currently prescribed for newborns with PPHN⁶³. Milrinone has undergone initial randomised control trials and appears effective at decreasing pulmonary arterial pressure; this study tested three conditions. Monotherapy with Milrinone was compared to Sildenafil, and a combination treatment with Milrinone and Sildenafil, the combination treatment was the most effective and recommended for resource-constrained centres. Further studies will need to be completed to understand possible side effects⁶⁴.

Extracorporeal Membrane Oxygenation

Extracorporeal Membrane Oxygenation (ECMO), involves drawing blood from the body to oxygenate it artificially; this was the most successful treatment for PPHN. However, ECMO has risks, including intracranial haemorrhage and arterial ligation⁶⁵. There is also the danger of overexpansion of the lungs, which may worsen PPHN due to damage caused to alveoli¹¹.

ECMO supports adaptation to normal breathing or stabilises a patient until appropriate treatment can be administered. Patients treated with ECMO for less than seven days have high rates of recovery ($\leq 85\%$), according to one study that looked at approximately 1500 patients between 2000 and 2010, while patients requiring fourteen or more days of ECMO had a $\sim 25\%$ survival rate. There was a strong correlation between the number of complications occurring and the number of days on ECMO⁶⁶.

It should be noted that 50% of patients that passed away after ECMO treatment had stopped due to recovery of the lungs, the cause of death for these patients was not recorded in the study due to limitations on access to information⁶⁶.

In most cases, ECMO has been replaced with iNO¹⁴, which gives similar outcomes in a more affordable, accessible way, while ECMO is still used as a last resort. The introduction of NO did not decrease overall mortality of PPHN though⁹, with approximately 50% of patients not responding or having a weak response to treatment⁵³. This suggests that new or combined treatments could improve outcomes.

1.1.8 A Gap in the Treatment Options for PPHN

Infants treated for PPHN at birth show increased risk for long-term issues such as chronic health problems, hearing loss and need for breathing therapy⁶³. In a review published in 2012, the survival rate of PPHN patients treated with ECMO between 2000 and 2010 was 81%⁶⁶. This is consistent with previous publications examining the death rate between 1989 and 1998⁶⁷. This is unfortunate as it would be expected that treatments and management of conditions would have improved in the preceding decade.

Figure 3. describes the various routes treatments use to induce a vasodilatory response. iNO has reduced effectiveness, preventing vasodilation via a sGC response. AC activity is reduced under hypoxic conditions; a treatment such as a prostacyclin used to activate AC would have reduced effectiveness, as it acts through a GPCR. PDE inhibitors require previous activation of AC or GC to be effective, as there would be decreased levels of cGMP/cAMP to convert to GMP/AMP, again reducing their effect on PDE activity.

PPHN could be treated with a specific activator of AC6. Forskolin binds to an allosteric binding site between the C1 and C2 domains, causing a conformation shift and increasing cAMP production. Inhibition of AC is partly due to nitrosylation reducing binding with G α s; forskolin can activate AC independently of G α s, allowing for a vasodilatory effect downstream of AC without G α s mediated activity. This treatment could be used independently or with other medications to treat PPHN.

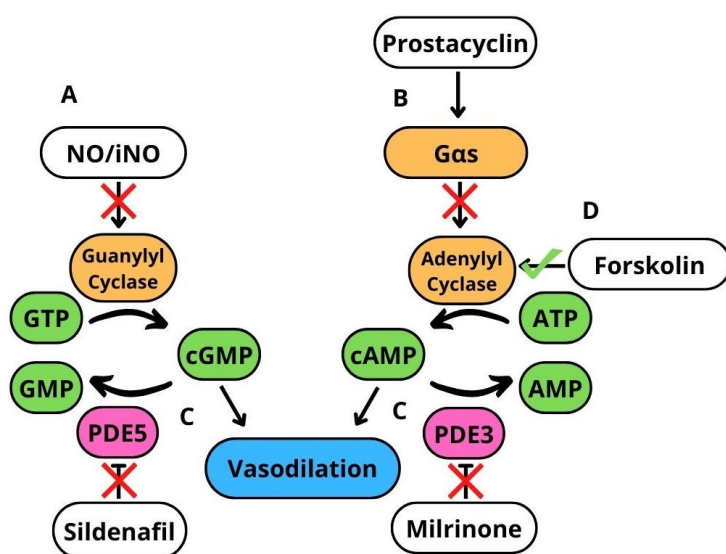


Figure 3. Routes to vasodilation that are inhibited under PPHN conditions. **A)** Nitric oxide does not cause a response in 40% of cases. NO activates guanylyl cyclase producing cGMP, causing vasodilation. **B)** Under hypoxic conditions activation of adenylyl cyclase is inhibited, this is in part due to nitrosylation of residues on AC preventing full activation by G α s. **C)** While PDE inhibitors such as Milrinone and Sildenafil extend the vasodilatory response, they require the presence of cGMP or cAMP to have this effect. **D)** Forskolin activates AC by an allosteric binding site, unlike other methods of activation this is expected to not be inhibited.

The currently available treatments for PPHN have been discussed; while they are effective in many cases, there is an opportunity to improve outcomes. Allosteric activation of, specifically, AC6 is an exciting option for treating PPHN as it uses a novel pathway, potentially bypassing the observed mechanisms of poor treatment response.

1.2 Adenylyl Cyclase (AC)

Adenylyl cyclase is an enzyme that catalyses the conversion of adenosine triphosphate (ATP) to cyclic adenosine phosphate (cAMP). cAMP is a universal signalling molecule, activating cAMP-dependent protein kinase (PKA); this will cause a change in Ca^{2+} concentration resulting in a downstream response. By this process, adenylyl cyclase regulates functions such as airway smooth muscle control⁶⁸ cell growth, protein expression and gene transcription⁶⁹.

Depression⁷⁰, inflammation⁷¹ and myocardial atrophy⁷² are associated with the cAMP pathway, indicating the diversity of responses to adenylyl cyclase activation.

AC plays a role in fundamental cellular processes in all life domains. Six distinct classes of adenylyl cyclase exist; only the third class (III) is present in mammalian genomes⁷³. Despite how structurally different these classes of enzymes are, they still carry out the function of converting ATP to cAMP⁷⁴.

1.2.1 AC activation pathway

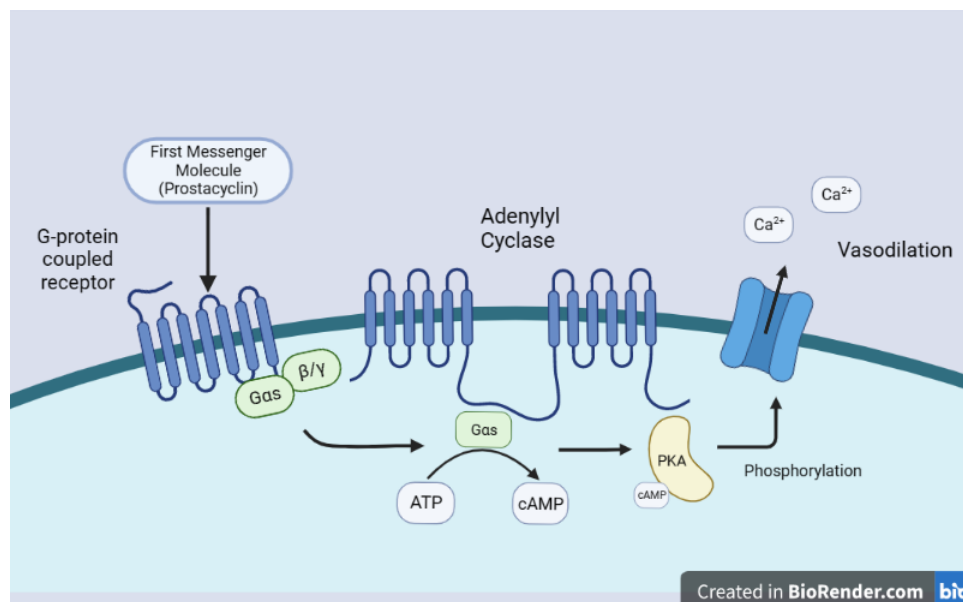


Figure 4. Depiction of adenylyl cyclase cascade, upon activation by first messenger molecule GTP will bind to G α s, G α s will then dissociate from the GPCR complex and bind to adenylyl cyclase. AC then converts ATP to cAMP, cAMP binds to and activates protein kinase A (PKA), PKA phosphorylates local calcium ion channels, inducing a vasodilation response. Created with BioRender.com.

Adenylyl cyclase is activated by a G-protein coupled receptor (GPCR). GPCRs comprise three independent units, a receptor, a heterotrimeric G protein (G α , G β and G γ proteins) and an effector⁷⁵.

Independent subunits allow for the specific regulation of each

component. A conformation change occurs once a signalling molecule activates the G-protein coupled receptor. This shift allows $G\alpha$, currently bound to guanosine diphosphate (GDP), to disassociate from the GDP molecule, replaced by guanosine triphosphate (GTP)⁷⁵. $G\alpha$ can then bind to and activate enzymes or ion channels; in this case, it binds to and activates AC. Activation molecules depend on location and AC isoform; AC6 is activated by prostacyclin⁷⁶, while others are activated by epinephrine⁷⁷.

Once AC is activated, ATP is converted into cAMP. cAMP activates Protein Kinase A (PKA) that can phosphorylate other proteins, affecting their function. When AC6 is activated, PKA phosphorylates Ca^{2+} ion channels, causing a reuptake of calcium ions, which results in vasodilation⁷⁸.

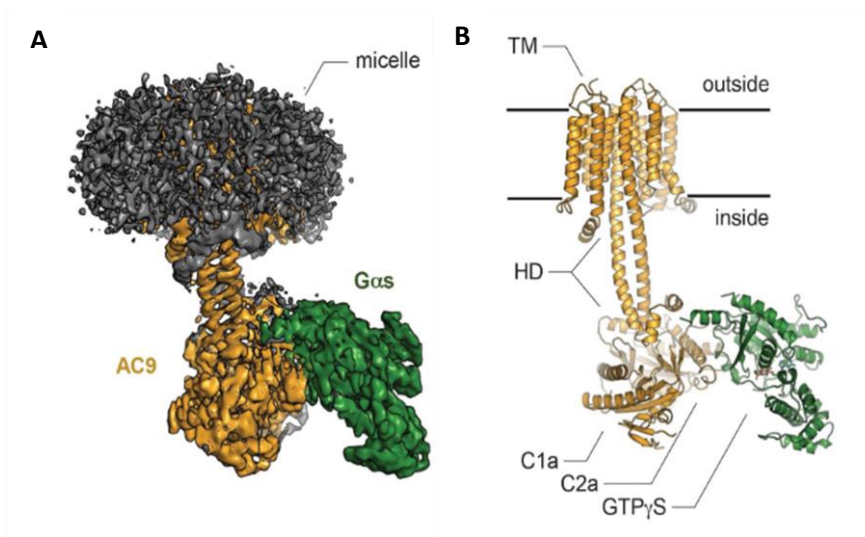


Figure 5. A) Crystal structure of complete bovine AC9 bound to $G\alpha_s$. **B)** Simplified structure showing complete protein. Transmembrane domain (TM), helical domain (HD), connection the cytoplasmic domains (C1a and C2a) to the TM. Image was adapted from doi: 10.1126/science.aav0778.

1.2.2 Adenylyl Cyclase Structure

Transmembrane-bound isoforms of AC consist of six transmembrane helices, followed by catalytic domains C1a and C1b, a second transmembrane domain with six helices, and a second catalytic domain C2a and C2b. Both C and N-terminus are cytosolic⁷⁹. The C1b and C2b domains are less conserved across isoforms, potentially playing an isoform-specific role in the activation

This response is terminated when GTPase hydrolyses GTP to GDP, causing the subunit to reassociate with the $\beta\gamma$ -complex, returning to its original conformation, ready to repeat the cycle when activated⁷⁵.

or inhibition of activity⁷⁴. G α s binds to the C2 domain, while the binding sites for forskolin and ATP sit between the two cytoplasmic domains⁸⁰.

The transmembrane domain has several functions; one study suggests a constant interaction between the two transmembrane clusters, which is associated with trafficking the enzyme to the plasma domain⁸¹. However, they are not required for the conversion of ATP to cAMP.

1.2.3 Adenylyl Cyclase Isoforms

There are ten known AC isoforms in humans, nine membrane-bound isoforms and an additional soluble isoform. These isoforms carry out the same function, converting ATP to cAMP, but their expression levels and response to cAMP vary significantly depending on location. For example, AC2 is observed in the brain, heart and skeletal muscle, while AC7 is expressed throughout the body⁸². These TM isoforms are predicted to have the same overall structure based on their amino acid sequence⁷⁹. Eight of the nine membrane-bound isoforms are consistently sensitive to forskolin, which binds to an allosteric binding site present between the two cytoplasmic domains.

Adenylyl cyclase isoforms are split into four groups based on how they are regulated. Group 1 consists of AC1, 3 and 8; these isoforms have increased activity in the presence of Ca²⁺ and are inhibited by G $\beta\gamma$.

Group 2, AC2, 4 and 7 are stimulated by the presence of G $\beta\gamma$, while AC5 and AC6 (Group 3) are both stimulated by G $\beta\gamma$ and inhibited by G α_i and Ca²⁺. Groups 2 and 3 show significant overlap in their protein sequences.

The final group, group 4, consists of only AC9; it was believed to be forskolin insensitive⁸². However, its characterization may change now that it has been determined to be forskolin sensitive. Regardless of forskolin activity, it does not meet the criteria set to fit into another group.

While all isoforms have functions associated with them, they are yet to be fully explored or understood; knock-out studies carried out on mice have been used to improve our understanding. An example of this is the removal of AC1 and AC8, affecting the formation of

long-term memories⁸³. Knockout of AC3 caused a decrease in pheromone-mediated behaviours in male mice, unable to detect odours⁸⁴.

While there are nine currently known transmembrane isoforms of AC in humans, as time goes on and more studies are carried out, there will probably be more isoforms or another factor that complicates the picture further. An example of this is the alternative splicing of AC8. Three distinct “versions” of AC8 have been found in rat brain cells, the fully intact isoform, another missing a glycosylation site, allowing for different activation methods and a third produced without a calmodulin regulation site, preventing regulation by Ca^{2+} ^{85,86}.

Table 1. List of adenylyl cyclase isoforms grouped by regulatory properties. Included is the main locations in the body the isoform is present, the associated activators and inhibitors for each isoform AC3, 5, 6 and 8 are found in the human heart. *Currently **debated**, evidence seems inconclusive

Adenylyl Cyclase Isoform	Predominant Isoform location ⁸³	Activators			Inhibitors		
		G Protein ⁸²	Protein Kinases ⁸²	Calcium ⁸⁷	G Protein ⁸²	Calcium ⁸⁷	Nitric Oxide ⁸⁸
Group 1							
AC1	Brain, adrenal gland	Gas	PKCα	CaM	Gai, Gβγ	-	-
AC3	Brain, olfactory epithelium, heart	Gas	PKCα	CaM*	Gβγ	-	-
AC8	Brain, lung	Gas	-	CaM	Gβγ	-	-
Group 2							
AC2	Brain, skeletal muscle, lung	Gas, Gβγ	PKCα	-	-	-	-
AC4	Brain	Gas, Gβγ	-	-	-	-	-
AC7	Brain, platelets	Gas, Gβγ	PKCα	-	-	-	-
Group 3							
AC5	Heart, brain	Gas, Gβγ	PKCα	-	Gai, z	Ca ²⁺	NO
AC6	Widespread, neonatal heart	Gas, Gβγ	-	-	Gai, z	Ca ²⁺	NO
Group 4							
AC9	Brain, skeletal muscle	Gas	-	-	-	-	

AC9 Forskolin Sensitivity

Forskolin is described as a non-specific adenylyl cyclase activator, with only AC9 being insensitive to forskolin⁸⁹. The literature has been mixed, stating that AC9 is sensitive to forskolin once Tyr1082 has been converted to a lupine residue⁹⁰ or that AC9 is conditionally activated by forskolin⁹¹. Another study observed an increase in AC9 activity in the presence of forskolin, with a moderate increase in activity when forskolin and G α s are present. This study included an analysis of a crystal structure, and it appears the C2b domain acts as a regulator, which is not observed in other isoforms. After activation, the C2b chain sits within the binding site of ATP and forskolin, preventing the overproduction of cAMP by AC9⁷⁹, this additional regulatory step may account for the inconsistent observations of forskolin sensitivity.

Conservation of Residues

While there is variation in the amino acid sequence within the transmembrane domains, the cytoplasmic domains, containing the allosteric binding site, appear highly conserved across isoforms. This may cause additional challenges, as creating a derivative that can selectively activate will be complex with slight variation between isoform AC5 and 6, sharing 91% identity when comparing only the catalytically active cytoplasmic domains⁹².

1.2.4 Isoform Study Selection

When selecting an isoform, we seek an enzyme that can activate and cause vasodilation, reducing pulmonary vascular resistance without adversely affecting health. Three isoforms are present in the heart and surrounding pulmonary arteries, AC5, AC6 and AC8, with the potential to treat PPHN. AC3 has also been found in vascular smooth muscle cells, playing a significant role in Ca²⁺-mediated signalling⁹³.

When AC3 was depleted from VSMCs, there was a decrease in cAMP accumulation; this is studied by measuring cAMP accumulation after adding forskolin. cAMP production was reduced by approximately 30% compared to a control sample, suggesting that AC3 may contribute to a vasodilatory response⁹³.

AC5 is the isoform found predominately in the adult heart⁹⁴ but does not appear to be involved in the signalling cascade producing a vasodilatory effect⁹⁵. Knock-out studies of AC5 in mice

models resulted in several beneficial effects. Old and young mice showed increased exercise endurance, improved resistance to pressure overload in the heart and a 30% increase in life span⁹⁶. Expression of AC5 and AC6 decrease in cases of heart failure⁹⁷, the decrease in AC5 is considered cardioprotective⁹⁸ but the overexpression of AC6 has been observed to improve heart function in cardiac injury⁹⁹. When specifically examining the relaxation of vascular smooth muscle cells, depletion of AC5 was observed to have no impact on β -AR-mediated cAMP accumulation⁹⁵.

Due to this and its lack of association with vasodilation and the high risk of unintended side effects, AC5 would not be an appropriate target for specific activation to treat PPHN.

AC6 is found throughout the body⁸³ and is present in high concentrations in neonatal heart tissue, specifically the right atrial appendage, left internal artery and coronary arteries. An increase in AC6 abundance is observed under hypoxic conditions⁴³. Increased expression of AC6 provides numerous beneficial effects such as improving cardiac function, reducing heart atrophy and improving survival rates in mice with cardiomyopathy^{100, 101}. When examined in the same study as AC3 and AC5, VSMCs depleted of AC6 only produced 40% of the cAMP compared to a control sample. AC6 was also the principal vasodilator in vascular myocytes⁹⁵. Due to this, AC6 would be an appropriate target.

AC8 is present primarily in the brain, lungs and heart, with much lower expression levels in the heart⁸³. In the brain, its function relates to the central nervous system. Knock-out studies of AC8 in mice found impaired object recognition memory, but it appeared to have little effect on spatial recognition memory¹⁰². Further studies suggest a reduction in anxiety-like and stress-induced anxiety behaviors¹⁰³ and increased growth of the adrenal glands¹⁰⁴. Mice with overexpressed AC8 in the heart show an increase in heart rate and increased heart rate stability¹⁰⁵.

Another study involved overexpression of AC8 in mice (AC8TG) heart showed no detrimental effects on overall heart health and function, despite a significant increase in AC turnover in the heart. This increase is believed to be due to the presence of AC8 as inhibition of AC5/6 by micromolar Ca^{2+} concentrations that do not affect AC8's activity¹⁰⁵, preventing regulation of AC8

by Ca^{2+} . When AC8TG mice were studied, they were found to have reduced life span and increased risk of heart failure, and this was due to early cardiac remodelling and increased expression and activity of PDEs. At 12 months, AC8TG mice showed increased lung and liver masses, indicating cardiac dysfunction¹⁰⁵.

Mice overexpressed with AC5 or AC8 show improved cardiac function, with increased cardiac contractility improving exercise (treadmill) performance¹⁰⁶. Studies of AC8's function seem to tie more to healing processes, migration of cells, and increased proliferation of vascular smooth muscle cells.

Cardiac tissue expressed with AC8 appears to have an enhanced contraction response to Ca^{2+} concentration¹⁰⁷. Although found in the heart, abundance is significantly lower than the other isoforms discussed, indicating that activating AC8 to treat PPHN would be inappropriate. Specificity away from AC8 could be beneficial, but it is difficult to judge the effect of AC8 with the currently available information.

AC3 and AC6 seem to be appropriate targets for specific activation by forskolin. As previously mentioned, AC6 is the primary vasodilator in VSMCs, and its activation would result in a vasodilatory response. In contrast, AC3 activation may contribute to a vasodilation response that would require further investigation to confirm and is the less abundant isoform in pulmonary tissue. AC6 has therefore been chosen as the isoform of interest, and it is the target selected for specific activation by a forskolin derivative.

1.2.5 Inhibition of AC by NO

While nitric oxide activates sGC in pulmonary myocytes to cause vasodilation, it also can inhibit both $\text{G}\alpha\text{s}$ and forskolin-stimulated AC6 activity. As NO inhibits both $\text{G}\alpha\text{s}$ and forskolin activity, the inhibition occurring is unrelated to the GPCR $\text{G}\alpha\text{s}$ pathway. Otherwise, forskolin activity would be unaffected¹⁰⁸.

Nitric oxide is a reactive species that can covalently bond to proteins acting as post-translational modification (PTM). Nitrosylation of cysteine residues is specific, requiring proximity to a NO source and the correct residues surrounding the cysteine residue to support the reaction. Once bound, NO will cause a conformation shift, induce further PTMs or alter protein-protein interactions¹⁰⁹.

Pulmonary arterial (PA) adenylyl cyclase activity and regulation were studied under hypoxic PPHN conditions in a porcine model. PPHN was induced in newborn pigs, and relaxation of pulmonary arterial rings was then studied after forskolin and sodium nitroprusside (SNP, NO donor, cGMP activator) were introduced.

Hypoxia decreases cAMP production, impairing adenylyl cyclase's ability to induce relaxation in pre-contracted pulmonary arteries in PPHN-exposed piglets. Hypoxia reduces both the response to forskolin and SNP-mediated relaxation, and this reduction is unrelated to ATP levels or hydrolysis of cAMP. This response is reduced despite an increased expression of AC6 under hypoxic conditions⁴⁴.

Another study showed that it is possible to reverse this inhibition; when NO-inhibited membranes are washed and incubated without NO, there is no decrease in inhibition. However,

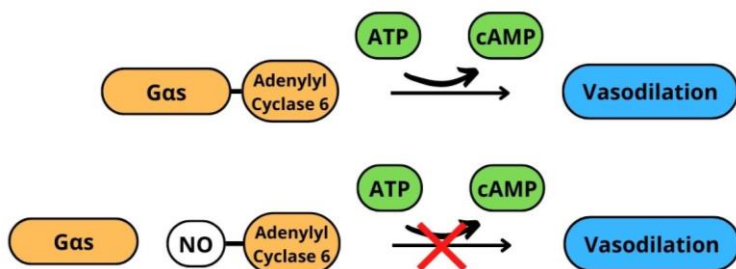


Figure 7. Gas binds to AC6, converting ATP to cAMP, causing a downstream vasodilation response. When nitrosylated AC6 cannot bind to Gas, preventing regulation by Gas.

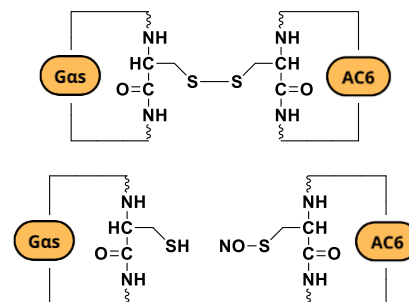


Figure 6. Cysteine residues are susceptible to nitrosylation (PTM), S-NO cannot bind with another cysteine residue preventing formation of disulphide bonds.

inhibition is reduced when the same membranes are washed with a reducing agent such as DTT. This suggests that nitrosylation, nitric oxide binding to cysteine residues, is the cause of inhibition¹¹⁰.

The impairment of AC6 activity was associated with a dose-dependent increase in nitrosylation. This effect was observed in an induced hypoxia porcine arterial model, and AC6 expressed in HEK293 cells exposed to hypoxic conditions for 72 hours. Nitrosylation of cysteine residues directly inhibits AC6 activity⁴⁴.

1.2.6 Adenylyl Cyclase Regulation

Ca²⁺ and Its Effects on Calmodulin

Intracellular Ca²⁺ can control cAMP production within the heart, epithelium and olfactory. This regulation stems from CaM kinase II (Calmodulin (CaM)-dependent protein kinase II). CaM activates CaM Kinase II once a Ca²⁺ ion is bound; CaM Kinase II can then phosphorylate susceptible proteins¹¹¹.

HEK293 cells were used to produce group 3 (AC5/AC6) adenylyl cyclase; in the presence of Ca²⁺, AC was inhibited. It was predicted that the most likely site for phosphorylation was Ser1076 due to an arginine residue in proximity, which supports the phosphorylation. To determine if this residue was the cause of inhibition, Ser1076 was mutated to an Ala residue; in this mutant, ATP turnover to cAMP was unaffected, and Ca²⁺ no longer inhibited the enzyme. The inhibition also did not occur for the wild-type in the presence of CaM Kinase II inhibitors, again supporting that phosphorylation is the mechanism for inhibition¹¹¹.

Calcium ions play an extensive regulatory role in AC. Non-physiological concentrations of Ca²⁺ inhibit all isoforms of AC due to competition in the active site with Mg²⁺⁸⁷. Ca²⁺ inhibits AC5 and AC6 at physiological concentrations; this is used to finely control heart rate and contractions, as Ca²⁺ ion concentration increases when AC6 is active, creating a method of self-regulation. Crystal structures of AC6 suggest a high-affinity Ca²⁺-pyrophosphate complex forming, locking the enzyme in an open (inactive) conformation and, as with other isoforms, direct displacement of Mg²⁺ ions reduces activity¹¹². This would only reduce phosphorylation-mediated inhibition and would not be prevented by adding CaM Kinase II inhibitors.

1.2.7 Adenylyl Cyclase Expression

Adenylyl cyclase is a transmembrane-bound protein, so it is difficult to express as a complete soluble protein. The two main cytoplasmic domains act in concert to catalyse the conversion of ATP to cAMP without the need for the transmembrane or helical domains¹¹³. Due to this AC has been produced in several systems both as a complete protein and as two separate cytoplasmic domains responsible for conversion of ATP to cAMP. In 1983 AC was purified from *E. coli*. However, it was estimated that even in high-expression AC strains, AC made up 0.0035% of the total protein present¹¹⁴. Despite the difficulties associated with purification, this protein was functional, and AC activity could be studied under several different conditions; it was observed that protein stability decreased as purity increased¹¹⁴.

When adenylyl cyclase is expressed as a transmembrane protein, it tends to have low expression levels, solubility and is difficult to study in detergent¹¹⁵.

In 1991 adenylyl cyclase type I was expressed in Sf9 (insect) cells; the two cytoplasmic domains formed a functional protein when combined in solution but had no activity as individual domains¹¹⁶. This was also observed when cytoplasmic domains were expressed in *E.coli*¹¹⁵.

The C1 and C2 domains have also been expressed with a linker between, again forming a functional protein^{114, 115}; these proteins exhibited the expected regulatory properties and were independently and synergistically activated by forskolin and G α s. This work was also done in *E. coli*.

For this project, *E. coli* will produce AC6; the two cytoplasmic domains will be connected by a section of the helical domain. As the Sorensen Group has previous experience producing and expressing proteins in *E. coli* it was decided that that would be the most appropriate system. A plasmid was purchased from GenScript, and the plasmid contains the N-terminus (AA 1-151), C1 (AA 306-673) and C2 (AA 914-1168) domains directly connected. This protein will be used in HPLC assays to determine AC activity.

Human Embryonic Kidney (HEK) Cells will be overexpressed with various AC isoforms for in vivo whole-cell assays. This protein will also be used as an unpurified lysate in HPLC assays to

determine AC activity. This protein will also be used for Homogeneous Time-Resolved Fluorescence (HTRF)-cAMP assays, a commercially available cAMP assay kit from CisBio (62AM4PEB).

1.2.8 Adenylyl Cyclase Activation Assays

Radioactive labelling

Assays on adenylyl cyclase activity have been carried out since the 1960s; these earlier studies focus on using a radio-labelled substrate as a detection method. In this study, crude extracts of rat cortex were used as a source of AC. ATP labelled with H^3 , C^{14} or P^{32} at the alpha phosphate can be used in the assay to produce labelled cAMP. However, as the ATP is labelled, along with any ADP or AMP that was produced, will also produce a signal separation of the nucleotides was needed.

Once the assay is complete labelled product can be isolated by chromatography on Dowex 50- H^+ , followed by precipitation of nucleotides and inorganic phosphates with the addition of $ZnSO_4 \cdot Ba(OH)_2$. This leaves 99.9% labelled cyclic AMP in the supernatant for analysis¹¹⁷. The precipitation step removes ATP, ADP, AMP, and inosine but does not precipitate adenosine or cAMP. While the method was effective, it is time and resource intensive as each assay must be carried out with replicates and individually purified.

The assay consists of Tris-HCl, $MgSO_4$, NaF (phosphodiesterase inhibitor), theophylline (phosphodiesterase inhibitor), labelled ATP and enzyme. The reaction was terminated by adding unlabelled cAMP, followed by boiling and centrifugation. Purification was then done before dilution and quantification with a liquid scintillation spectrometer¹¹⁷.

The most cited and adopted method was published in 1974, noting the difficulties of producing a sensitive assay when 0.05% conversion of ATP to cAMP is the norm¹¹⁸. This method aimed to increase the sensitivity of the experiments by producing a higher purity product, reducing background noise produced by ^{32}P .

Non-Radioactive Labelling

Another method of measuring AC activity is observing the decrease in ATP concentration; commercially available cAMP Glo Assay (Promega) is an example of this.

The concentration of ATP decreases as cAMP concentration increases; ATP concentration is inversely proportional to cAMP concentration. Once the assay is complete, ATP concentration is determined by adding a luciferase-based reagent, converting the remaining ATP into AMP, oxyluciferin. A concentration curve of ATP/cAMP concentrations can then be determined, allowing for a rapid measure of cAMP concentrations and AC activity¹¹⁹.

This method would not be appropriate for an impure lysate assay as we know ATP is converted to products other than cAMP. For example, ADP/AMP, would decrease the ATP concentration without cAMP production.

Another assay involves the reduction of nicotinamide-adenine dinucleotide phosphate (NADP⁺) by glycogen phosphorylase and glucose-6-phosphate dehydrogenase. To begin this process several cleaning steps are carried out to remove other nucleotides present in the sample, leaving only cAMP present. Once purified cAMP is hydrolysed to AMP, which will stimulate glycogen phosphorylase A, causing a cascade of reactions resulting in the reduction of NADP⁺ proportional to the amount of AMP present. The assay was then compared with the radiolabelled assay and results were seen to be consistent across both methods¹²⁰.

High Throughput Methods

High throughput and non-labelling methods will be used for this project to quantify cAMP production. The high-throughput kit from CisBio (HTRF cAMP dynamic 2 kit) completes these assays in 96 well plates with or without a forskolin derivative. This will allow for rapid initial screening of derivatives against multiple AC isoforms. AC3, AC5, AC6, AC7 and AC9 will have their activities determined. Once an assay is complete, a stopping solution is added containing labelled cAMP; the unlabelled cAMP (produced by AC) will compete with the labelled cAMP to bind with a cAMP Cryptate-antibody. The unlabelled cAMP will produce no fluorescence signal, reducing the overall signal; this can then be quantified^{121, 122}.

Non-Labeling Methods

The second method used to measure cAMP production will use an HPLC for detection. Once an assay is complete, the samples will be denatured with acid or heat and centrifuged, and they will then be analysed by HPLC. cAMP will be separated from other nucleotides on the column then the UV absorbance will be measured and used to determine concentration against a calibration curve of cAMP.

This method reduces the need for purification as it is completed automatically by the HPLC and measures the cAMP concentration rather than requiring further reactions or purification steps. It also does not use an indirect measure of cAMP concentration, such as measuring pyrophosphate production.

1.3 Forskolin - Adenylyl Cyclase Activator

Forskolin is a small molecule found in the *Plectranthus barbatus* plant (also referred to as - *coleus forskolii*) native to India¹²³. It has been and still is used as a medical treatment in Hindu and Ayurvedic medicine; in 1974, it was examined for activity by the Indian Central Drug Research Institute (CDRI)¹²⁴. The plant extract was found to have properties reducing blood pressure and antispasmodic effects; the activity was determined to be almost entirely due to the presence of forskolin¹²⁵.

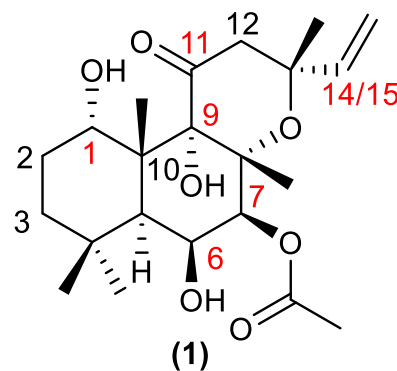


Figure 8. Adenylyl cyclase activator forskolin, labelled with key positions in red.

Forskolin can independently activate all nine transmembrane-bound isoforms of adenylyl cyclase by binding to an allosteric site; this effect is synergistic with G α s activation of AC. Forskolin is commonly used to support the study of adenylyl cyclase due to the low basal activity and the complex regulatory nature of AC. The interface between the two cytoplasmic domains creates a binding pocket for forskolin⁸⁰.

As forskolin is a non-specific activator of AC, modifications will need to be made to the structure to selectively activate AC6. AC6 activation will cause vasodilation in the heart and lung tissue,

allowing the transition from pre-birth to post-birth breathing without the side effects of activating other isoforms of AC.

Forskolin has recently undergone clinical trials as a treatment for glaucoma; it was found to be a safe and effective alternative to beta blockers, which can have adverse effects on heart and lung function¹²⁶.

1.3.1 Review of Forskolin Derivatives in the Literature

Forskolin derivatives have been synthesized to activate adenylyl cyclase since the 1980s; here, I will describe some of the work done, the results and what conclusions can be drawn about AC6.

Early studies of adenylyl cyclase do not distinguish between isoforms as they had yet to be observed/defined. Instead, adenylyl cyclase from the body part of interest was extracted and used as a crude preparation; for example, the first study used AC from rat cerebral cortex. It can be challenging to draw relevant conclusions from these studies, as previous studies have not examined AC6. In these cases, the source of the adenylyl cyclase will be noted along with the potential isoforms observed.

1.3.2 Structure-activity relationships for activation of adenylate cyclase by the diterpene forskolin and its derivatives (1982)

A study has been carried out on forskolin to understand its structural relationship with the AC binding site. As this work was completed in 1982, adenylyl cyclase isoforms are not discussed; crude membranes from rat cerebral cortex were used as a source of AC¹²⁷. The main isoforms

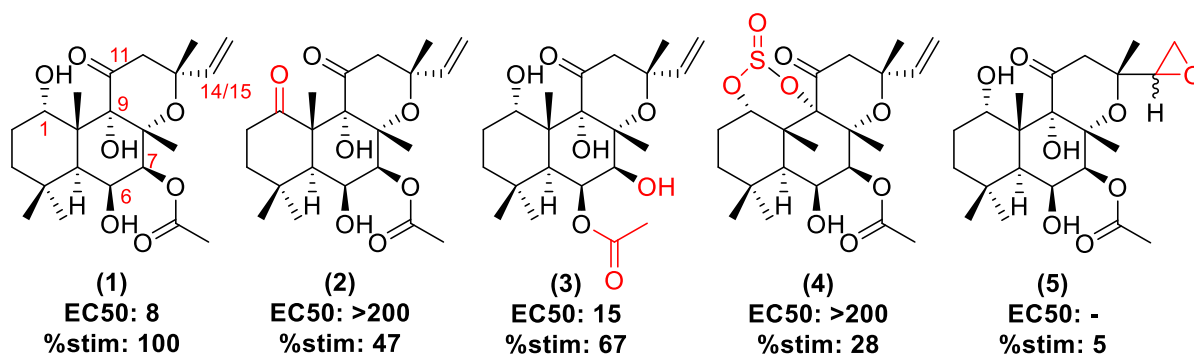


Figure 9. Forskolin with key positions labelled. EC50 (μM) and stimulatory response (% of forskolin) for several example compounds. (1) Forskolin (2) 1-keto (3) 7-deacetyl-6-acyl (4) 1,9-sulphate (5) 14/15 epoxy, with undefined stereochemistry.

expected in mammalian brain tissue would be AC1/AC3 and AC2⁸², members of group 1 and group 2, respectively, while AC6 is part of group 3. EC50 (0.01 to 200 μ M) values and a stimulatory response (% compared to forskolin activity at 5 μ g) were determined to compare compound activity. This study aimed to determine which structural moieties produce forskolin activity. 20 compounds were synthesized, with modifications to the 1, 6, 7, 9, 11 and 14 positions.

Eliminating the alcohol at the 1 position by conversion to a methyl group abolished activity, while conversion to a ketone group reduced activity by 50%. Introducing an acyl group at this position reduced activity to 20% of forskolin. This suggests that the 1-OH is essential for activity.

Migration of the C7 acyl group to the C6 position reduced activity to 65% of forskolin. The introduction of various groups to replace the acyl group at C7 only reduced activity, including forming a carbonate ring between the C6 and C7 positions.

Removal of the alcohol at the C9 position to form 9-deoxy forskolin reduced activity by 50%, while 1,9-dideoxy forskolin had zero activity. Reduction of the ketone at C11 to an alcohol group either eliminates or significantly reduces activity, depending on the stereoisomer formed. Reduction of the double bond at the C-14/15 position causes a moderate reduction in potency, while forming an epoxide at the same position eliminates activity¹²⁷.

Due to the presence of multiple isoforms in brain tissue, this study provides little information on isoform selectivity, it did, however, highlight the importance of the C1 and C9 OH groups for AC activity. Modifying either of these positions to isosteres or other small hydrogen bond donating moieties was not examined, so they could be potential candidates for modified but retained activity. Alterations to the C6/C7 positions generally caused a decrease but did not eliminate activity, suggesting these sites are potential targets for modification. The lack of activity in the epoxide has exciting potential as a modification to improve selectivity with a relatively minor change abolishing activity. An isoform with a differently shaped binding site may be able to accommodate the modification, and other modifications of the C14/15 positions would need to be explored further.

1.3.3 The Antihypertensive and Positive Inotropic Diterpene Forskolin: Effects of Structural Modifications on Its Activity (1983)

In this study, 31 compounds were screened for blood pressure-lowering activities in anaesthetized rats. Arterial blood pressure was measured (Δ BP, mmHg) before treatment and two hours after the final dose was administered once a day for five days (25 mg/Kg). These compounds had modifications at the same positions as the previous study, 1, 6, 7, 9, 11 and 14.

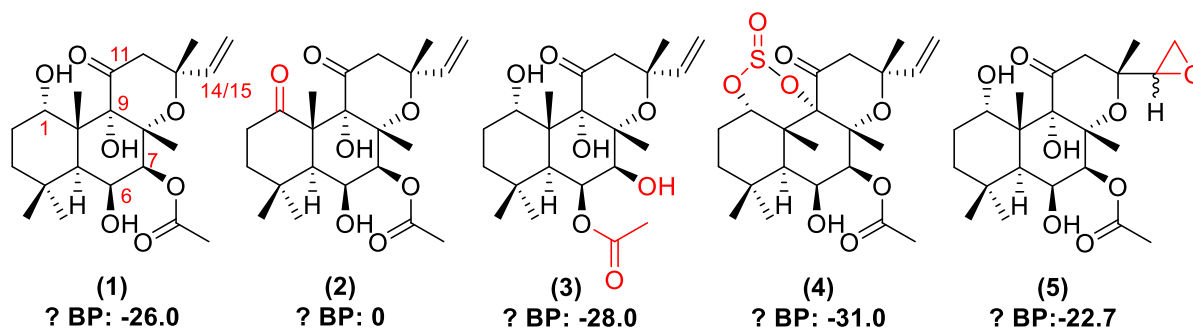


Figure 10. Forskolin with key positions labelled. Change in blood pressure used as a measure of activity (Δ BP mmHg). **(1)** Forskolin **(2)** 1-keto **(3)** 7-deacetyl-6acetyl **(4)** 1,9-sulphonate **(5)** 14/15 epoxy with undefined stereochemistry. Removal of the 1-OH bond by introduction of a ketone reduced activity to 0, while the sulphonate **(4)** also removes this H-bond activity increases over that of forskolin.

Forskolin caused a 26.0 mmHg decrease in blood pressure. Similar blood pressure decreases were observed with several compounds: 6-acetyl, 7-deacetyl, 1,9 sulfonate, 6,7-carbonate and the 1-methyl-6-acetyl-7deacetyl. This data conflicts significantly with the data examining AC from rat cerebral cortex; this may give direction for the design of isoform-selective derivatives. Introducing an epoxide at the C14/C15 position eliminates activity in the cerebral cortex adenylyl cyclase¹²⁷ and intraocular eye pressure¹²⁸. The epoxide had a minor effect on forskolin's antihypertensive properties, suggesting that it would still activate group 3 AC (AC5/6) but not group 1 and 2 (AC1-4,7,8).

Compounds such as the 1-methyl, 1-keto **(2)** and 1,9-dideoxy lost activity, supporting the previous study in that the C1 and C9 alcohol groups are essential for binding with AC.

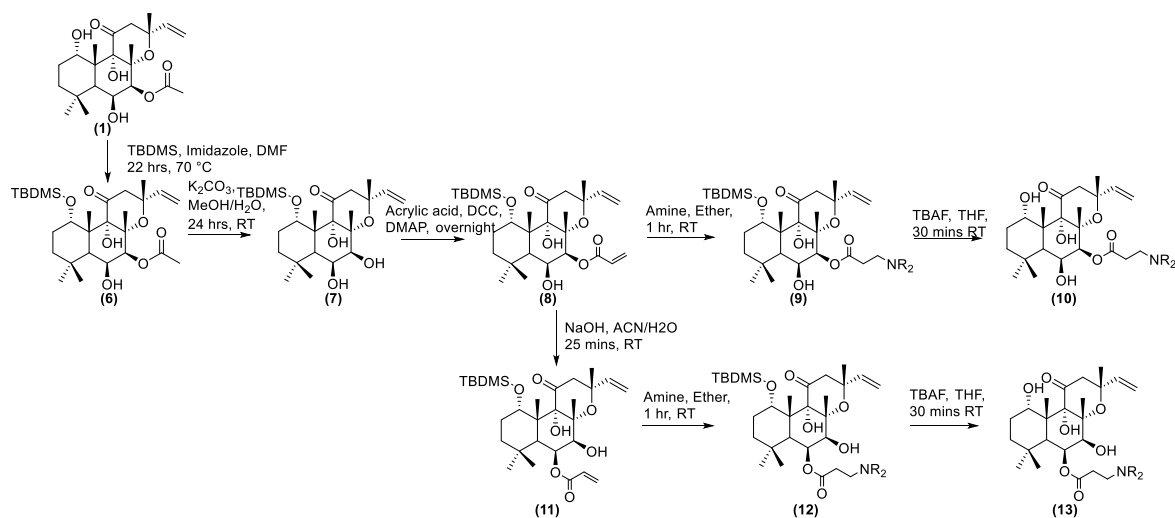
Derivatives were also tested for positive inotropic activity; the derivatives performed poorly with little activity at 100x forskolin concentration in a guinea pig atrium method. There was little

discussion or detail on this result other than forskolin was found to have an EC₅₀ of 2.5×10^{-8} M, while none of the derivatives showed significant activity at 0.1×10^{-6} g/mL.

This study provides some direction for the initial derivative library, with compounds such as the 6-acetyl, 6-acetyl-7-deacetyl, 14/15 epoxide and several carbonates. These compounds can then be studied with activation assays to determine if they retain their ability to activate AC6; this can then be compared with the computational docking studies to validate the model.

1.3.4 Cardiovascular Effects of New Water-Soluble Derivatives of Forskolin (1988)

Due to forskolin's poor water solubility, this study examined synthesizing water-soluble derivatives that retained vasodilatory and positive inotropic properties. This study focused on the C6 and C7 positions and producing water-soluble derivatives by conversion to HCl salts. The general synthetic route to generate these compounds is shown below.



Scheme 1. Synthetic route for C6/C7 amino acyl derivatives via the acrylic acid path. Starts with protection of the C1 alcohol, followed by deacylation of the 7-position (7). Once the acyl group is introduced for the 6-acyl derivatives the migratory step is carried out, shifting the acyl group from the C-7 to C-6 position (11). A Michael addition reaction is then carried out with a variety of amines (9/12). The protecting group is then removed to give final compounds (10) and (13).

Mammalian hearts were used in this study with no isoforms present defined. As mammalian cardiac tissue was being examined, it would be expected that the isoforms present would be predominantly AC5⁹⁷ with a lower presence of AC6⁸². The results from this study are more relevant than the previously discussed studies.

Synthesis was carried out by protection of the C1 alcohol and removal of the C7 acyl group (**7**). This protected deacyl derivative was then treated with either chloroacetyl chloride or acrylic acid (**8**); both groups allow the addition of an amine forming various aminoacyl derivatives at the C6 and C7 positions. In this study, the 7-acyl group was not reintroduced for the C6 aminoacyl derivatives. These compounds were then converted to the HCl salt by dissolving the compound in methanol, which was then added to dry diethyl ether saturated with HCl gas at 0 °C.

Once synthesised, positive inotropic activity was determined using guinea pig atria, the tissue was suspended in solution, and the tissue was exposed to a 95% oxygen 5% carbon dioxide mix. EC₅₀ of forskolin was recorded to be 0.022 µg/mL.

The hypotensive activity was studied in anaesthetized cats; arterial pressure was measured before and after administering a forskolin derivative. The effect on blood pressure was determined by measuring rats' blood pressure before and after each compound's application.

The final model used was the anaesthetized dog model; femoral artery blood pressure was measured before and after the application of compounds, and this was carried out on a select group of more successful compounds.

A total of 73 compounds were synthesized; almost every compound contained one or more amine group and a ring as part of the added chain at the C6 or the C7 position. In the study of positive inotropic activity, there was a total of 21 compounds that showed potent positive inotropic activity (defined as 0.16-3.0 µg/mL). Compounds of interest in this study would be those modified but still retain activity, particularly those that performed poorly in other studies but well in AC extracted from heart tissue.

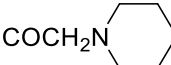
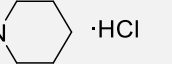
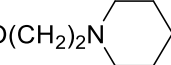
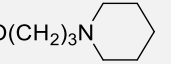
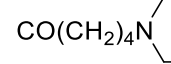
Compound	R ⁶	R ⁷	EC ₅₀ Guinea Pig Atrium (µg/mL)	ED ₂₀ Cat (mg/kg)
Forskolin 1	H	COCH ₃	0.02	0.038
14	COCH ₂ N  ·HCl·H ₂ O	H	1.8	3.0
15	H	COCH ₂ N  ·HCl	>30	10
16	CO(CH ₂) ₂ N  ·HCl·1.5H ₂ O	H	0.5	1
17	CO(CH ₂) ₃ N  ·1.5HCl·1.5H ₂ O	H	NA	3
18	CO(CH ₂) ₄ N  ·HCl	H	NA	NA

Table 2. NA=Not Active. Table of EC₅₀ and ED₂₀ data from Khandelwel et al, showing improved activities with the aminoacyl chain in the C6 vs the C7 position (**14 vs 15**). Also showing the increase in chain length from one to two methyl groups improves activities (**14 vs 16**), while increasing to three (**17**) or four (**18**) methyl groups significantly worsens activities. While most sets of derivatives followed these rules there were exceptions.

While several compounds were synthesized that retained activity, there was not enough to draw structural-activity solid relationships. Generally, compounds containing straight-chain aminoalkyl groups performed poorly. Conversely, cyclic amino groups performed better, with improved activity at the 6 position over the 7. Introducing branching chains generally produced poorly performing compounds, despite some compounds (**(19,20) Figure 11.**) containing large bulky substituents.

When comparing derivatives containing the same moiety but different chain lengths (**Table 2.**) two carbons appear to be the ideal amount; this is not consistent across all compounds but demonstrated well when comparing (**14-18**). The 6-piperidinoacyl (**16**) produced by the acrylic acid synthetic route produced a significantly more active compound than when synthesized via the acetyl chloride method (**14**). It was noted that a further increase in chain length did begin to affect activity negatively, this is observed with compound (**16**) when compared to (**17**) and (**18**).

In these samples, there did not appear to be a set rule that the aminoacyl chain is superior at the 6 or 7 position. While the 6 position was generally better, that was not always the case, and

no concrete pattern appears. However, it highlights the importance of various chain lengths; again, no ideal chain length is set, and the ideal chain length is also position dependent.

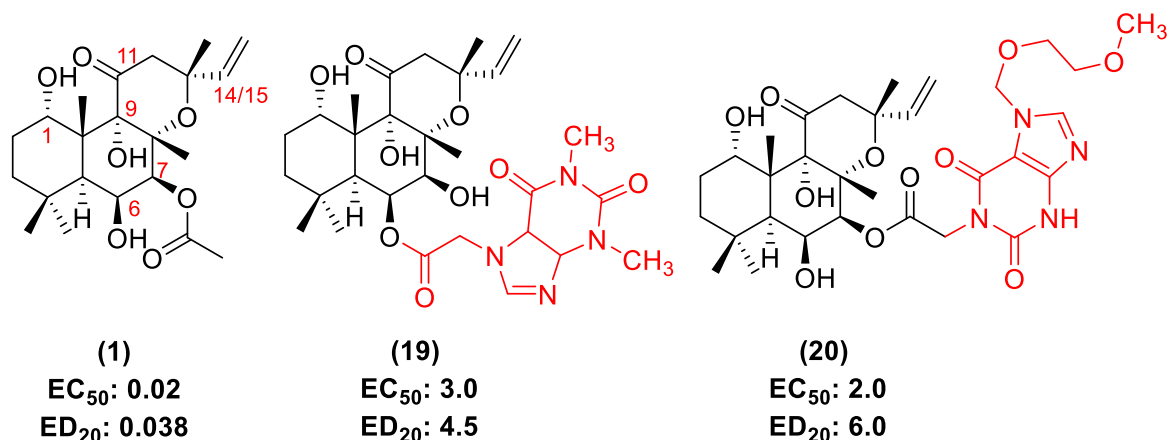


Figure 11. Example forskolin derivatives with large steric bulk at the C6/C7 position that retain strong adenylyl cyclase activity. EC₅₀ refers to EC₅₀ Guinea Pig Atrium (μg/mL) and ED₂₀ refers to ED₂₀ Cat (mg/kg).

1.3.5 Adenylate Cyclase Stimulation and Intraocular Pressure Reduction by Forskolin Analogs (1989)

Forskolin analogues were assayed for AC activation in vitro as a crude extract from rat corpora striata and effects on intraocular eye pressure in Albino New Zealand rabbits were measured by direct application to the eye. There is no discussion of isoforms or types of adenylyl cyclase. This study found a correlation between in vitro AC activation and reduction in pressure in vivo, except in one case where the derivative had poor in vitro activity but higher than forskolin in vivo activity. This was predicted because the molecule acted as a prodrug, providing poor activity in the assay but was metabolised to forskolin (**1**) by esterases within the eye.

Similar activity levels are seen in the 7-deacyl and 6-acyl-7deacyl derivatives, supporting a space in the AC binding pocket in this area and some flexibility in the positioning of these groups. Modifications of the C1 position significantly reduced the effect on IOP except for the previously mentioned compound, which is expected to act as a prodrug. The presence of the epoxide at C14/15 abolishes activity. However, this compound also had a carbonate link between C1 and C9. These two modifications were not tested independently, so it is impossible to determine if that is due to the epoxide or removal of H-bonding to C1/C9 and the binding site. Modifications

introduced caused a significant reduction in activity, as expected from other studies discussed. This supports that the focus of derivatization should be on the C6/C7 positions, with possible minor modifications to other areas to improve selectivity.

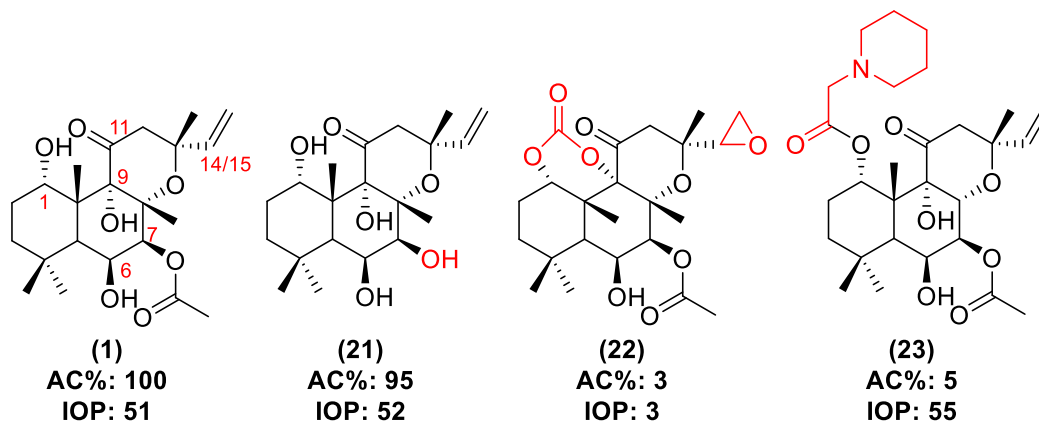


Figure 12. Forskolin with key positions labelled. Example forskolin derivatives of interest, **(21)** retains activity despite removal of the acyl group supporting that it does not play a major role in binding, although this is isoform dependent. **(22)**'s poor activity is unsurprisingly, this compound lost activity. A derivative containing only the epoxide was not tested, this is unfortunate as the epoxide is a compound of interest. Compound **(23)** has poor activity against the AC assay, however it had a significant effect on IOP, this is suspected to be because it acts as a prodrug. AC% is the % activity of AC when compared to forskolin. IOP refers to the reduction in eye pressure based on a reduction in outflow pressure.

1.3.6 Type selective regulation of adenylyl cyclase (2001)

The first successful isoform-specific forskolin derivatives were published in this article in 2001; it is stated that over 200 new compounds were synthesized based on virtual screening of ligands. The structures of these compounds are not presented, but six isoform-specific activators are shown, (**Figure 13**). Activation values are approximations. This is the first of the studies discussed to isolate and purify specific isoforms (AC2, 3, 5) rather than using unpurified cell extracts. AC5 and AC6 are most similar in terms of amino acid sequence and are group 3 isoforms, so the impact of structural changes to forskolin are expected to be similar in both isoforms⁸³.

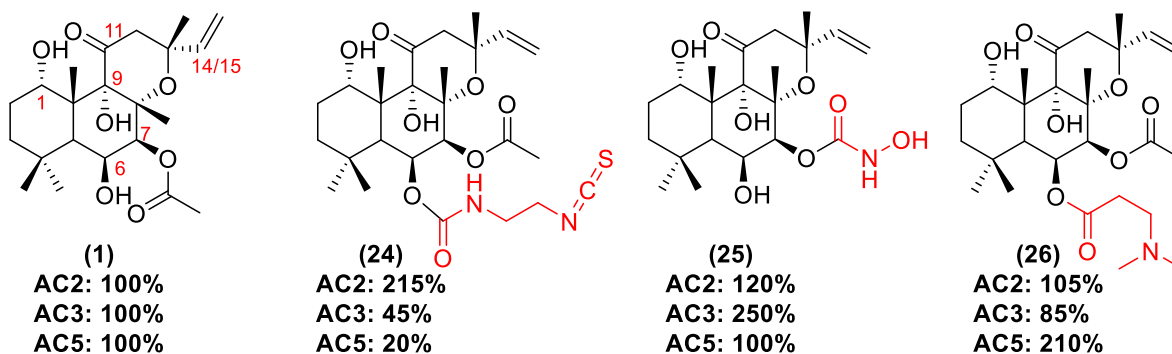


Figure 13. Forskolin labelled at key positions. **(24, 25 and 26)** are specific activators of AC2, AC3 and AC5 respectively. AC activity is stated as a percentage of forskolin activity. Compound **(26)** is NKH477. Values are approximate.

From the collection of compounds studied it was concluded that modifications to the C1, C9, C11 and C14/15 positions should be avoided as they generally result in loss of activity. The exception is reduction to an alcohol group at the C11 position, which would present some isoform selective activity, with 44%, 19% and 55% of forskolin activity in AC2, 3 and 5, respectively.

To achieve specific activation of AC2, an isothiocyanate group was introduced at the C6 position. This was suspected to increase selectivity due to highly nucleophilic amino acid groups present in AC2, in this case, a lysine residue not present in other isoforms.

For AC3, it was found that the introduction of polar groups at the C7 position increased selectivity. Dehydroxylation at the C6 position to give a double bond between the C5 and C6 carbons also increased selectivity for AC3.

Alteration at the C6 or C7 positions to include a positively charged group increased ACV activity without decreasing activity in other isoforms. NKH477 (**Figure 13, (26)**) has been used as heart medication due to its ability to stimulate cardiac adenylyl cyclase activity in patients with heart failure. Reduction of the C14/C15 double bond resulted in higher selectivity for AC5, which was combined with the positively charged groups at C6 to increase selectivity for AC5.

This study can direct the library of compounds to be made. The focus will be on the C6/C7 residues for increasing activity for AC5/6. This will be combined with modifications such as reduction of the double bond or conversion to an epoxide to improve selectivity of other

isoforms. Dehydroxylation of C6 will be avoided as this increases specificity to AC3 while decreasing activity in AC5.

1.3.7 Forskolin Carbamates: Binding and Activation Studies with Type I Adenylyl Cyclase (1996)

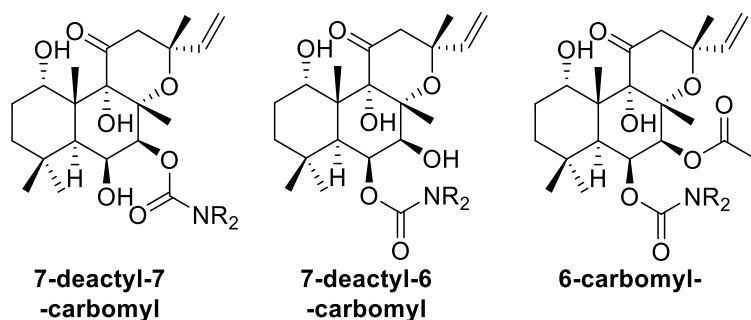


Figure 14. Forskolin derivatives synthesized with carbamate functional groups.

Several forskolin carbamate analogues were tested for binding affinity and activation of isoform 1 adenylyl cyclase extracted from Sf9 insect cells. For each molecule, the binding IC₅₀ (nM), activation EC₅₀ (μM) and % maximal (of forskolin)

activation. 6-carbamoyl-7-deacyl, 6-carbamoyl, and 7-deactyl-7-carbamoyl derivatives were synthesized, general structures are shown in **Figure 14**.

In the 7-deactyl-7-carbamoyl series, there was a trend of decreasing binding affinity as bulk of chains and hydrophobicity increased. Phenyl derivatives exhibited weak affinities compared to forskolin but significantly higher than those containing a cyclohexane ring. This is predicted to be due to the planar nature of phenyl rings alongside potentially π - π stacking interactions. This correlates with previous studies that found alkyl chains performed worse than cyclic counterparts with or without branching. The phenyl derivatives activity improved when a 4-hydroxy or 4-amino group was introduced to the ring.

In both binding and activation, 6-carbamoyl-7-deactyl derivatives performed worse consistently than the 7-carbamoyl equivalents. Except for the CH₂CH₂N(CH₃)₂ chain, which had similar binding but much higher maximal activation, this is the side chain in NKH477.

The 6-carbamoyl derivatives exhibited poor binding if they contained hydrophobic residues, whereas derivatives containing a primary amine or a ring with amine bound well. Polar amines performed better than the equivalent derivative from the 6-carbamoyl-7-deacyl compounds, with polar non-hydrophobic compounds performing best.

Overall, they concluded that bulk was poorly tolerated at the C7 position but acceptable at C6, with or without reacylation of the 7 position. The introduction of small, linear substituents at the 7 position was better tolerated than at the C6 position.

1.4 Conclusions

Although there have been numerous studies on forskolin derivatives effect on AC activation, it is challenging to draw rules or conclusions from the results. Activity varies significantly between isoforms, and one group at the 6 position can have a very different result to the same functional group at the 7 position. To understand which compounds will activate AC well, synthesising compounds and testing their activity in AC6 will be necessary. Once that is complete, it may be possible to conclude compound selectivity.

A few rules were consistently observed or observed in the studies looking at more significant numbers of compounds. It appears that steric bulk can be well tolerated if planar, as shown in (**Figure 11.**) and in the carbamate study, where phenyl groups significantly outperformed cyclohexane derivatives. Even small branching groups can impact activation, and generally should be avoided. When comparing 6-carbamoyl and 6-carbamoyl-7-acetyl compounds, there appears to be little consistency in which performs better. Activity depends on the structure of the chain at the 6 position, it will be necessary to synthesize both versions to compare activity. Although not studied, the epoxide may have significantly higher activity in group 3 ACs compared to other groups due to its retained activity in heart isoforms compared to brain isoforms; this could be a route to isoform specificity.

As none of these studies were carried out on Isoform 6 conclusions drawn are speculative and will need to be tested computationally and experimentally.

The complications of PPHN have been described and discussed, with the possible treatment options and the pitfalls associated with them. The appropriate target for forskolin as a specific adenylyl cyclase activator to treat PPHN was also discussed. A short literature review was then carried out to examine previously made forskolin derivatives and how modifications in structure

affected activity. Previous reviews have supported the need for new PPHN treatments due to the short and long-term risks associated with PPHN⁶⁶.

1.5 Project Overview

To complete this project, first, it will be necessary to produce a functional AC6 protein that is relatively pure and to design an activation assay to quantify cAMP production. A computational model will be built to dock forskolin derivatives and test how well they bind to AC. Based on this and the literature, a library of compounds will be designed and synthesized. The AC activation assay can then measure how well they activate AC. None of the compounds produced may retain activity when this step is complete, as even minor changes can abolish activity. If this is the case, previous steps must be repeated until appropriate forskolin analogues can be found. Other isoforms of adenylyl cyclase will then be produced in the activation assays. By testing the forskolin derivatives with multiple isoforms, it will be possible to determine whether each derivative can selectively activate AC6.

Once a lead molecule has been selected, multiple rounds of derivative synthesis, assays and computational modelling will likely need to be completed to refine features that provide selectivity and make progress towards a selective AC6 activator. Derivatives that selectively activate other isoforms would also be of interest as there may be several treatment possibilities through selective AC activation.

Chapter 2 – Chapter Methods

2.3 Materials and Methods Relating to Chapter 3

Materials and Methods for Chapter 3 Can be found in the supporting information Chapter 9.

2.4 Materials and Methods Relating to Chapter 4

Methods for creating the homology model and ligand docking of forskolin derivatives are described in Chapter 4.1 – Page 54.

2.5 Materials and Methods Relating to Chapter 5

Reagents and Equipment Used:

Adenosine-5'-diphosphate monopotassium dihydrate, adenosine, creatine phosphokinase from rabbit muscle, formic acid (ACS grade), methanol (HPLC grade), phosphoric acid (ACS grade), and potassium phosphate monobasic (KH_2PO_4) were purchased from Sigma Aldrich. Adenosine triphosphate disodium salt hydrate and cyclic adenosine monophosphate were purchased from Tokyo Chemical Industry Co (TCI). Adenosine-5'-monophosphate was purchased from Acros Organics. 3-isobutyl-1-methylxanthine (IBMX) were purchased from EMD Millipore. Potassium phosphate dibasic (K_2HPO_4), Tris base and MgCl_2 was purchased from Fisher Scientific. Miliq water was prepared using a Thermo Scientific Barnstead Easypure II (resistivity $18.2+ \text{M}\Omega \times \text{cm}$). HPLC experiments were carried out using a Waters Alliance 2695 Separations Module with Waters 2996 Photodiode Array Detector. An xbridge phenyl column ($4.6 \times 150 \text{ mm}$, $5 \mu\text{m}$) was used for separation. A Spectrafuge 24D was used to centrifuge assay samples at $14,000 \text{ g}$ for four minutes. Waters total recovery clear glass HPLC vials were used for assay samples.

2.5.1 HPLC Material and Methods

HPLC Separation Method:

Column: Xbridge Phenyl $5 \mu\text{M}$, $3.0 \text{ mM} \times 150 \text{ mM}$.

Buffers: Buffer A: $80 \text{ mM KH}_2\text{PO}_4$ (pH 6.0 w/ 20% KOH). Buffer B: MeOH (100%). Buffer C (Wash only): Miliq water (0.1% FA).

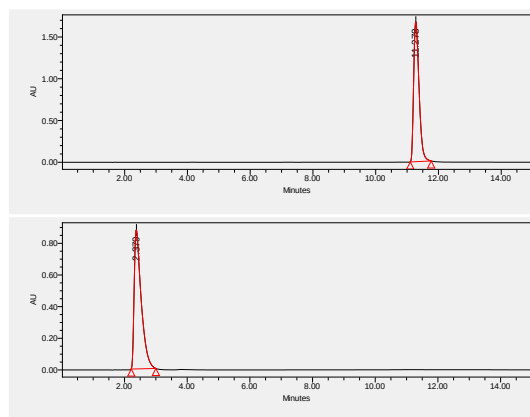


Figure 15. HPLC Chromatograms of cAMP and ATP Standard respectively. cAMP has a retention of 11.3 minutes and ATP has a retention time of 2.8 minutes. Each standard was run at 0.5 mM concentration.

Separation Method: 0 min, A (100%). 2 min, A (100%). 8 min, A (85%). 13 min, A (85%). 15 min, A (100%). 20 min, (A) 100%. Flow rate: 0.5 mL/minute.

Retention times: ATP - 2.8 minutes cAMP – 11.3 minutes.

The column is equilibrated with potassium phosphate buffer for 15 minutes before a run is started. Once the HPLC method set is complete a wash method is used to remove any remaining phosphate buffer present in the column before returning to 100% methanol for storage conditions. 0 min, A (100%). 10 min, A (100%). 20 min, B (5%), C (95%). 30 min, B (5%), C (95%). 35 min, B (50%) C (50%). 30 min, B (50%) C (50%). 45 min, B (100%). 60 min, B (100%).

2.5.2 Bacterial AC6 Assay Methods

Assay Reagent Standard Concentrations (before stopping solution addition): MgCl₂ (100 mM, 20 µL), ATP (3.5 mM, 20 µL), Fsk (1mM, 2 µL), AC6 protein (20 µL) tris buffer (50 mM pH 7.4, to 200 µL total volume).

All stock solutions were made using tris buffer (50 mM, pH 7.4), except forskolin, forskolin derivatives and IBMX which were dissolved in DMSO.

Final Concentrations (before stopping solution addition): MgCl₂ 10 mM, ATP 0.35 mM, Fsk 10 µM, IBMX 1 mM.

Protocol: On ice add MgCl₂, ATP, Tris buffer and forskolin to a 1.5 mL Eppendorf tube. Add AC protein to Eppendorf tube, invert 5 time to mix, place into water bath set to 37 °C for 15 minutes. After 15 minutes removal samples from water bath, add 50 µL 5% formic acid ([final] 1%) to each assay, invert five times to mix, centrifuge at 14,000g for 4 minutes. Pipette top 200 µL of solution into a Total Recovery HPLC vial, analyse by HPLC. A calibration curve can then be used with the area of curve of cAMP peaks to quantify cAMP production. The calibration curve can be found in **Figure 16**.

2.5.3 Bacterially Produced AC6 Expression and Purification Methods

DNA sequences are available in the supporting information.

AC6 was expressed in *E. coli* with a pET 30a plasmid, the cells used were arabinose induced, grown in LB media with kanamycin. The culture was grown at 37 °C until an OD of 0.6-0.8 was reached, at which point the culture was induced with ITPG (1 mM), and expressed at 16 degrees overnight. The cells were pelleted at 17,000 xg for 15 minutes. The cells were then resuspended in tris buffer (25 mM, 300 mM NaCl, 5M GuHCl, pH 8.0) and sonicated for 15 minutes to lyse the cells. Cell debris was then removed by centrifuging at 20,000 xg for 45 minutes. To refold the protein was dialyzed into assay buffer, any precipitation was then removed by centrifugation at 20,000 xg for 45 minutes.

Initial assays were completed with unpurified protein, this was to determine if the protein had folded properly and was functional.

Attempts at using a flag tag resin for purification were unsuccessful, with the protein not binding to the resin and appearing in the flow through despite west blot confirmation of the presence of the flag-tag. More recent protein modelling using alphafold (not shown) suggested that the flag tag was buried within the protein, which would explain the lack of affinity.

Ion exchange chromatography (IEX) was used for protein purification, the protein was dialyzed into IEX starting buffer (Tris 20 mM, pH 8.0), the elution buffer (Tris 20 mM, 1M NaCl, pH 8.0). Protein was purified using an anionic exchange (Capto HiRes Q 5/50) and cationic exchange (Capto HiRes S 5/50). Fractions from both columns were grouped based on an SDS gel, these fractions were then tested for activity, once fully functioning protein is produced adenylyl cyclase protein will be purified further and an appropriate concentration of AC for HPLC assays will be determined.

2.5.4 Bacterially Produced G α s Expression and Purification Methods

The DNA sequence for human G α s was codon optimized for expression in *E. coli* with a His-tag, and cloned into a pET30a vector, purchased from GenScript (Piscataway, NJ, USA). The plasmid was transformed into BL21, grown in TB medium with kanamycin, the culture was then grown at 37 °C until OD₆₀₀ reached 1.2. The culture was then induced with ITPG (1 mM) at 15 °C for 16 hours, the cells were then pelleted by centrifugation (17,000 xg) for 15 minutes. The cells were resuspended in phosphate buffer (Na₂HPO₄ 50 mM, 300 mM NaCl, 5 mM imidazole, pH 8.0) and sonicated for fifteen minutes. The lysate was then vortexed and centrifuged to remove debris. G α s was purified by Cobalt NTA resin. Fractions used in assays were fraction 21 – **G α s #1** and 24 – **G α s #2**, shown in SDS gel in Supporting Figure 1. **G α s #3** was produced by Harjasmin using the same method. G α s was used as is, in a partially purified state. Initial assays were carried out to determine if G α s affected AC6 activity, which it did. Once production of AC6 is finalized and confirmed to be functional expression, purification and determining an appropriate concentration of G α s will be completed.

2.5.5 Quantification of cAMP:

For quantification of cAMP first a calibration curve needs to be established. At least 6 different concentrations should be used across the range of concentrations produced by the assay. As ATP consumption should remain below 10%, 0.035 mM can be used for the highest concentration then remaining samples should cover the remaining range of concentrations.

Once complete this data can be plotted and the area under the curve of cAMP in an assay sample can be compared to the calibration curve and a concentration determined.

When replicating ATP, protein concentrations should be adjusted so that less than 10% of ATP is consumed, as the initial reaction rate is being measured. If cAMP production is too low assay duration can be increased.

A calibration curve was created to quantify cAMP production. Samples were made for 6 different concentrations. As 0.35 mM of ATP is used in these assays and ideally no more than 10% of ATP will be consumed the curve was designed for a 0.035-0.0035 mM cAMP range. These samples were then run on the HPLC, with repeat injections. An average of the injections was taken to create the calibration curve, shown below (**Figure 16**). Once all conditions are finalized and an appropriate ATP concentration selected, the concentration curve will be reproduced at the correct concentration range.

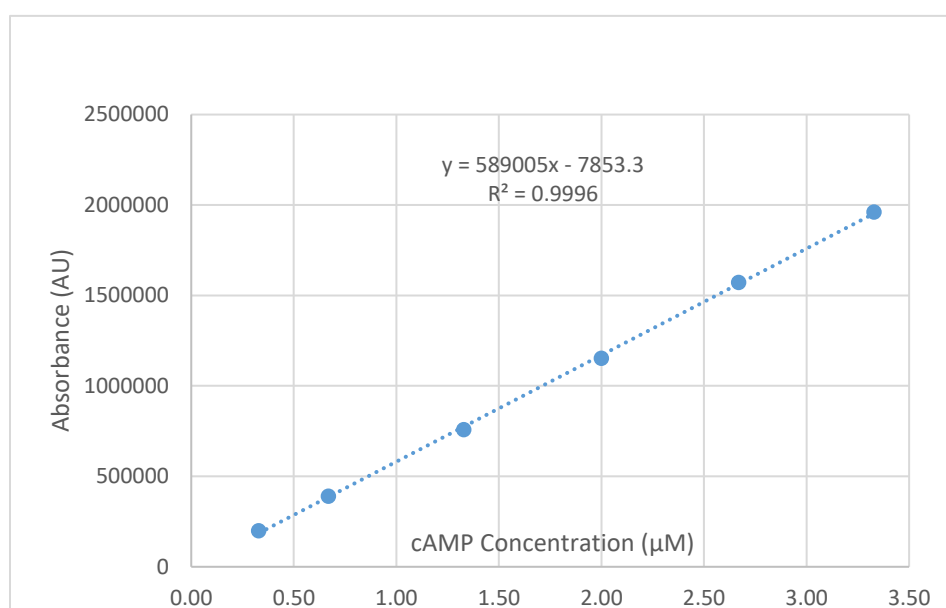


Figure 16. Calibration curve created by plotting known concentrations of cAMP against the area of the curve. This produces a linear graph. Because of this cAMP concentrations will not be determined for every experiment.

2.6 Chapter 6 – HEK HPLC Assay

Reagents and Equipment Used:

Adenosine-5'-diphosphate monopotassium dihydrate, adenosine, creatine phosphokinase from rabbit muscle, formic acid (ACS grade), methanol (HPLC grade), phosphoric acid (ACS grade), and potassium phosphate monobasic (KH_2PO_4) were purchased from Sigma Aldrich. Adenosine triphosphate disodium salt hydrate, cyclic adenosine monophosphate and tetrabutylammonium hydrogen sulfate were purchased from Tokyo Chemical Industry Co (TCI). Adenosine-5'-monophosphate was purchased from Acros Organics. Creatine phosphate disodium salt, 3-isobutyl-1-methylxanthine (IBMX) were purchased from EMD Millipore. Potassium phosphate dibasic (K_2HPO_4), Tris base and MgCl_2 was purchased from Fisher Scientific. Miliq water was produced using a Thermo Scientific Barnstead Easypure II (resistivity $18.0+ \text{ M}\Omega \times \text{cm}$). HPLC was carried out using a Waters Alliance 2695 Separations Module with Waters 2996 Photodiode Array Detector. An xbridge C18 column (4.6 x 150 mm, 5 μm) was used. A Spectrafuge 24D was used to centrifuge samples at 14,000 g for four minutes. Waters total recovery clear glass HPLC vials were used for assay samples.

2.6.1 HPLC Materials and Methods

HPLC Separation Method: Buffer A: Potassium phosphate 65 mM (KH_2PO_4 26 mM, K_2HPO_4 39 mM, TBAHS 4 mM, pH 6.0), Buffer B 65 mM (KH_2PO_4 26 mM, K_2HPO_4 39 mM, MeOH 25%, pH 6.0). pH adjusted with phosphoric acid. Flow rate set to 1 mL/min throughout the run. Injection volume 50 μL , blank injections containing buffer A were run between each sample and standard. No run over observed in blank samples. To equilibrate the column with TBAHS buffer A was eluted through the column for thirty minutes. Before shut down a method was used to wash the column

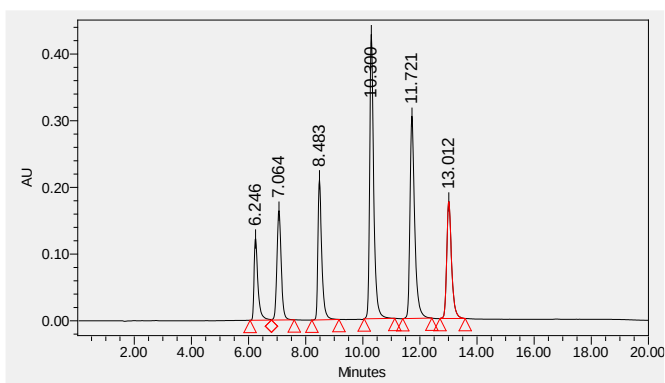


Figure 17. Standard run of AMP (6.25, 8.48), adenosine (8.48), ADP (10.38), ATP (11.72), and cAMP (13.01) (0.1 mM) from left to right. Significant separation between adenosine and cAMP is observed with this method.

with Milipore water (0.1% formic acid) for thirty minutes before returning to 100% methanol for storage. Wavelength at 254 nm recorded for quantification.

Column Equilibration: 100%, switch to 100% milipore w/ formic acid. Wet prime both buffer lines. Wet prime water again, start with 100% methanol then switch to water before buffers.

Separation Method: 0 min. A (100%), 3 min. A (70%). 7.5 min, A (20%). 15 min, A (5%). 16 min, A (5%). 18 min, (A) 100%. 20 min, (A) 100%. Flow rate – 0.5 mL/minute.

2.6.2 HEK AC Assay Methods

Assay Reagent Standard Concentrations (before stopping solution addition): MgCl_2 (100 mM, 20 μL), ATP (3.5 mM, 20 μL), Fsk (1mM, 2 μL), IBMX (100mM, 2 μL), phosphocreatine (20 mM, 20 μL), creatine kinase (3 units), AC6 protein (20 μL) tris buffer (50 mM pH 7.4, to 200 μL total volume).

All stock solutions were made using tris buffer (50 mM, pH 7.4), except forskolin, IBMX (DMSO) and creatine kinase (tris buffer (50 mM, pH 7.4), 0.1 M imidazole). For ATP concentration curves the volume of ATP added to each sample was varied.

Final Concentrations (before stopping solution addition): MgCl_2 10 mM, ATP 0.35 mM, Fsk 10 μM , IBMX 1 mM, phosphocreatine 2 mM, creatine kinase 3 units.

Protocol: On ice add MgCl_2 , ATP, Tris buffer, IBMX, forskolin, phosphocreatine and Ckinase to a 1.5 mL Eppendorf tube. Add AC protein to Eppendorf tube, invert 5 times to mix, place into water bath set to 37 °C for 15 minutes. After 15 minutes removal samples from water bath, add 50 μL 5% formic acid ([final] 1%) to each assay, invert five times to mix, centrifuge at 14,000g for 4 minutes. Pipette top 200 μL of solution into a Total Recovery HPLC vial, run samples on HPLC. A calibration curve can then be used with the area of curve of cAMP peaks to quantify cAMP production.

2.6.3 HEK Cell Produced AC Expression and Purification Methods

Protein Production:

The N-terminal FLAG epitope-tagged human adenylyl cyclase (AC) isoforms 5, 6, and 7 were codon-optimized for expression in mammalian cells and cloned into the KpnI- NotI restriction site of the pcDNA 3.1/Hygro (+) expression vector. The isoforms were carried by expression vector pcDNA 3.1/Hygro (+) and were commercially synthesized by GenScript (Piscataway, NJ, USA). Protein production, purification, and assay methods (CisBio cAMP Kit) have previously been published¹²⁹.

2.6.4 Quantification of cAMP

See Section 2.4.4, Figure 16

2.7 Chapter 7 – Derivative Selection Synthesis

2.7.1 – Synthetic Methods

Synthetic Methods: Synthetic method can be found in the supporting information section, Chapter 9.

2.7.2 – Analysis of Forskolin Derivatives

Analysis. LC-MS analysis was carried out using a Bruker Compact Q-TOF instrument with an electrospray ionization source. Separation was achieved through Intensity Solo C18, 100mm x 2.1 mm column with a 0.4mL/min flow rate, eluent A was 0.1% formic acid in water, and eluent B was 100% acetonitrile. The gradient was 20-60% B at 0-10 min, hold at 60% B from 10-20 min, 60-20% B from 20-25, 20% B for 25-30 min. MS was acquired using parameters: capillary voltage 3.5 kV; gas temperature 200 °C; drying gas, 3.0 l/min-1 and nebulizer pressure of 0.3 bar, injection volume was 1-2 µL. Data was acquired with a mass scan range of 50-1200 m/z. Forskolin and its derivatives eluted between 15 and 23 minutes. The expected mass for each compound was calculated by $[M-H]^+$ or $[M-H+Na_2]^+$ as these are the most commonly observed adducts. (-) ion mode used.

NMR analysis was performed using a Bruker Avance 500 spectrometer. The primarily used NMR solvent was CDCl₃, if a molecule showed poor solubility in chloroform then acetone-d₆, methanol-d₄ or DMSO-d₆ were used instead, depending on the molecule's properties. Experiments carried out on final molecules were ¹H NMR, ¹³C NMR, COSY, HSQC and HMBC. If required further experiments such as NOESY were completed.

Below the preparation of protein samples, analysis of protein and HTRF-cAMP kit assay methods are described. Synthesis methods for the molecules tested are described in the supplementary information section, Chapter 9.

2.7.3 Assay Materials

Human embryonic kidney (HEK) 293T cells were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in a cell-culture medium composed of DMEM-F12 (Gibco, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (MilliporeSigma, Burlington, MA, USA) and 1% penicillin-streptomycin (100mg/ml) (Thermo Fisher Scientific). Hygromycin (200 µg/ml; Sigma- Aldrich) was used in the selection medium to generate stable cell lines. All chemicals were of analytical grade. The cAMP-Gs dynamic bioassay kit was obtained from CisBio, PerkinElmer, USA. Forskolin and IBMX were purchased from Sigma Aldrich, Canada. HRP- conjugated mouse monoclonal anti-FLAG antibody (Abcam, ab49763) was purchased from Abcam, and anti β- actin mouse monoclonal antibody was from Invitrogen, Sigma-Aldrich, Canada.

2.7.4 Molecular Biology and Cell Culture

The N-terminal FLAG epitope-tagged human adenylyl cyclase (AC) isoforms 5, 6, and 7 were codon-optimized for expression in mammalian cells and cloned into the KpnI- NotI restriction site of the pcDNA 3.1/Hygro (+) expression vector. The isoforms were carried by expression vector pcDNA 3.1/Hygro (+) and were commercially synthesized by GenScript (Piscataway, NJ, USA). According to the manufacturer's instructions, the gene constructs of AC isoforms were transiently expressed in HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA). We generated stable HEK293T cells expressing the isoforms 5, 6, and 7 in the selection

medium containing 200 µg/mL hygromycin as described previously¹³⁰. AC isoform 3 and 9 was kindly gifted from Prof Joachim E. Schultz, Pharmazeutisches Institut, Germany, and AC3/6 isoforms knockout HEK293T cell line was generously gifted by Dr. Val J. Watts, Professor of Medicinal Chemistry and Molecular Pharmacology at Purdue University. The stable cell lines used in the study were characterized using western blot analysis and AC enzymatic activity.

2.7.5 Western Blot Analysis

20µg of whole cell extracts were separated on SDS- PAGE and transferred on a nitrocellulose membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). All the selected AC isoforms are flag tagged. After transfer, the membranes were first blocked by incubation with 3% fish gelatin prepared in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 1 hr, and the membrane was incubated with antibodies against anti-FLAG (1:1000), β -actin (1:10,000) at 4°C for 12-16 hr. Membranes were washed thrice with TBST for 10 min and for β -actin, incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-mouse (Bio-Rad) for 2 h. Anti-FLAG antibody was HRP conjugated; therefore, secondary antibody incubation was not required. Blots were washed with TBST three times and developed with the clarity max ECL system (Bio-Rad) according to the manufacturer's instructions.

2.7.6 cAMP Measurement Assay

cAMP generation was measured using an HTRF-based cAMP kit (CisBio) as described previously (PMID: 24531140, 29165978). The detection range of this assay is 150– 718,000 pM. The experiments were planned per the manufacturer's instructions. Briefly, 2500 cells of each AC isoform and HEK293T cell lines were added in wells in white 96-well plates (low volume white microplate, CisBio) in the presence of 10µM forskolin and its derivatives. The dose of forskolin was optimized using a different luminescence-based cAMP assay¹²⁹, and the same concentration was used for all the synthesized derivatives. 3-isobutyl-1-methylxanthine (IBMX) was added as a PDE inhibitor (100 µM) to prevent cAMP degradation during 15 min stimulation in the cell culture incubator at 37°C. HTRF was measured using a Flex station 3 plate reader (Molecular Devices, USA). Fluorescence resonance energy transfer (FRET) ratios (665/615 nm) were

extrapolated to cAMP concentrations using a standard curve according to the manufacturer's instructions.

Chapter Three – Isolation and Purification of Forskolin

This work was published in Bioorganic and Medicinal Chemistry Letters, 44 (2021) 128119, in collaboration with the Herbert and Dakshinamurti groups (University of Manitoba).

3.1 The Isolation, Purification and Complete Characterization of the Diterpene Forskolin from Nutritional Supplements

Forskolin's ability to activate adenylyl cyclase (AC) independently of G α s means it is frequently used to study AC activity. The Web of Science search returns 16,700 journal articles containing forskolin in the title. Forskolin is very expensive, with prices ranging between CAD295¹³¹ and CAD600¹³² per 10 mg, making it unusable for synthetic work. An alternative would be to synthesise forskolin, several complete syntheses have been carried out in various ways. First completed in 1987, then in 1988 and 1989, other synthetic routes were published¹³³⁻¹³⁶. Most recently in twenty-four steps¹³⁷, this is an expensive and time-consuming method of acquiring forskolin. Due to its high cost and impractical synthesis alternative means of production are required.

3.2 Forskolin for Weight Loss

Forskolin has been used as a health food product to assist weight loss, forskolin has been studied in mice and humans to determine its effect on body weight. The study in mice showed a decrease in blood glucose levels and fat cell diameter¹³⁸, while the study on female humans did not show weight loss but did seem to prevent weight gain¹³⁹. A study in human males showed a significant decrease in body weight; this may be related to an increase in free testosterone, which promotes muscle gain and fat loss¹⁴⁰. The effect on testosterone may explain why the effect varies by sex so strongly, both trials in humans had 30 or less subjects and lasted twelve weeks, making it unwise to draw conclusions from them. The supplement bottle states that weight loss will be achieved by taking the capsules regularly alongside increasing exercise and decreasing calorie intake.

Extraction from these supplements is a cheap and effective way of acquiring highly pure forskolin, both for synthesis or AC assays. This method could also be applied to other forskolin-containing products or crude root extracts with slight modification. As each capsule contains a crude plant extract, the identity of any compound extracted and purified must be confirmed.

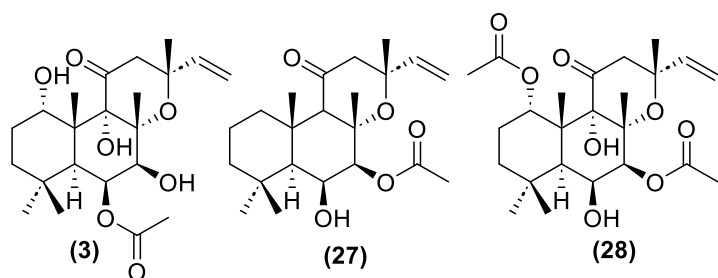


Figure 18. Three structurally related diterpenes extracted from *Coleus forskolii*. 6-acetyl-7-deacetylforskolin (**3**) 1,9-dideoxyforskolin (**27**) and 1-acetylforskolin (**28**).

Single crystal x-ray diffraction, NMR, IR and Mass Spec were carried out. Several related diterpenes have been extracted from the roots of *Coleus forskolii*, and described in the literature^{141, 142} (**Figure 18**).

3.3 Coleonol

There has also been some historical confusion over a molecule known as coleonol (**Figure 19**). A number of different compounds are reported in the literature with the name coleonol, in one article the structure is shown to be the same as forskolin except for the alcohol and acetyl groups at positions 6 and 7 being trans with respect to each other¹²⁵ (**29**). Another article lists the stereochemistry as forskolin except for undefined stereochemistry at the C-1 position¹⁴³ (**30**),

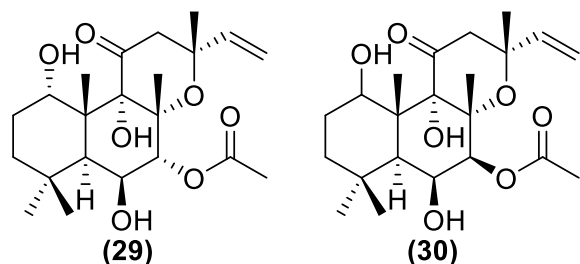


Figure 19. Structure of coleonol as described in the literature.

while a third uses the names forskolin (**1**) and coleonol interchangeably¹⁴⁴. This inconsistency in the literature supports the need for characterization of forskolin from the root or capsule extract.

When comparing the physicochemical properties, forskolin and coleonol were consistently found to be different. M.P.: 220-222 °C (coleonol) 230-232 °C (forskolin), Circular dichroism (c.g.s): -2.16×10^{-40} (coleonol) -2.7×10^{-40} (forskolin), IR, NMR data and GLC retention time were all different. Four derivatives of each were synthesized, a hydrogenated, 1-acetyl, 1-keto and metal hydride reduction product, all have

different melting points, suggesting each reaction formed different derivatives, supporting that coleonol and forskolin are different molecules¹⁴⁵. Coleonol's pharmacological properties were also studied and found to be different to forskolin¹²⁵.

This confusion was clarified when the X-ray crystal structure of coleonol was published in 1985¹⁴⁶, stating that the 6- and 7- substituents are cis with respect to the ring system, identical to the X-ray structure of forskolin that was published in 1980¹⁴⁷.

This stereochemistry was supported by a base-catalyzed rearrangement reaction of forskolin, also published in 1985¹⁴⁸. By treating forskolin with ethanolic KOH, a rearrangement product is formed **(31)** consistently and as the only product. Below is the mechanism proposed for the rearrangement. When coleonol is treated under the same conditions, the same product is formed, and the identity of **(31)** has been confirmed by crystal x-ray analysis and NMR. This again supports coleonol and forskolin is the same molecule.

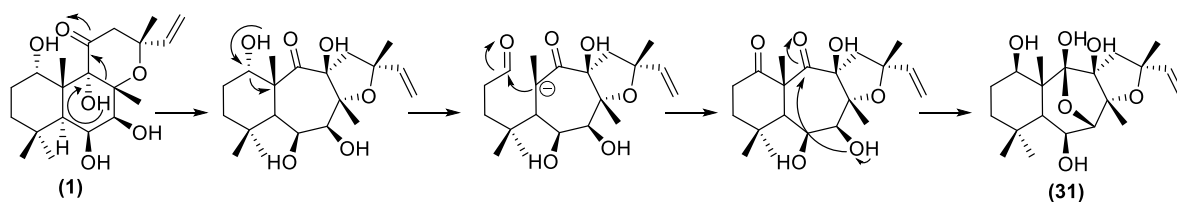


Figure 20. Base-catalyzed rearrangement of forskolin and coleonol, if the stereochemistry is different two different products should form/no reaction would occur. Both molecules underwent the same transformation, forming the same final product, therefore they are the same.

Although using the names coleonol and forskolin interchangeably is correct, it should not be encouraged for clarity's sake, especially as pharmacological studies have been completed on coleonol and are highly cited¹²⁵.

3.4 Results and Discussion

Forskolin was successfully extracted and purified from forskolin-containing weight loss supplements. Each bottle contains 60 capsules; the calculated maximum recovery is 3 g of pure forskolin per bottle. According to this calculation, approximately 56% of the forskolin has been recovered from the capsules, indicating further recovery should be possible. However, due to diminishing returns and solvent costs, purchasing more capsules and starting the process over

may be preferable to repeating recrystallizations on the mother liquid. Previously investigation of forskolin weight loss supplements has been carried out to determine the quantity of forskolin present compared to the label claim. Although they did not study “Herbal Slim Forskolin” the eight supplements investigated did meet the requirements that the content of forskolin was 91-111% of the claimed value¹⁴⁹.

The NMR matches previously published NMR data¹⁵⁰ and X-ray crystal structures¹⁴⁷. Forskolin has the chemical formula $C_{22}H_{34}O_7$ and a calculated mass of 410.2304. The molecular ion peak $[M + H]^+$ has a mass of 411.2382, corresponding to the experimental mass acquired of 411.2356 m/z.

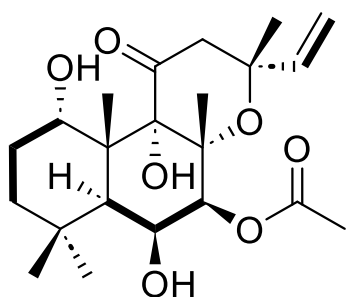


Figure 21. COSY connectivities present in forskolin show as a bold line.

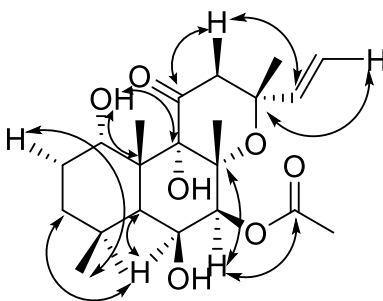


Figure 22. Key HMBC Connections.

COSY and HMBC NMR techniques were also used to corroborate that the molecule extracted was forskolin and that the structure matched what was expected.

Number	¹ H NMR	¹³ C NMR	¹ H NMR Lit. ¹⁵⁰	¹³ C NMR Lit. ¹⁵⁰
1	4.57, m	74.4	4.54, ddd (3.0)	74.4, d
1α-OH	3.05, m		3.4, d (2.5)	
2α	2.16, m		2.16, dddd, (15.0)	
2β	1.41, m	26.6	1.42, dddd, (15.0)	26.6, t
3α	1.10, m	36.1	1.09, ddd, (13.4)	36.1, t
3β	1.77, m		1.76, ddd, (13.4)	
4	-		-	
5α	2.18, m	42.8	2.15, dd (3.2)	42.8, d
6α	4.46, m	70.0	4.44, ddd (3.2)	69.9, d
6β-OH	1.82, s		1.9, dd (2.8)	
7α	5.47, d (4.2)		5.46, d (4.3)	
8	-	81.4	-	81.4, s
9α-OH	6.06, s	82.6	6.2, s	82.7, s
10	-	43.0	-	43.0, s
11	-	205.4	-	205.6, s
12α	2.47, d (17.1)	48.7	2.38, d (17.3)	48.8, s
12β	3.19, d (17.1)		3.17, d (17.3)	
13	-		-	
14	5.94, dd (17.1,10.6)	146.3	5.93, dd	146.3, d
15 cis	4.98, dd (10.7, 1.1)	110.8	4.97, dd (10.5)	110.7, t
15 trans	5.29, dd (17.1, 1.1)		5.28, dd (16.8)	
4α CH3	1.03, s		1.02, s	
4β CH3	1.26, s	24.3	1.25, s	24.3, q
8β CH3	1.71, s	23.6	1.70, s	23.6, q
10β CH3	1.44, s	19.8	1.42, s	19.8, q
13 CH3	1.34, s	31.5	1.33, s	31.5, q
Acetyl CH3	2.16, s	21.2	2.16, s	21.2, q
Acetyl CO	-	169.7	-	169.8, s

Table 3. Observed ¹H and ¹³C NMR data of forskolin in CDCl₃. ¹H and ¹³C NMR spectra were measured at 500 MHz, the experimental and literature chemical shifts of the spectra are shown in δ (ppm). Multiplicity of signal is indicated after chemical shift and parenthesis contain the coupling constants (Hz).

3.5 Conclusion

A novel method for acquiring and purifying forskolin has been found and confirmed by NMR, Mass Spec and X-Ray diffraction. This method can open opportunities and access to forskolin to researchers who may not have previously accessed it. This method will acquire forskolin in gram quantities to synthesise forskolin derivatives.

Chapter Four – Homology Modelling of AC6

This work was published in Molecular and Cellular Biochemistry 446, (2018) 63-72, in collaboration with the Dakshinamurti and Chelikani groups (University of Manitoba).

4.1 Generation of an AC6 Homology Model

4.1.1 Computer-aided drug design

Computer-aided drug design is a commonly used method to reduce the time and costs associated with development of a new drug. Structural Based Virtual Screening (SBVS) attempts to emulate the structure and properties of a target molecule or protein and the interactions that would occur between it and a ligand. In this case examining a wide variety of forskolin derivatives and predicting how well they would bind to the AC6 forskolin allosteric site. Based on these interactions the ligands can be given a score and ranked from best to worst activator¹⁵¹. Drugs discovered using these methods include the antibiotic norfloxacin, isoniazid, used to treat tuberculosis and STX-0119 for treating lymphoma¹⁵².

Once a library of compounds is established that are predicted to have high affinity for the binding target, they can be synthesised and then tested in vitro, in this case using enzyme produced and purified from *E. coli*. This will allow for validation of the in-silico modelling. If no appropriate ligands are found more modelling can be carried out with a focus on the most successful ligands, with repeated rounds of in vitro and in silico testing until an appropriate activator is found.

The computational work completed before the first round of forskolin derivative activity testing will be minimal, once the first round is complete validation of the computational model can be completed. This will be followed by more in-depth comparison of the forskolin binding sites in various isoforms.

4.1.2 Computational Model Creation

To improve our understanding of the interactions between G α s, forskolin and AC6 a homology model was built. A homology model uses the protein's amino acid sequence to be modelled (in this case Human AC6), compares this sequence to similar proteins with a crystal structure

published. These sequences are then aligned to predict a structure for the protein of interest. At the time of construction, there was no crystal structure of AC6, or a complete structure for any transmembrane-bound AC isoform of mammals. In this study homology model of AC6 was built using Discovery Studio 4.5. A Basic Local Alignment Search Tool (BLAST) search compared the human AC6 protein sequence (NP_056085) with known crystal structures. The most similar result showing a 93% match in the Protein Data Bank (PDB) was 1CJK. This crystal structure is an amalgamation of the C1 domain from canine AC5 and the C2 domain from rat AC5, with bovine G α s, expressed separately in *E. coli* then combined in solution to form a functional AC protein⁸⁰.

To convert 1CJK into an appropriate template, it was subjected to the prepare protein algorithm, removing water molecules and replacing any missing residues. This was then used the template, aligning the AC6 sequence over the template to generate a 3D model. The ligands, forskolin and ATP were then copied into the AC6 model, using the “copy from template” function. Discovery Studio has a “build homology model” function that was used to generate 20 models. These were then optimized and analysed based on their Probability Density Function Energy (PDF) score and Discrete Optimized Protein Energy (DOPE) score. A lower score from both functions indicates a better model. To validate the binding position of forskolin, it was removed from the structure and redocked into the binding site, the energy minimization function was then used, and the interactions between the forskolin and the binding pocket were examined. There was no difference in the involved residues before and after redocking.

The amino acid side chains were then refined using the Chemistry at Harvard Macromolecular Mechanics (CHARMM) force field, a computational method that calculates forces between atoms and finds an optimized position based on those calculations.

The final structure was checked using PROCHECK, software for determining the quality of stereochemistry in a protein model¹⁵³. Using a Ramachandran plot, a visualisation of allowed regions based on the protein backbone determined by dihedral angles of individual amino acids, it was found that 98.8% of residues were within the allowed regions.

4.1.3 Nitrosylation as Cause of AC6 Inhibition

When this work was completed, the cause of AC6 inhibition was suspected to be due to nitrosylation of cysteine residues, however that had not been confirmed.

AC6 is sensitive to changes in its catalytic rate by nitric oxide¹¹⁰, this suggests a degree of regulation by nitrosylation, which also occurs commonly under hypoxic conditions¹⁵⁴ and with treatment by iNO¹⁵⁵. Nitrosylation occurs when a NO group binds to a cysteine residue to form an S-NO bond, this has been shown to regulate GCPR complexes¹⁵⁶.

The effects of hypoxia on AC signalling pathways have been discussed in chapter, under hypoxic conditions the decrease in AC activation is due to G α s rather than cAMP degradation²³.

It is important to understand the clinical relevance of AC6 nitrosylation as this may be the cause of hypoxia, nitrosylation of the cysteine residues may affect protein function or stability, potentially inhibiting action. While most post translational modifications require enzyme activity nitrosylation of cysteine residues can occur in the presence of local charged residues, this may allow for site specific S-nitrosylation.

Previous studies have suggested that S-nitrosylation may be controlled by numerous factors such as high exposure of the sulphur atom, hydrophobic regions or low pK_a. More recently a local acid-base motif has been suggested as a mechanism for S-nitrosylation. These motifs support protein-protein interactions contributing to the nitrosylation¹⁵⁷. The likelihood of nitrosylation can be predicted through computational modelling and analysis of the surrounding amino acid sequence. It should be noted that this method is based entirely on the primary amino acid sequence, not on the structure¹⁵⁸. This method generates a score for a target cysteine residue based on adjacent residues¹⁵⁹.

It has been hypothesized that the presence of cysteine residues with nitrosylation potential on AC6 or G α s might play a role in the function of AC6 and due to high homology among AC isoforms there may be conservation of these cysteine residues across multiple isoforms.

4.1.4 Nitrosylation Site Prediction

To determine if there are appropriate targets for nitrosylation on AC6 two different pieces of software were used, GPS-SNO 3.0¹⁵⁹ and NSO 1.0¹⁶⁰.

NSO 1.0 predicted twenty residues as having nitrosylation potential, while GPS-SNO 3.0 predicted four residues, with only two residues, C772 and C1004, appearing in both sets of results.

For G α s NSO 1.0 predicted seven potential residues, while GPS-sNO 3.0 predicted a single residue as a location for nitrosylation.

As only the C1 and C2 domains, not the complete protein, is included in the model, this study of nitrosylation is limited to the cytoplasmic domains, and there could be other nitrosylation sites on the transmembrane domains.

Looking at the locations of selected residues on the model it was determined that residue C1004 is most likely to reduce activity of AC if nitrosylated as it exists at the interface between the C2 domain and G α s. C174 on G α s was predicted to be a nitrosylation target, this residue exists close to the C1004 residue on ACC2.

It is possible that under normal conditions, these two residues interact, forming a disulphide bond, increasing how much the proteins interact, causing a conformation change, and contributing to the activation of AC6. This fits with a decreased but not stopped activation of AC6 by G α s under hypoxic conditions. If the seemingly favoured nitrosylation of either cysteine residue prevented this interaction, we would expect a reduced interaction between the proteins.

In this model, there is 6.2 Å distance, between the residues, however, that should not be a reason to rule out the formation of a potential disulphide bond under physiological conditions.

As this is based on a homology model and calculations, it is not conclusive but can and will be tested experimentally by replacing either residue with another. This would be expected to have a similar effect on AC activity as the nitrosylation of the specified residue.

4.2 Homology Model of Human AC6 Bound to G α s

Figure 23. shows the homology model built with Discovery Studio. The C1 and C2 domains are shown in beige and cyan/teal, respectively, while G α s is displayed in green. The ATP binding site is highlighted, the forskolin binding site and the interface between G α s and the AC6 C2 domain, where nitrosylation of cysteine residues may be the mechanism preventing activation of AC6 under hypoxic conditions.

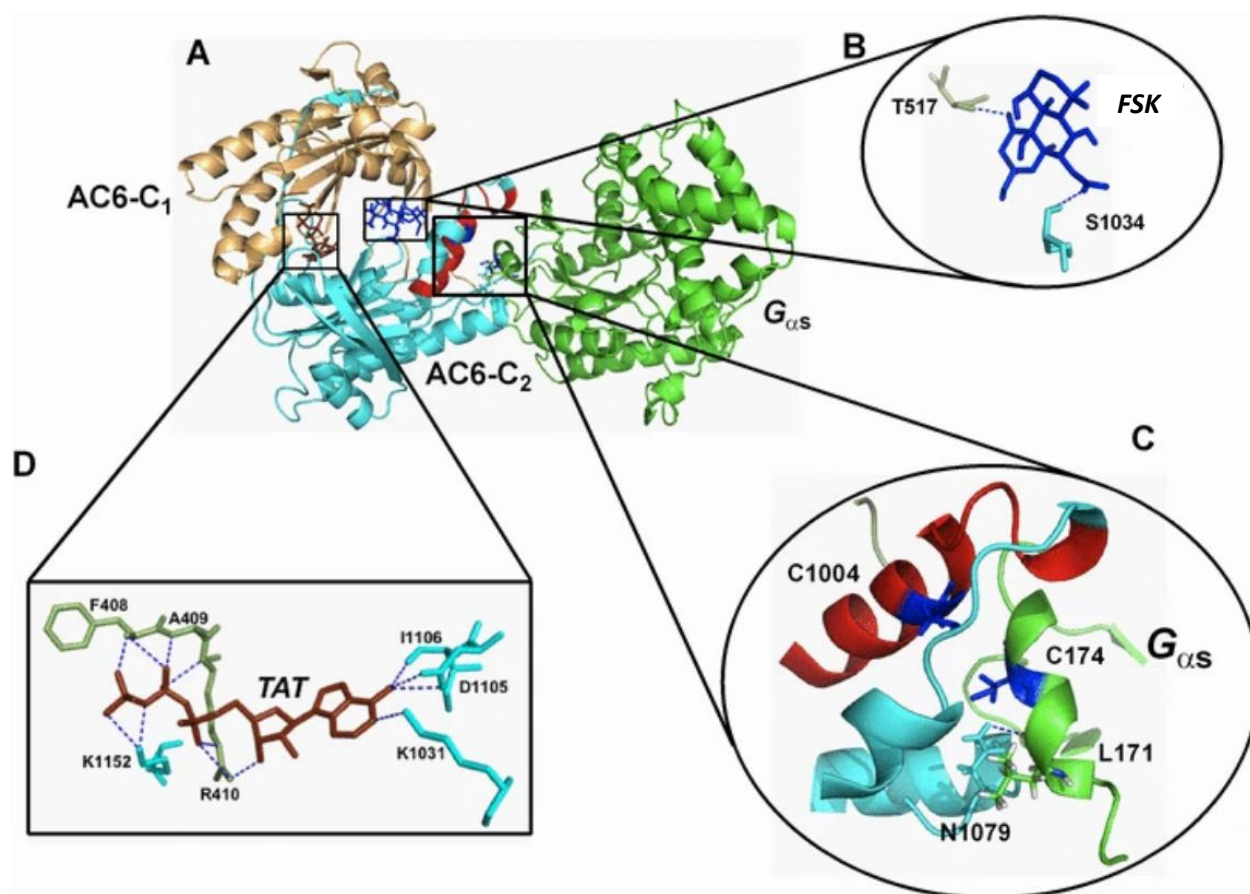


Figure 23. (A) Computational model of human AC6, with truncated transmembrane domain. Cytoplasmic domain 1 is shown in beige, C2 shown in cyan, while G α s is shown in green. (B) The binding site for both forskolin and ATP site between the two cytoplasmic domains. Forskolin (FSK), shown in dark blue, interacts with a threonine and serine residue as expected from the crystal structure the homology model is based on. (C) Possible sites for nitrosylation and their relative locations at the interface between C2 and G α s, C1004 located on C2, while C174 is on G α s. There is a 6 Å distance between the residues, suggesting the possibility of a disulfide bond forming. (D) ATP analog adenosine-5-Rp-alpha-thio-triphosphate (TAT) shown in red with interactions that occur in binding site. This image is reprinted from doi: 10.1007/s11010-018-3273-4.

4.3 Virtual Ligand Screening

This study identified 1840 compounds based on a minimum similarity of 70% to forskolin within the ZINC database. The ZINC database is a publicly accessible database containing over 120 million purchasable compounds, ready to use for virtual ligand screening¹⁶¹. All molecules above the similarity threshold were prepared for analysis by Discovery Studio, generating 3D coordinates and fixing bad valencies. 1809 compounds were determined to be appropriate for analysis; their energies were then minimized using CHARMM and compounds not meeting Lipinski's Rule of Five were removed.

This left a total number of 1502 compounds for docking with LibDock, part of Discovery Studio software. Three different scoring functions were used to rank the compounds: LibDock Score, Lig Score 1 and Lig Score 2.

For each score a compound was ranked ranks were then combined for each compound, the molecule with the lowest score has the best overall binding across the three different scoring mechanisms (Table 4). These scores are

Compound	Lib Dock	Lig Score 1	Lig Score 2	Combined Score
37	129.4	6.6	7.2	4
38	120.6	6.3	7.3	8
26	123.8	6.5	6.9	8
41	127.7	5.6	6.5	14
36	109.8	6.1	7.2	14
43	99.9	6.3	6.8	17
32	95.6	6.3	6.3	20
38	104.5	5.7	6.4	20
35	110.2	4.5	6.5	20
33	93.4	6.5	6.2	21
40	114.5	5.3	5.9	21
44	56.0	6	6.3	24
34	103.6	3.5	5.8	29
39	83.5	4.4	6.2	30

based on Vander Waals interactions, H-bonding, pi interactions between the binding pocket and ligand.

Table 4. Lib Dock, Lig Score 1 and Lig Score 2 for the top binding compounds when docked with a homology model of AC6. For each score a ligand is given a ranking, these rankings are then combined to give a final score, with the lowest score predicted to be the strongest binding ligand.

Forskolin derivatives expected to perform well are ranked most highly, with NKH477 being the second highest rated and the N'N-Et derivative (**37**) performing best. Interestingly Compound (**39**) (NKH477 with the reduced double bond at C14/15) was the lowest ranked despite the insignificant structural change. The structures are shown below, in **Figure 24**.

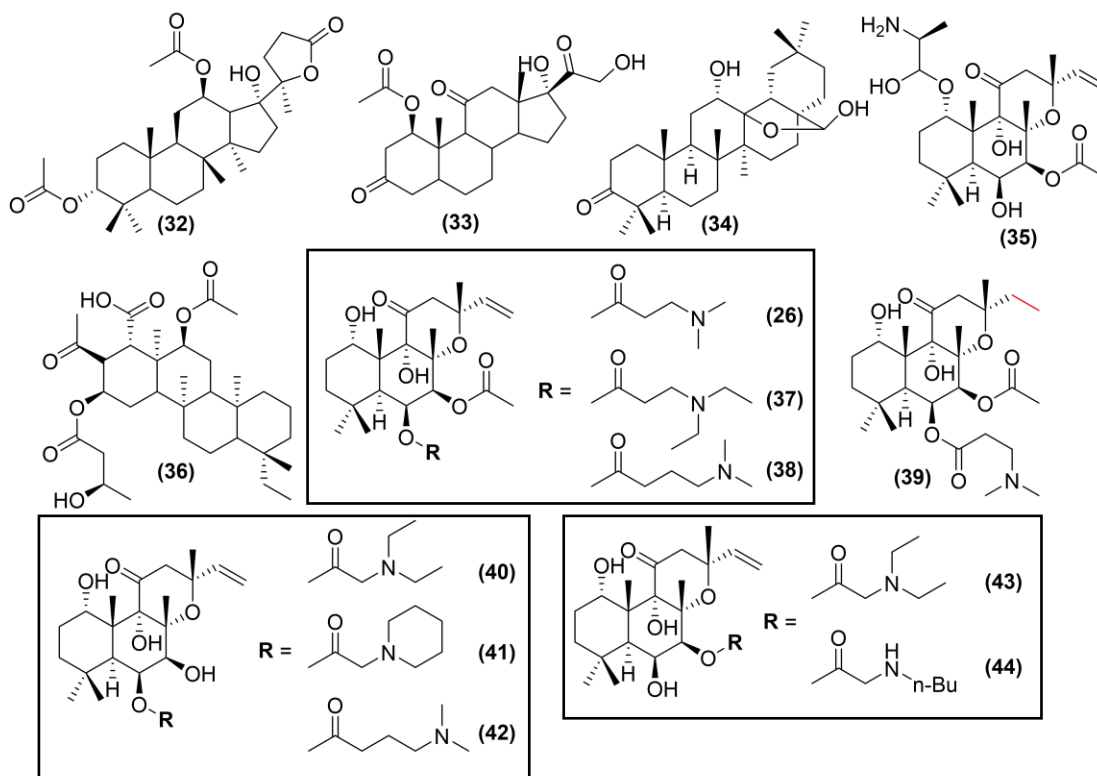


Figure 24. The structures of the most highly rated molecules out of the original 1500 molecules assessed, this is based on the predicted energies of interactions between the ligand and receptor site. Compounds (32)-(36) were generated through examining structures similar to forskolin in the ZINC database and compounds (6), (37)-(44) were generated manually.

4.4 AC6 Model #2

A second model of AC6 (AC6 #2) was made as part of a course (BGEN7020, Proteins), this model consisted of the two cytoplasmic domains of AC6 bound to G α s. This model was made using the same method as the previous model, the amino acid sequence of AC6 underwent BLAST alignment to find the most closely related crystal structure. This matched with 1CJK, so 1CJK was again prepared as a template to which the sequence of AC6 was applied. This was used to generate 10 optimised models, and the best model was selected for docking studies based on PDF and DOPE scores.

The “predict binding site” function was then used, to determine where binding of forskolin is most likely to occur in the structure. Shown in **Figure 26**. This

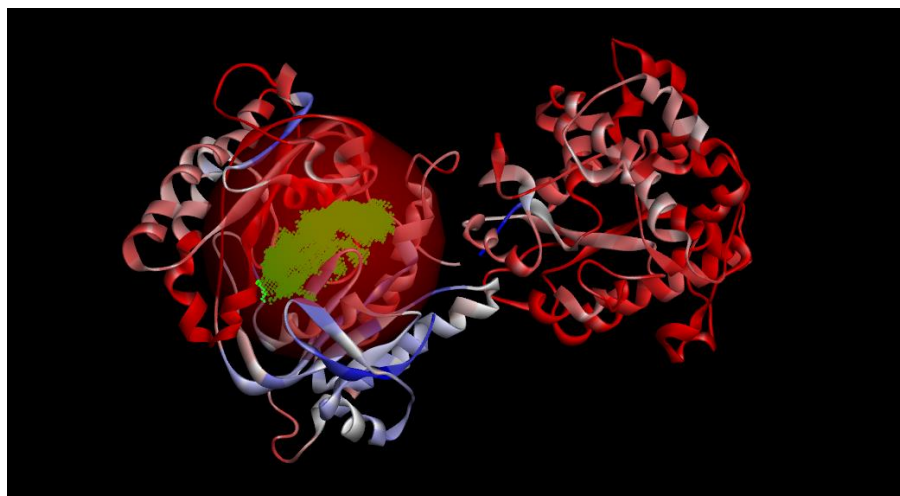


Figure 26. The predicted binding region of forskolin, within AC6 #2, is shown by the red sphere, with the green texture showing positions forskolin could sit in, this includes the ATP binding site. This predicted binding site aligns with the binding site found in crystal structure 1CJK.

binding site aligns with the ATP and forskolin binding

site, the green area

highlights regions of possible binding.

Forskolin sits towards the upper right portion, while ATP binds in the lower left. As before forskolin was removed from its binding site, and redocked within the binding site to validate its position. Forskolin interacts with the expected residues as before after redocking.

4.4.1 AC6 #2 Ramachandran Plot

The model was analysed by Ramachandran Plot, with PROCHECK. This tests the stereochemical quality of the model, determining if the model would not be possible due to steric clashing with itself. This model had no residues in disallowed or generously allowed regions, with 90% of

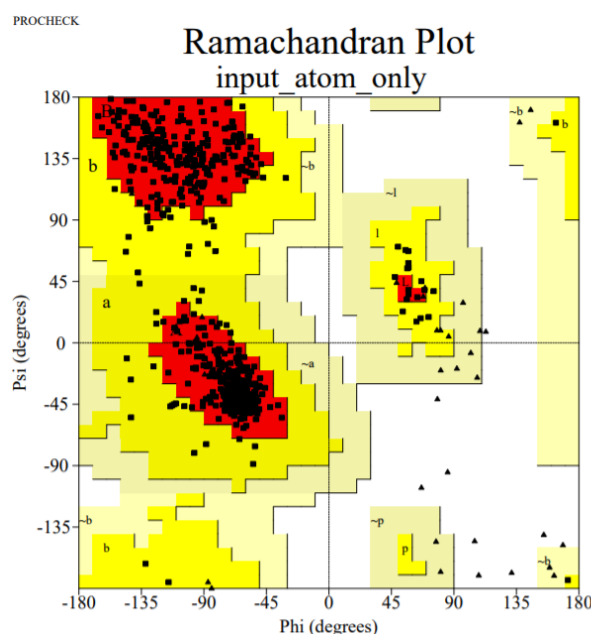


Figure 25. A ramachandran plot of AC6 model #2 this figure shows the Psi and Phi angles of the protein to predict whether a protein structure is viable. The red areas show residues with no steric clashes, yellow are in an acceptable conformation and white regions are disallowed. Triangles represent glycine residues that can have phi and psi angles outside these regions and still be in an acceptable conformation.

residues in the most favoured regions and 10% in allowed positions, with no residues in disallowed regions^{153, 162}.

The peptide chains that make up a protein consist of many carbonyl groups that can delocalize their electrons into the backbone of the protein, this gives a rigid structure. The phi angle represents the dihedral angle of the bond between the nitrogen group and alpha carbon, while the psi angle represents the bond between the alpha carbon and carbonyl.

In the Ramachandran model atoms are considered hard spheres the size of their van der Waals radius. This gives limited range to the number of positions a peptide chain can be in, defined by their phi and psi angles. Outside of these specific angles a residues Van der Waals sphere will overlap with another sphere, causing a steric clash and putting it into the disallowed category.

While this does not confirm the quality of the model, it does show that the protein is not occupying an impossible conformation.

Red areas of the chart represent areas where there is no steric clashing between residues, normally, these residues will be in alpha-helices or beta-sheet conformations. Yellow regions represent those residues sitting in an acceptable conformation using a slightly shorter van der Waals radius for each atom in the calculation, allowing the residues to sit closer to each other.

White regions are disallowed as there is too much steric hindrance to allow the conformation, there is an exception for glycine residues as they do not have a side chain, so do not have the same steric issues.

4.4.2 Ligand Docking AC6 Model #2

Once the model was complete and performed well in structural analysis it was used to determine binding energies with a small set of molecules, all forskolin derivatives with amino-containing moieties. The –CDOCKER Energy and –CDOCKER Interaction Energies were compared between molecules to determine which had the highest binding affinity. Based on these scores, The molecules were then ranked from 1-10. Scores from the CDOCKER Energy and CDOCKER Interaction energy were combined then the molecules were ranked from lowest score (highest binding energies) to highest (lowest binding energies), shown in **Table 5**.

CDOCKER energy is calculated based on the interaction energy between the ligand and receptor and the internal strain of the ligand. CDOCKER Interaction Energy examines the energy of non-bonded interactions between ligands and protein.

Molecule	Structure		-CDocker Energy Kcal/mol	-CDocker Interaction Energy Kcal/mol	Total Ranking
	C7	C6			
(45)	OCOCH ₃	NHCO(CH ₂) ₂ NMe ₂	14.8 (2)	66.3 (4)	6
(46)	OCOCH ₃	OCO(CH ₂) ₂ NPro ₂	14.0 (3)	68.5 (3)	6
(47)	OCOCH ₃	OCO(CH ₂) ₈ NEt ₂	14.0 (4)	68.7 (2)	6
(37)	OCOCH ₃	OCO(CH ₂) ₂ NEt ₂	13.8 (5)	72.0 (1)	6
(48)	OCOCH ₃	OCOC(CH ₂) ₈ NMe ₂	16.7 (1)	63.5 (6)	7
(49)	OCOCH ₃	OCO(CH ₂) ₂ N(C ₅ H ₁₀)	10.3 (6)	65.6 (5)	11
(26)	OCOCH ₃	OCO(CH ₂) ₂ NMe ₂	8.7 (7)	58.3 (10)	17
(50)	OCOCH ₃	β-NHCO(CH ₂) ₂ NMe ₂	8.1 (8)	61.2 (7)	15
(51)	OH	β-NHCO(CH ₂) ₂ NMe ₂	7.9 (9)	60.1 (9)	18
(52)	OH	β-NHCO(CH ₂) ₂ NEt ₂	6.5 (10)	60.7 (8)	18

Table 5. A more limited selection of compounds was then docked within the binding site and their binding affinity was studied with two measures, CDOCKER energy and CDOCKER interaction energy. Both of these functions examine interactions between a molecule and the binding site, rating their ability to bind to the enzyme. The top ten compounds are listed in table x. Each compound is given a ranking 1-10 based on the –CDocker Energy and –CDocker interaction energy, these rankings are then combined to give a total ranking, the lower the score the better. Compound (50), (51) and (52) are marked with a β sign as these molecules have inverted stereochemistry at the C6 position.

From this docking analysis several compounds expected to perform well did. NKH477 (26) and similar compounds (an additional methyl in the amino chain or replacement of N’N-methyl groups with N’N’-ethyl groups) perform the best. One of the conditions tested was various chain lengths, surprisingly, compounds with an 8-carbon chain at the 6-position performed well. This may be due to modelling limitations, as the long mobile chain would allow the N(Me)₂ group to be placed in many locations that would provide strong binding energy. This, however would not be a realistic conformation for the molecule to adopt. As compounds above 3-4 carbon chain length have worsening binding properties, performing poorly in assays, compounds with these chain lengths will be synthesized as part of the second derivative library. Replacement of the 6-OR with a nitrogen atom to form 6-NR₂, appears to have improved binding affinities. The likelihood of synthesising this compound seems low, the 6 position tends to be very unreactive, requiring migration of acetyl group from the 7 to the 6 position. The 6- position may be

susceptible to bromination, which may allow synthesis of these compounds. The same applies for compounds **49-51**, containing the inversed stereochemistry at the 6 position.

Amination of the 7-position will also be included in future synthetic and computational libraries, as these derivatives may show improved binding, and with the protection of the 1-OH, it seems reasonable synthetically.

Forskolin derivatives with altered stereochemistry are yet to be studied, further computational studies will be completed on these molecules before synthesis. It may be worth synthesising these compounds regardless of the results, as they are currently unknown and no predictions can be made from previous in vitro/vivo studies.

A surprising result from this data are compounds **(46)**, **(47)** and **(37)**, it seems odd that the replacement of N'N' methyl with N'N' propyl would increase binding affinity. Again this may be a limitation of the software, groups with increased steric bulk being positioned in an ideal, unrealistic conformation. In vivo, the increased chain length may cause steric clashing or prevent a molecule entering a binding site.

This docking study also supports reintroducing the 7-acetyl group, performing consistently better than the 7-deactyl derivative. Previous studies (examining other isoforms) found that the acetyl groups value depended on the chain at the 6-position, results from the initial testing may give direction on this issue. Once in vitro and in vivo studies are complete the results can be compared to computational binding energies, allowing validation of the model and indicating how reliable the ligand binding energies are.

4.5 Conclusions

Two homology models of AC6 were made; both were based on an old crystal structure that was a mix of multiple isoforms. Fortunately, since the project started a cryo-EM structure of AC isoform 9 was captured, further modelling will be completed with a homology model based off of that crystal structure.

There has also been an increase in the number of available methods for creation of a protein model, that do not rely on homology modelling. An example is AlphaFold, an AI-based modelling system using DeepMind, which produces accurate protein models¹⁶³.

Until computational results can be compared with experimental data they are of limited use, as there is no in vitro validation until that has been completed.

This is further complicated by factors such as solubility and cell permeability, a structure could score excellently in computational studies but poorly in whole cell cAMP assays, due to being unable to enter the cell and affect cAMP activity. This highlights the advantages of having multiple assay methods, as a purified enzyme assay would be unaffected by cell permeability issues. Due to this computational data is treated cautiously, but should not be dismissed because it does not match with the first set of in vivo study.

Once the models have been validated, additional models of various isoforms will be created, allowing for the study of the forskolin binding site. Comparison of AC isoforms will be used to highlight the difference in sequence and structure that may allow for specific activation of AC6.

Chapter Five – Design and Preparation of Bacterially Produced AC6 Enzyme Activation Assay

5.1. Adenylyl Cyclase 6 produced in *E. coli* activation assays

This section describes assays carried out using HPLC to analyze the production of cAMP by adenylyl cyclase expressed and purified from *E. coli*. By quantifying cAMP production, we can determine how well a forskolin derivative activates AC6 then compare activity with other AC isoforms to determine specificity. This will aid in finding an appropriate specific activator of adenylyl cyclase 6 to treat PPHN.

While assays carried out using HEK produced AC6 are expected to more accurately represent PPHN conditions, the bacterial AC6 can be produced in larger quantities and purified, oppose to using an impure lysate or whole cell. This allows for more precise examination of the protein without interference from impurities and other active enzymes. The flexibility of this assay also allows other conditions to be studied in the future, such as the addition of a nitrosylating reagent to study the effect of hypoxia/PPHN in bacterially produced AC. Isolation of the pure protein will also allow for LCMS proteomics experiments, to study the binding interactions between forskolin and adenylyl cyclase.

These features make the HPLC assay an effective screening tool for forskolin derivatives, their activity can be tested quickly and cheaply to reduce the number of compounds tested using in vivo assays methods. Initially only AC6 will be examined, when development is finished other AC isoforms can be produced for studies determining specificity.

As previously discussed adenylyl cyclase is a transmembrane domain protein which causes difficulty in the purification and expression of proteins, due to their poor solubility. This makes it more desirable to overexpress the enzyme in another system, without the transmembrane domain to produce usable quantities of soluble protein.

To produce adenylyl cyclase a truncated version was designed and a plasmid was purchased in a pET-30a (+) vector from GenScript USA Inc, sequence shown in the supporting information. This plasmid was expressed in *E. coli* by a collaborator, the protein was produced and purified, and then all assay work was completed by myself. This protein went through several iterations, the Flag-Tag was removed and replaced with a His-Tag, to aid with purification. At the start of each experiment description the protein used will be included.

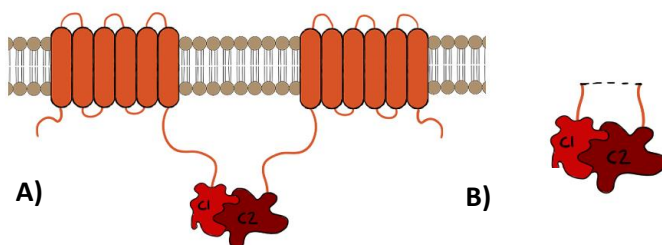


Figure 27. Adenylyl cyclase contains transmembrane domains that are unnecessary for the conversion of ATP to cAMP, these domains make protein expression difficult. **A)** A complete AC protein representation **B)** The truncated version of the protein with transmembrane domains removed.

G α s was produced and purified by myself, then later on by the same collaborator (Harjasmin). G α s was also produced using a pET-30a (+) vector, and purified by His affinity column. Production and purification methods can be found in Chapter 2 - Methods.

5.2 Preparation of Bacterially Produced Adenylyl Cyclase Assay Method

A standard assay will contain tris buffer, MgCl₂, ATP and AC protein, then forskolin and G α s will be added as required. The assay will have a total volume of 200 μ L, with 20 μ L of protein as the initial testing volume. The assay will run for 15 minutes at 37 °C before heating and centrifugation to end the assay. Each sample will then be analyzed by HPLC and quantified by a calibration curve of cAMP concentrations. The calibration curve can be found in Chapter 2, **Figure 16**. As the relationship between cAMP concentration and UV absorption is linear for developmental assays results will be expressed as area of the curve (AOC).

Below is a description of the development work completed on the HPLC BAC assay. The HPLC separation method and final assay method are described in Chapter 2.

5.2.1 BAC Assay Set One:

Protein Used: BAC6 (#1), preparation is described in Chapter Two, this set of assays used the refolded, unpurified lysate. **G α s #1** was used.

Aim: Determine if the protein produced from *E. coli* is functional, including turnover of ATP to cAMP, and sensitivity to G α s and forskolin. The protein has not been purified, initial testing will be done to determine if the protein is active before beginning protein purification.

Conditions: Each assay is run in a 1.5 mL Eppendorf tube, first tris buffer is added, followed by MgCl₂, ATP, forskolin, G α s. AC6 protein is then added, tubes are closed and inverted five times to mix, then placed in a 37 °C water bath for 15 minutes. At the end of 15 minutes the Eppendorf tubes are either placed in a heat block set to 95 °C or boiling water for three minutes. They are then spun down at 12,000g for 3 minutes.

200 μ L assay solution is then pipetted into a total recovery HPLC vial and area of cAMP peak (AOC) is used to determine cAMP production. For publication this area will be converted to cAMP (moles) produced per protein (μ g) per minute, or similar alternative. For the following assays below concentrations of reagents were used, if altered will be noted in the assay conditions. For these assays 20 μ L protein will be used, this volume is unadjusted for protein concentration and purity until protein has been shown to be functional as expected. G α s will have a consistent volume of 10 μ L for initial experiments.

Final concentrations (Standard Conditions): MgCl₂ (10 mM), ATP (1 mM), Fsk (10 μ M), Tris buffer (50 mM pH 7.4). Std is a

standard assay sample, containing MgCl₂, ATP and Tris, any other component added will be listed.

#	Condition	AC6 (μ L)	Forskolin	G α s	AOC
1	Std AC6	10	+	-	679,895
2	Std AC6	30	+	-	1,897,653
3	No Protein	0	-	-	112,570
4	Std + G α s	20	-	+	5,179,375
5	Std + G α s + Fsk	20	+	+	8,560,010
6	Protein Only	20	-	-	-

Discussion: The protein appears to be active, now

Table 6. Assay results from BAC assay set one. Protein used - **BAC #1**
Results from first assay set to examine AC6's functionality.

that has been confirmed further assay conditions will be run. A sample without G α s or forskolin will be included in the next assay set to determine how it affects activity.

AC6 #1 is sensitive to G α s. Once purified there will need to be repeat experiments with forskolin alone and observation of protein turnover without activators, so that it will be possible to determine if forskolin impacted turnover. Future experiments will include duplicates of each experiment so that consistency between samples can be determined.

5.2.2 BAC Assay Set Two:

Protein Used: BAC6 #1. G α s #1 was used.

Aim: To confirm the functionality of AC6 with comparison of standard assays $\pm$ forskolin. Duplicates have been included to determine consistency between assay samples. Samples with IBMX have been included to determine if impurities present in protein solution are causing hydrolysis of cAMP to AMP. Assays will be run without MgCl₂ and without ATP to confirm that AC6 is causing turnover.

Conditions: The concentration of ATP was decreased to half the original concentration from 1 mM to 0.5 mM. This was due to small amounts of ATP appearing in the following blank injection. The final forskolin concentration was increased from 10 μ M to 100 μ M, this concentration is too high for regular assays but useful for determining if the protein is active in the presence of forskolin. Other assay conditions, reagents and stopping method remains the same. Isobutyl methylxanthine (IBMX, final concentration 1 mM), a PDE inhibitor was added to each assay sample, to prevent degradation of cAMP, as the protein is unpurified.

#	Condition	Forskolin	G α s	AOC #1	AOC #2
1	Std	+	-	819,602	848,289
2	Std - MgCl ₂	+	-	-	-
3	Std - ATP	+	-	-	-
4	Std - IBMX	+	-	722,478	1,124,917
5	Std - Fsk	-	-	1,090,117	1,019,163
6	Std + G α s - Fsk	-	+	1,574,597	378,400
7	Std + G α s + Fsk	+	+	2,530,713	2,315,896

Table 7. Assay results for BAC assay set two. AC6 Protein used XYZ. Results from the second assay set, $\pm$ forskolin and G α s. Assays run without essential components, the protein is not functional without MgCl₂ and no cAMP is produced without ATP present. The presence of forskolin appears to decrease cAMP production.

Discussion: The most immediate issue is the lack of increase in turnover in the presence of forskolin, cAMP production is higher in samples that did not contain forskolin (Assay 1 vs Assay 5). This is the opposite of the expected effect, in future assays a sample will be included that has DMSO (2 μ L) with no forskolin to ensure that presence of DMSO is not affecting turnover. Forskolin does increase turnover in the presence of G α s (Assay 7). This could be explained by improper folding, forskolin alone does not influence activity as the two cytoplasmic domains are too distant, once G α s is introduced the conformation of AC6 changes, bringing the two cytoplasmic domains closer together, at which point forskolin can support, the conformation change.

These results were inconsistent between duplicates, this would make sense with improper or inconsistent folding, in future experiments assays will be carried out in duplicate or triplicate and variation between samples monitored.

There was an observed significant decrease in turnover with G α s compared to previous assays, this reason for this is currently unknown. G α s was stored at 4 °C between experimentations and may have begun to degrade over time, decreasing its ability to activate AC. It will be necessary to repeat assays with these conditions to determine why the results are inconsistent, and to see if the protein is activated by G α s/forskolin.

The assay without MgCl₂ produced no cAMP, this is expected as Mg²⁺ or an alternate divalent ion is a required co-factor for AC activity. The assay without ATP also produced no cAMP. This was also expected, but important to determine if ATP present in protein samples could interfere with assay results, either by increasing starting ATP concentrations or affecting final cAMP concentration.

5.2.3 BAC Assay Set Three:

Protein Used: BAC6 (#2) and G α s #2 were used.

Aim: To determine if the most recent production of protein is functional. Checking for forskolin and G α s sensitivity. Experiments done in triplicate. New G α s and AC6 were used.

Conditions: Assay conditions were unadjusted for this set of assays. The stopping method was changed to involve adding 50 μL of formic acid (5%) to give a final concentration of 1% formic acid and a total assay volume of 250 μL . IBMX was added to one sample (Assay 3), but not included in other samples.

#	Condition	Forskolin	G α s	IBMX	AOC 1	AOC 2	AOC 3
1	Std	+	-	-	41770	32834	48841
2	G α s + Fsk	+	+	-	54111	61577	64609
3	IBMX	+	-	+	42191	48386	40880
4	G α s	-	+	-	50475	54284	42452
5	Std + Fsk*	+	-	-	34365	35132	32572

Table 8. Assay results from BAC assay set three. Assay results examining the effect of forskolin, G α s and IBMX on cAMP production. *Denotes 10x concentration.

Discussion: The same issue is observed again, the presence of forskolin seems to decrease or have negligible effect on activity, not increasing it. When the concentration of forskolin is increased 10-fold there is a further reduction of activity. This is unexpected as the same amount of DMSO (2 μL) is used, forskolin purity has been checked and the sample is pure so there is not an increase in impurities when increasing concentration, indicating that forskolin is inhibiting activity in some way. Again, this points towards a poorly folded binding site, when forskolin is bound there is an impact on cAMP production.

G α s increases activity, as with experiment set two, the increase is not to the expected degree. Despite forskolin's poor activity affect alone, it still increases AC activity when paired with G α s, as was observed in previous experiments.

IBMX appears to have little effect on cAMP measured, as protein is partially purified, PDE enzymes may have been removed or deactivated, so a significant effect was not expected, for the time being IBMX has been removed from bacterial AC assays.

5.2.4 BAC Assay Set Four:

Protein Used: IEX column fractions.

Summary: These assays were carried out to on nine various protein preparations, from the flag-tagged AC6 protein. The protein used was produced using the same method. Includes fractions from IEX column, and samples from both the soluble and insoluble fraction. Depending on the fraction activity ranged from less than 0.1% turnover to complete consumption of ATP. This was used to determine which fractions contained active protein. These protein samples are labelled Ion exchange (IEX) fraction number (A4).

5.2.5 BAC Assay Set Five:

Protein Used: IEX A4 – relabelled as **BAC #3**, was not activated by forskolin but showed moderate activity, this experiment was run while waiting for the next protein preparations to be ready.

Aim: The aim of these experiments was to determine an appropriate length of assay, for these assays to be successful they need to observe the initial rate of reaction, as this will be linear and unaffected by decreasing ATP concentrations. Due to this generally less than 10% of the starting substrate should be consumed. To determine if this is the case a set of assays will be completed with assays stopped at a range of times from 0-20 minutes.

#	Condition	Forskolin	Gas	AOC #1	AOC #2	Average AOC
1	0 mins	+	-	606,392	612,321	609,357
2	4 mins	+	-	596,427	939,853	768,140
3	8 mins	+	-	1,705,144	1,705,796	1,705,470
4	12 mins	+	-	922,717	1,953,339	1,438,028
5	16 mins	+	-	1,952,572	2,076,806	2,014,689
6	20 mins	+	-	2,436,001	3,036,696	2,736,349
7	20 mins *	+	-	797,787	1,165,805	981,796

Table 9. Assay experiments examining various stopping points. Also examines the stopping conditions of the assay. * Denotes the samples that used pre-boiled protein, if denatured fully zero cAMP production is expected.

Conditions: Conditions were unaltered apart from the stopping times. For the zero-minute time point once protein was added the Eppendorf tubes were placed in a water bath at 95 °C for three minutes then centrifuged. For the 20-minute sample with boiled protein before the assays were started 50 µL of protein was added to an Eppendorf and boiled for three minutes, the assay was carried out as normal from that point onwards. All samples contained forskolin and none contained Gas.

Discussion: From these results it appears that the method of stopping reaction is not completely denaturing the protein, as cAMP production occurred in the 0-minute time point and the boiled protein assay still produced cAMP. The assays are currently stopped by combination of heat to denature the protein followed by centrifugation to prevent protein being pipetted into an HPLC vial and blocking the column. From this heat alone for 3 minutes does not fully denature the protein, unlike in the HEK produced AC, where heating alone prevented all activity. A different method of stopping reactions will be tested, most likely by addition of acid or SDS at the end of specified time.

Although there is some inconsistency in the results and these experiments will need to be repeated with finalized protein, they show that 15 minutes may be too long to study the initial rate of reaction, with this protein and ATP concentration. For the time being 15 minutes will remain the standard time, due to the issues with stopping the reaction it is difficult to draw conclusions from the data.

5.2.6 BAC Assay Set Six:

Protein Used: IEX A4 - BAC6 #3

Aim: A small selection of assay conditions was run to test the stopping conditions of the assay. Two different acids will be used, formic acid and HCl.

Conditions: Assays were run in duplicate and the stopping condition was changed to use acid instead of heat. A 0- and 7.5-minute experiment were also run. A final volume of 1% for formic

acid and concentration of 0.2 M for HCl. Samples will then be centrifuged at 14,000 g for 4 minutes.

#	Condition	Duration (minutes)	AOC #1	AOC #2
1	Std 1% formic	15	246,768	299,325
2	Std 0.2 M HCl	15	256,424	263,949
3	Std 1% formic	0	0	0
4	Std 1% formic	7.5	107,493	105,488
5	No Protein	15	0	0

Table 10. Results of experiments examining the stopping conditions of the assay. Two different acids were used to denature the protein present, as an alternative to heating which did not completely stop enzyme activity.

Discussion: From these experiments it seems that either 1% formic acid or 0.2 M HCl would act as an appropriate stopping reagent combined with centrifugation at 14,000g for 4 minutes. Unlike in previous experiments the zero-minute time point generated 0 cAMP, suggesting that the protein was denatured immediately and completely, preventing production of cAMP. From this point onwards 1% formic acid will be used to stop assays. Further testing will be carried out by rerunning several samples at the end of the run, this should give a few hours between initial and second injection of the assay sample, if there has been a noticeable increase or decrease in cAMP it would suggest further changes need to be made

5.2.7 BAC Assay Set Seven:

Protein Used: Two samples from a new production of AC6 Flag-Tagged protein (A3 - **BAC#4** and A10 - **BAC #5**) were examined, with an AC6 construct that replaced the Flag-Tag with a His-Tag - **BAC#6**.

Aim: Forskolin sensitivity was tested in three new productions of protein; AC6 Flag Tag A3 **BAC #4**, AC6 Flag Tag A10 - **BAC #5**, and AC6 His tag combined elution - **BAC #6**. Protein purity and concentration varies between samples and is unadjusted.

Conditions: Conditions remain unchanged.

Results: Summarized in **Table 12**.

5.2.8 BAC Assay Set Eight:

Proteins Used: Flag-Tag A3 (**BAC #4**) diluted to 5% of original concentration **BAC #7**.

Aim: To validate the assay it will be necessary to complete an ATP concentration curve so that an appropriate concentration of ATP will be used in the assays. This will need to be repeated once protein is finalized, however I wanted to run a test set of assays to get a range of appropriate ATP concentrations to use and a practice of the experiment.

Conditions: Conditions remain unchanged. ATP concentrations range from 0.001 to 1 mM, each sample run in duplicate.

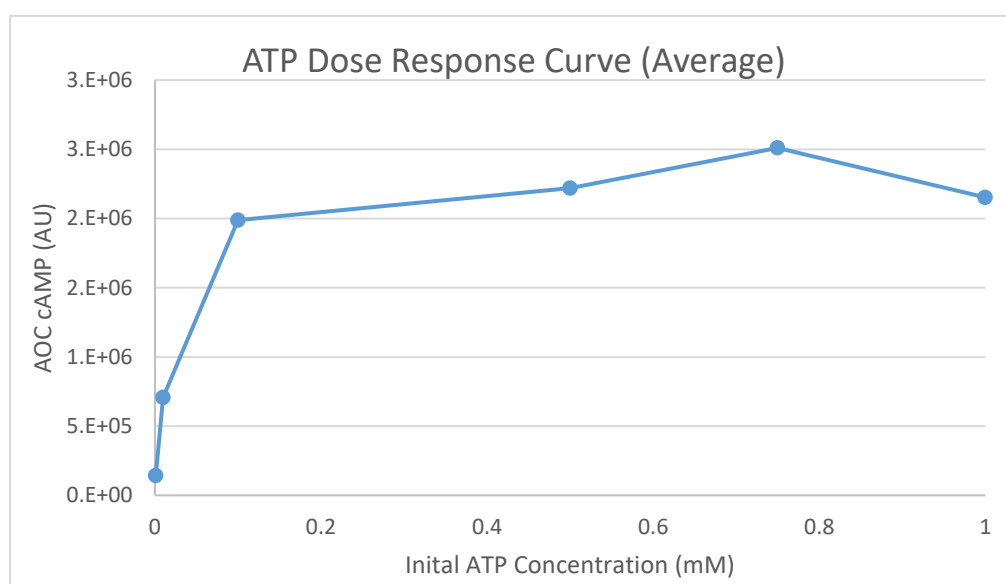


Figure 28. Experimental results from assay set eight, a dose response curve of ATP concentration against the area of the curve of cAMP, although the protein to be used is not finalised this experiment was done as a test run, to highlight any issues.

Results: cAMP production appears to level out at 0.1 mM ATP, experiments were repeated in duplicate, to give a dose response curve. This will be repeated once protein sample, concentration and purity are finalised.

5.2.9 BAC Assay Set Nine:

Protein Used: A3 - **BAC #4** further diluted to 0.5% of initial concentration **BAC #8** and AC6 His-tag combined elution **BAC #6**. **Gas #3** was used.

Aim: BAC #4 was diluted to bring AC activity into acceptable range and determine activity in the presence of Gas or forskolin to see if activity responds appropriately. Test increased concentrations of forskolin to see if higher concentrations affect activity.

Conditions: Reagents and conditions were kept consistent. These experiments were run as two sets of assays 1-4 and 5-12, in assays 5-12 forskolin final concentration was increased to 100 μ M. Experiments 1 and 3 were repeated, (9 and 11 respectively), to ensure consistent results and consistency between same day different assay runs.

	#	Forskolin	Gas	AOC #1	AOC #2
Protein #8	1	-	-	128,890	119,292
	2	+	-	135,105	104,834
	3	-	+	185,377	202,508
	4	+	+	196,781	197,370
	9	-	-	116,754	117,460
	10	+(10x)	-	113,599	110,711
	11	-	+	205,667	202,620
	12	+(10x)	+	212,899	217,952
	#	Forskolin	Gas	AOC #1	AOC #2
Protein #6	5	-	-	53,416	53,628
	6	+* (10x)	-	51,495	55,452
	7	-	+	73,255	72,581
	8	+*	+	59,739	75,195

Table 11. Assay results from BAC assay set nine. Protein used #6 and #8. Same day repeat experiments also carried out to determine consistency between results.

Discussion: The same results are observed, with Gas approximately doubling cAMP production in protein 5 and increasing by around 50% in protein 3. Forskolin did not further increase turnover. Increasing forskolin concentration also reduced turnover again. Repeat experiments in same-day assay sets were within 5% of each other.

5.2.10 BAC Assay Set Ten:

Results: Summarized in Table 12.

5.2.11 BAC Assay Set Eleven:

Protein Used: AC6-soluble His-tagged BAC #9, Gas #3 was used.

Aim: Carry out a set of assays in triplicate, calculate standard deviation and % difference between samples. Use NKH477 to confirm lack of binding at the forskolin bind site. Add 2 μ L DMSO as a blank to a standard assay sample, observe how it influences cAMP production.

Conditions: Full suite of experiments to determine activity in triplicate. Run with higher pH Tris buffer (50 mM, pH 8.5), this greatly increases turnover, cannot be compared to previous results directly.

Results: Summarized in **Table 12**.

#	Protein Used	Conditions Tested	Assay Results	Conclusions
1	Flag tagged-AC6 first production - BAC#1	Determine if AC protein is functional.	G α s + Forskolin increase activity, forskolin alone untested.	Repeat experiments to determine if forskolin sensitive.
2	Flag tagged – AC6 first production purified BAC#1	Is AC6 activated independently by forskolin?	Introduction of forskolin decreased activity.	Protein appears to be forskolin insensitive.
3	Flag tagged – AC6 second production BAC#2	Is AC6 activated independently by forskolin?	New G α s. Insignificant increase in turnover with all conditions.	Lower turnover than expected
4	Nine different fractions from IEX purification	Standard condition (w/ forskolin) all proteins, duplicate.	Fraction activity varies widely.	AC activities used to determine which fractions are active.
5	BAC#3	Time point experiments (0 min – 20 min)	cAMP increase is close to linear, successful test run.	Stopping condition doesn't completely stop activity.
6	BAC#3	Stopping Conditions	Formic acid (1%) or HCl (0.2 M) appears to prevent enzyme activity.	Use 1% formic acid as the stopping condition.
7	Flag A3 - BAC#4 Flag A10 - BAC#5 His - BAC#6 Flag A3 5% - BAC#7	Check protein activity, [ATP] curve	Turnover high, samples with/without forskolin small difference in activity.	Dilute protein concentration and repeat experiments.
8	BAC#7 G α s 3 – insoluble fraction refolded G α s 4 – Purified G α s	Testing fresh G α s, $\pm$ forskolin.	G α s 3 increases turnover, G α s 4, does not, at several different volumes. At highest volume cause significant decrease in turnover.	Forskolin does not increase turnover independently. G α s solution composition potentially reducing activity.
9	BAC#8, BAC#6	Same as before.	Same as before.	Same as before. Try forskolin derivative as an activator.
10	AC6 his soluble - BAC#9 AC6 his insol - BAC#10 BAC#11 AC6 flag refolded	Test three new proteins for activity.	Turnover increased insignificantly by G α s, forskolin causes an insignificant increase.	Protein doesn't appear to be functioning as expected. NKH477 activity similar to forskolin.
11	BAC #9	DMSO only, NKH477, [Fsk] 10x, triplicate. Denatured protein.	NKH477 higher turnover than fsk + G α s but still very low. Fsk, DMSO, fsk + G α s all give similar turnover.	Deviation of triplicate results less than 5%, well within acceptable limits. Production of cAMP is from AC6.

Table 12. Summary of all bacterial AC6 assay results.

5.3 Conclusion

The assay method is complete; it is possible to measure cAMP production from AC6-purified *E. coli*. This method is quick and relatively cheap, it is adaptable to other experimental conditions and cell permeability/solubility of compounds will not significantly affect results. Once a method to consistently produce functional AC is completed, it will be possible to produce several different isoforms by purchasing appropriate plasmids, then repeating expression and purification steps.

Due to the construct used, the allosteric binding site for forskolin is not properly folded, a new plasmid is being purchased with alterations that are expected to produce a functional protein.

Unfortunately, the AC6 construct that is currently being used does not produce functional AC6, as it is insensitive to forskolin. The two cytoplasmic domains may be sufficiently close together when forskolin binds, causing no impact on activity. In some cases, there was a decrease in activity in the presence of forskolin, it is possible that forskolin binding reduces activity as it prevents the cytoplasmic domains from reaching an ideal conformation. This inconsistency between results suggests multiple ways for the protein to fold; forskolin appears to bind and decrease activity. This decrease suggests that it prevents the two cytoplasmic domains from reaching an optimum conformation or prevents ATP from entering the binding site as they are adjacent.

However, all the steps have been completed to carry out these assays. To create a functional enzyme, a new construct of adenylyl cyclase should be created, with a short linker between the two cytoplasmic domains, between 12 and 36 residues. This modification will allow the two cytoplasmic domains to sit in a more natural conformation and hopefully be activated by both $G\alpha_s$ and forskolin. Once functional testing of forskolin derivatives and examination of AC activity will be completed.

This plasmid has been purchased and work to produce and purify the protein has begun. Once the protein is purified, its functionality can be tested, and the validation experiments are completed.

Once prepared it will be possible to determine the activity of each derivative when compared to forskolin in adenylyl cyclase 6. Different isoforms can then be tested to determine isoform specificity. These experiments will form the initial assessment of our interest in a particular derivative; many minor alterations to forskolin's structure can cause activity to decrease or be eliminated. The in vivo assays to assess activity use contracted smooth muscle tissue in a porcine model, meaning that it is not practical to test the activity of all molecules with this system, especially those expected to perform poorly.

Chapter Six – Design and Preparation of HEK-Produced AC6 Enzyme Activation Assay

6.1 Adenylyl Cyclase 6 Produced in HEK293 Activation Assays

This section describes the assays completed on adenylyl cyclase 6 produced in HEK293 cells (HEKAC6), all assays use an impure lysate with AC being a low percentage of total protein content.

The quantity of protein available was limited as it was produced in a collaborators laboratory and this work could not be completed in the Sorensen laboratory, reducing the number of experimental conditions and replicates run. This protein is the same as the ones used in the homogeneous time-resolved fluorescence (HTRF) based cAMP kit, method described in Chapter 2 and assay results shown in Chapter 7.

Several different protein isoforms (AC5, AC6 and AC7) were used in generating a functional assay, all protein samples are unpurified protein lysate from either wild type HEK293 cells (WTHEK), HEK293 over expressed with adenylyl cyclase isoforms will be labelled as HEKAC5, HEKAC6 and HEKAC7. Total protein concentration was standardized to approximately 0.5 mg/mL unless otherwise specified.

HEKAC assay results are analyzed by the area of the curve (AOC) of cAMP, this measure is based on UV absorption during HPLC analysis. For publication results will be converted from AOC to moles of cAMP produced, moles per protein (g) or % activation compared to that of forskolin, these are the most commonly used units in the literature. However, cAMP AOC is linear with concentration, so this step will not be taken until results are more consistent, and the method is ready. Calibration curve found in Chapter 2 (**Figure 16**).

Results between assay runs are difficult to compare as conditions and reagents were consistently changed to look for an ideal set of conditions, and a variety of proteins were used due to low volumes/concentrations of protein available. In some assays AC5 and AC7 were substituted for AC6 when the type of protein would not affect the aim/results of the assay set.

A summary of the results from these assays can be found in **Table 22**.

6.1.1 Method Development Aims

To design an assay that can measure the production of cAMP by adenylyl cyclase from ATP, this method needs to be consistent in the quantity of cAMP produced. Adenylyl cyclase should be activated by forskolin and G α s, this can then be used to determine how well forskolin derivatives activate AC6. This assay will require an ATP regeneration system as unpurified lysate is being used. This prevents degradation of ATP to adenosine which in turn inhibits adenylyl cyclase activity. This is also important to prevent reduction of cAMP production due to decreased availability of ATP.

Once a method is established it will be possible to study the effects of ATP, forskolin and G α s concentration on activity, with dose response curves. This will be done alongside a number of validation experiments, ensuring results are consistent across multiple runs, productions of protein and days of experimentation.

6.2 Method Development

The original assay method was adapted from the published method shown below.

The formation of [32 P]-cAMP from [α - 32 P] ATP was measured. The 100 μ L reaction mixture contained: Tris-HCl (pH 8.5), 25 mM; MgCl $_2$, 10 mM; [α - 32 P] ATP, 1 mM; cAMP, 1 mM; dithiothreitol (DTT), 1 mM; phosphocreatine, 5 mM; phosphocreatine kinase, 50 units; bovine serum albumin, 100 μ g; and enzyme. The reaction was initiated by the addition of enzyme and samples were incubated at 37 °C for 30 minutes. The reaction was halted by the addition of 100 μ L 2% SDS solution¹¹⁴.

As the method of detection was changed from measuring radioactivity produced by [32 P]-cAMP to UV detection by HPLC there was no need to use labelled ATP, which was replaced with unlabeled ATP. The addition of unlabeled cAMP to the literature assay would have no effect on results as it would not be detected, however it would be by HPLC, so it was removed. The cAMP is added to occupy phosphodiesterase enzyme binding sites, it was replaced with IBMX. The

addition of DTT inhibits enzyme activity that breaks down ATP into ADP and AMP. Phosphocreatine and phosphocreatine kinase regenerate ADP into ATP. The assay will be stopped with heat and centrifugation, this was later changed to addition of acid and centrifugation, increasing the volume of the solution with a stopping solution decreases overall cAMP concentration reducing the limit of detection.

Once it is confirmed that the enzymes are functional and a standard assay condition is found the ATP regeneration system will be introduced and tested. Before this addition there will be increased variation in cAMP production between replicates.

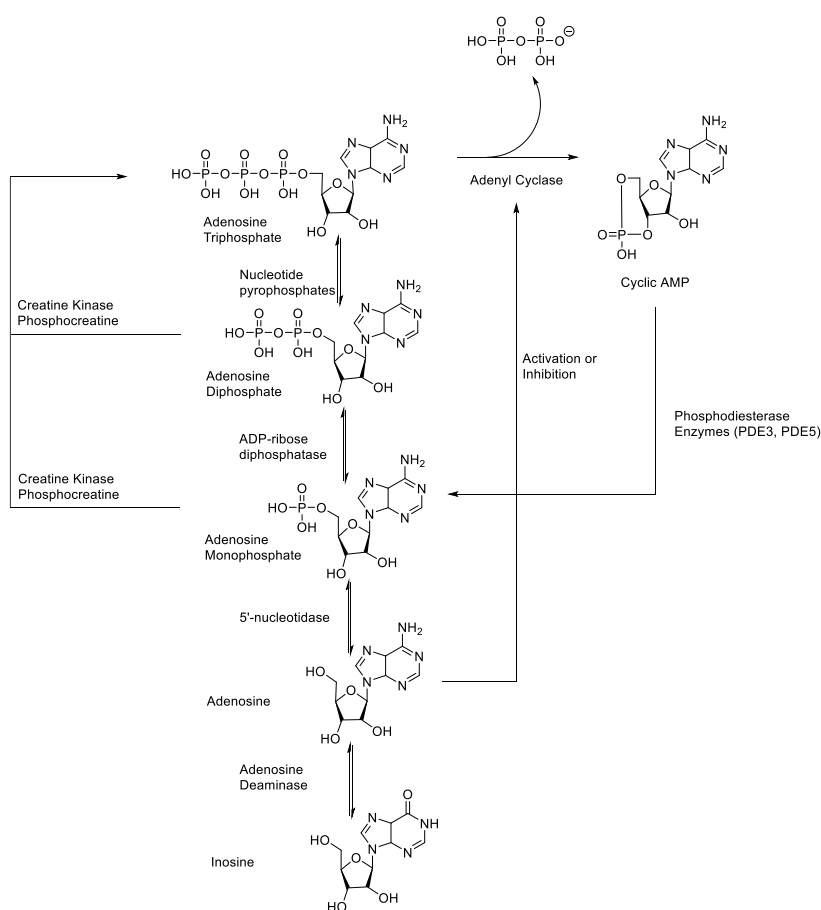


Figure 29 . Enzyme induced degradation pathway of ATP to adenosine and inosine, and hydrolysis of cAMP to AMP. Conversion of ATP to ADP can be reversed with the introduction of creatine kinase, and phosphodiesterase enzymes prevent cAMP degradation. These enzymes can be introduced to the assay to ensure consistent measurements of cAMP levels can be taken.

HPLC chromatogram that has similar retention to cAMP, making it more difficult to quantify.

6.2.1 ATP Regeneration

As this assay method uses impure protein there will be a number of active proteins present in the lysate. Enzymes of importance are mostly phosphodiesterase, enzymes that hydrolyze bonds between phosphate groups. This generates a number of different adenosine derivatives, ATP undergoing hydrolyses to ADP, then AMP, adenosine and finally inosine. The presence of adenosine causes issues for a number of reasons, the first being an additional peak in the

ATP undergoing reactions other than conversion to cAMP also decreases ATP concentration in solution, potentially reducing production of cAMP. Finally adenosine acts as a p-site inhibitor, directly inhibiting AC activity or indirectly by inhibitory or stimulatory receptors¹⁶⁴.

To prevent the generation of adenosine regeneration of ATP from ADP (or ADP from AMP), with creatine kinase and phosphocreatine (CK and PC) is used. Creatine kinase can reversibly transfer a phosphate group from phosphocreatine to ADP, forming ATP and creatine. An alternative is the use of pyruvate kinase and phosphoenolpyruvate, to complete the transfer of PP_i through the same mechanism from phosphoenolpyruvate to ADP. These systems cannot however regenerate AMP from adenosine, as the 5-nucleotidase step is not reversible.

6.3 Method Development Assay Results

6.3.1 HEKAC Assay Set One

Aim: The aim of this assay was to test for production of cAMP by AC6 produced in HEK cells. This included $\pm$ forskolin and samples containing no ATP to confirm production of cAMP is due to added ATP. Samples $\pm$ IBMX, and several different protein concentrations as the amount of cAMP turnover is currently unknown and will ideally remain below 10%.

Assay Conditions ([Stock], volume, [Final]): Protein: 50 μ L, MgCl₂: 20 mM, 100 μ L, 2 mM, ATP: 15 mM, 100 μ L, 1.5 mM, Forskolin: 1mM, 1 μ L, 10 μ M, IBMX: 1 M, 1 μ L, 1 mM, total volume): 1000 μ L. Run time: 10 minutes. Temperature: 37 °C. See Chapter 2 for finalised method.

Discussion: Separation of ATP, cAMP, ADP and AMP was achieved with standards, however the retention times of cAMP and shift slightly with assay samples. It is necessary to run assay samples to check for separation of cAMP and adenosine.

In this initial assay no turnover from ATP to cAMP observed. This may be due to low enzyme concentration or low enzyme activity.

In the next set of assays ATP concentration was decreased, as there was a small but noticeable run over observed in the HPLC blank injections. The volume of protein added to each assay will also be increased and duration of assay changed to 15 minutes to allow for more turn over. At

this point G α s will not be included as the protein had not been produced yet, G α s will be added once a base set of conditions are found.

HPLC Method:

Solvent A: methanol (100%), Solvent B: Miliq w/ phosphoric acid (430 μ M), Solvent C: KH₂PO₄ (80 mM, pH 6.0).

Time (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0.0	1.2	0	0	100
2.0	1.2	0	0	100
10.0	1.2	10	0	90
10.3	1.2	10	90	0
12.0	1.2	20	80	0
15.0	1.5	9	91	0
15.3	1.5	8	0	92
17.0	1.5	0	0	100
18.0	1.2	0	0	100
20.0	1.2	0	0	100

Table 13. The HPLC gradient for initial analysis of assay samples.

6.3.2 HEKAC Assay Set Two

Aim: Run a small set of assays to test if AC6 produced in HEK cells is active. This included samples spiked with cAMP, this is due to potential degradation of cAMP, it is currently unknown if in the previous assay samples, no cAMP was produced, or cAMP was produced but then hydrolyzed to AMP or consumed in another process.

Conditions: Protein (volume, μ L): 100-150. MgCl₂ (sol mM, volume, final mM): 20 mM, 50 μ L, 2 mM. **ATP ([stock] mM, volume, [final] mM): 15 mM, 5 μ L, 0.15 mM.** Fsk ([stock] mM, volume, [final] mM): 1mM, 2 μ L, 40 μ M. IBMX ([stock] mM, volume, [final] mM): 1 M, 1 μ L, 2 mM. **Total volume (μ L): 500** (250 added post assay).

The length of assay was increased to 15 minutes, the tris buffer changed to 100 mM pH 8.5, ATP concentration decreased tenfold to 0.15 mM, and the total volume of assay was reduced to 500 μ L to conserve protein. A pH of 8.5 has been observed as optimal for cAMP production by AC¹¹⁴, this may not always be the case but should provide increased turnover compared to 7.4. The pH

will be reduced to 7.4 in the future, initially it is more important to establish the protein is functional then refining optimum conditions.

As no cAMP was observed in previous experiment cAMP was added to several samples before the assay was started. Also, the retention time of cAMP is different in standard samples compared to assay samples, cAMP spikes were added to several samples, to confirm identity of cAMP peak. Added cAMP concentration was 0.1 mg/mL.

Assay samples will also be run without protein and other co-factors to ensure that production of cAMP is from the AC6 protein oppose to other mechanisms.

#	AC6	ATP	Forskolin	cAMP	AOC #1	AOC #2
1	+	+	+	-	322378	405494
2	+	+	+	+	959982 [285982]	1059828 [385828]
3	+ (x1.5)	+	+	-	637279	-
4	-	+	+	+	647953	677392
5	-	-		+	674254	-

Table 14. Assay results from HEK Assay Set Two. cAMP has been produced in quantifiable concentrations, cAMP spikes affected cAMP concentration as expected.

Discussion: Several of the experimental conditions were run in duplicate to determine consistency between samples. The consistency was poor (>10%) for some samples, this is an important issue to keep track of, it will be monitored as conditions are changed and new reagents introduced.

cAMP spike of 0.1 mg/mL as a standard equates to an AOC of 674,000. For spiked sample #2 adjusted cAMP concentrations are listed in square brackets. Due to the variation in results it is difficult to tell if the values vary as much as they do because of degradation of cAMP, but they are within the expected range, when compared to #1. cAMP is produced in all samples that were expected to produce cAMP, cAMP produced in the first assay set may have been too low to detect (>0.01% ATP turnover).

Assay samples run without protein and spiked with cAMP showed similar values, and no cAMP was produced in unspiked samples, confirming that any cAMP produced is due to enzyme activity within the protein sample.

The poor consistency between duplicates is most likely due to the impure lysate, with a lack of ATP regeneration system, this should improve reliability between results once finalized.

6.3.3 HEKAC Assay Set Three

Aim: To determine if HEK cells overexpressed with AC6 have higher rate of cAMP production compared to wild-type HEK cells (WTHEK). These cells have no overexpressed proteins so can act as a control comparison. HEKAC6 should contain significantly more AC, therefore producing more cAMP under the same conditions, while protein concentrations were different they are approximately 0.5 mg/mL. Various protein concentrations were used to determine if less protein could be used per assay.

Conditions: Unchanged

Discussion: AC6 overexpressed is producing significantly more cAMP than WTHEK cells at all concentrations, this suggests a higher concentration of AC present within the overexpressed lysate.

Results: The protein concentration appears acceptable at 100 µL in a 500 µL assay for the time being, this may be adjusted later as conditions and aims are altered. A reduced protein or total volume would be preferred.

#	Protein (µL)	Forskolin	AOC #1
1	AC6 (100)	+	462548
2	AC6 (110)	+	486182
3	AC6 (120)	+	433779
4	AC6 (130)	+	445915
5	AC6 (140)	+	678799
6	AC6 (150)	+	551620
7	HEK (100)	+	146633
8	HEK (120)	+	198974
9	HEK (150)	+	182508

Table 15. Assay results from Assay Set Three. 100 µL protein is an acceptable amount to use, this will reduce overall protein use. HEK cells overexpressed with AC6 produce significantly more cAMP than WTHEK cells.

6.3.4 HEKAC Assay Set Four

This set of assays used AC7 due to limited quantity of protein available.

Aim: A range of ATP concentrations was examined (0.12, 0.24 and 0.36 mM). The pH was reverted to 7.4, as this would be the most representative pH for study. Blank injections of IBMX were run to check if they were causing unknown peaks in the HPLC chromatograms. The forskolin concentration was also decreased to be the literature standard assay concentration (10 μ M). Sodium fluoride was also introduced as a potential phosphodiesterase inhibitor.

This was the first assay set to use acid as a stopping condition rather than heat. Access to heat block is limited due to being in another laboratory. This method was also found not to eliminate all activity in the bacterially produced AC assays, this was determined by rerunning HPLC analysis of assay samples over several hours. Alternate methods for stopping assays include adding acid instead, followed by centrifuging. Formic acid denatures enzymes, prevents further turnover of ATP to cAMP and prevents degradation of cAMP. 5% formic acid (50 μ L) was added to samples to give a final concentration of 1%, samples are then inverted 5 times to mix and centrifuged at 14,000 g for four minutes.

#	[ATP] mM	Forskolin	AOC #1	AOC #2
1	0.12	+	189,115	200,200
2	0.24	+	258,731	277,867
3	0.36	+	358,491	321,965
4	IBMX Inj	-	0	0

Table 16. Assay results from HEK Assay Set Four, increasing [ATP] increased cAMP production. Further increased [ATP] will be tested.

concentration is likely to change and continue to be experimented with until the assay conditions and protein are finalized.

Discussion: Increasing the quantity of ATP increased turnover to cAMP, for future experiments ATP concentration will be increased, to 0.75 mM double the maximum concentration used in this assay, this

NaF had no impact on duplicate consistency, introduction of NaF will be explored further when more assay conditions can be run. I expect that NaF's role may already be filled by IBMX as this also inhibits inhibition of cAMP hydrolysis.

The IBMX blank did not have a peak in the chromatogram. It was decided to stop running every experiment in duplicate for time being, running in duplicate gives too few experimental conditions, when required conditions will still be carried out in duplicate.

6.3.5 HEKAC Assay Set Five

Aim: To study the impact of introducing sodium fluoride (NaF) and phosphocreatine (PC) to assays on both HEKAC6 and WTHEK. IBMX has already been included to prevent the hydrolysis of cAMP to AMP, however addition of NaF has been observed to have similar effects. In place of an ATP regeneration system previous studies (source) have included phosphocreatine alone, as the required creatine kinase enzymes may be present in the impure lysate. This will allow for PC to phosphorylate ADP to ATP without the addition of another enzyme to the assay. Protein concentration was also reduced to increase the number of conditions run.

Conditions: Unchanged.

Results: IBMX already acts as a phosphodiesterase inhibitor, introduction of NaF unnecessary. Phosphocreatine alone does not prevent production of adenosine.

6.3.6 HEKAC Assay Set Six

Aim: To test protein activity with and without forskolin over a sixty-minute time period. Experiments not duplicated.

Conditions: Unchanged

Discussion: There is a steady and consistent increase in cAMP in samples containing forskolin, equating to an approximate 300,000 increase in cAMP AOC every 15 minutes. Further time points, for example 75 minutes, would not show an increase in cAMP as by 60 minutes almost all ATP present had been consumed, or converted into ADP or AMP. These results are close to linear, allowing for adjustment of the assay length if need be, coupled with an increase in ATP concentration and adjusting protein concentration would allow for variation in the length of experiment if needed. The same increase is not observed in samples without forskolin, which

only increases a small amount after the initial 15 minutes. This was confirmed to be from the assay with a protein injection without ATP, there was no cAMP present at the beginning or end of the assay.

cAMP concentration was quantified by comparison to calibration curve and converted to cAMP produced in μM .

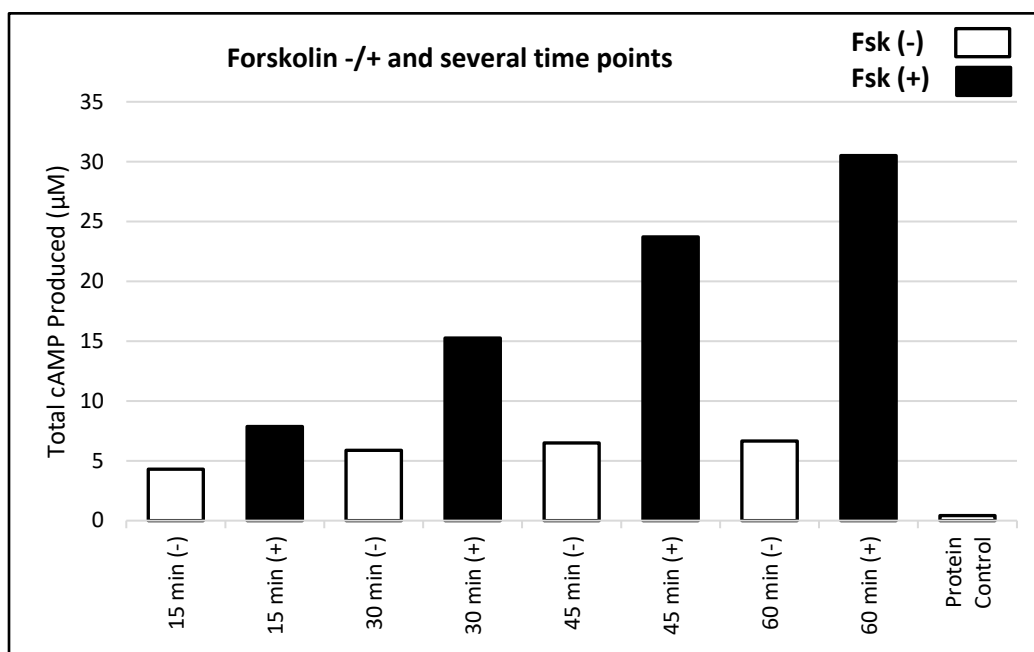


Figure 30. Tabulated assay results from Assay Set Six. When forskolin is added cAMP production increases almost linearly until 60 minutes. Samples without forskolin produce little additional cAMP after the initial fifteen minutes. If needed the assay length can be increased.

6.3.7 HEKAC Assay Set Seven

Protein used: AC6HEK and WTHEK.

Aim: Analyze forskolin activity against two forskolin derivatives, NKH477 (**26**), well known strong activator of AC5, and expected to activate AC6 well and 1-TBDMS-7-deacyl forskolin (**7**), due to loss of H-bonding at the 1-position and removal of the acetyl group at the 7-position, this derivative is expected to have very low activity. 1-TBDMS-7-deacyl forskolin was used as a negative control, the activity should be similar to assays without forskolin. This should allow

determination of the sensitivity of the assay, if forskolin and NKH477 have similar activity it may be difficult to acquire an accurate representation of how well a derivative activates AC6.

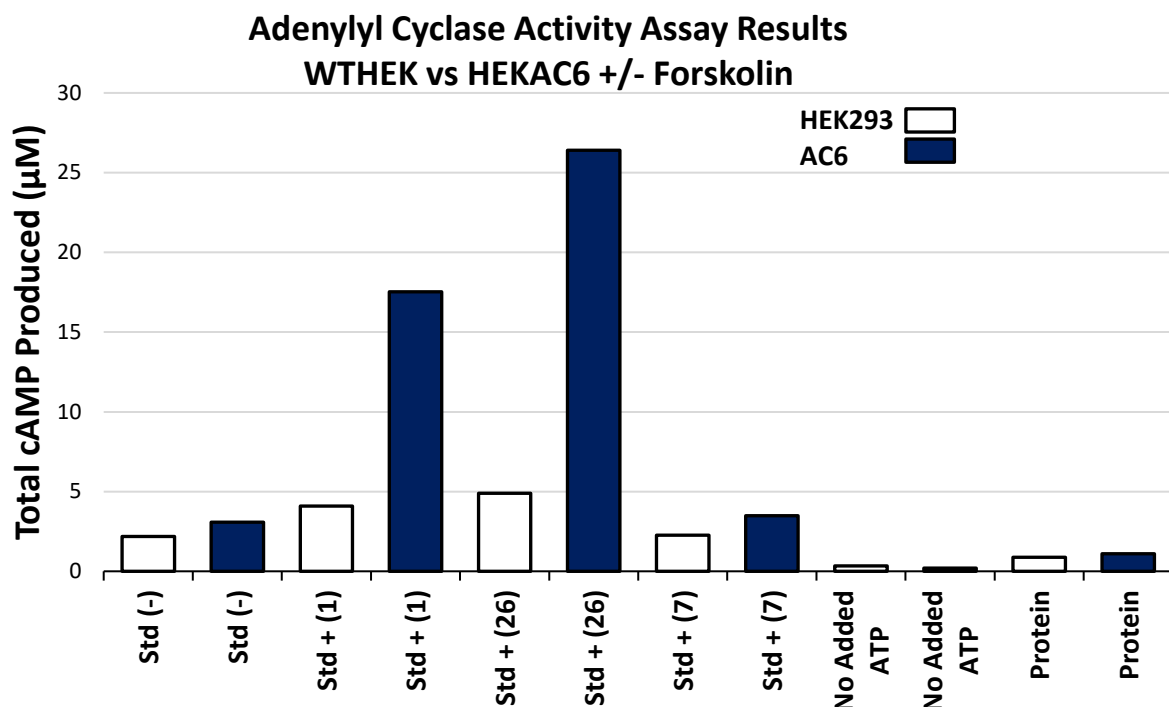


Figure 32. Graph comparing cAMP produced by HEKAC6 and HEKWT when forskolin and several forskolin derivatives are introduced, the results are as expected, forskolin **(1)** shows a significant increase in cAMP production. **(26)** Shows a larger increase in cAMP, while **(7)** had almost no impact on activity.

Discussion: In **Figure 32** activity of AC6 (blue) and WTHEK (white) are compared under various conditions. The standard assays show similar levels of activity, with a slight increase in cAMP production

in the AC overexpressed cells, this is expected as basal AC turnover is low. This is increased almost 6-fold in the AC6HEK, with a small increase in WTHEK in the presence of forskolin. Turnover is further increased in the presence of NKH477 **(26)**, as expected. An insignificant increase is observed when **(7)** was added, increasing cAMP production to 3.4 μM from 3.0 μM

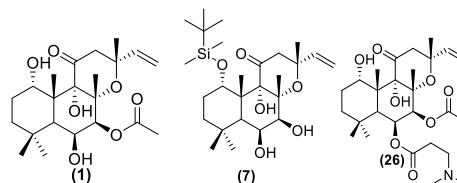


Figure 31. Structures of relevant compounds, **(1)**, **(7)**, **(26)**, being forskolin, NKH477 and 1-TBDMS-7-deacylforskolin respectively.

in the assay containing no derivative. When no ATP is added to the assays cAMP presence is close to zero.

6.3.8 HEKAC Assay Set Eight

Aim: The aim of these experiments was to incorporate an ATP regeneration system to counteract the production of ADP, AMP and adenosine. Phosphocreatine has previously been added, alone, in this set of assays creatine kinase will be introduced. The total volume of each assay was also reduced to 100 μ L.

Conditions: Protein: 10 μ L, $MgCl_2$ 10 mM. ATP 1 mM. Fsk 10 μ M. IBMX 1 mM. Phosphocreatine: 2 mM. DTT: 1 mM. Creatine Kinase: 5 units. Total volume: 100 μ L (50 added post assay).

Assay solutions were made up to 100 μ L with Tris buffer instead of 500 μ L. This reduced protein required for each assay, allowing for more conditions to be run. Decreasing the volume will increase the margin of error, and consistency between duplicates will be more difficult to maintain. Total recovery HPLC vials were used, as this was the cause of limitation of assay volume. Even though needle draws from bottom of vial issues start to arise using less than 750 μ L sample in a HPLC vial. Experiments were complete in triplicate.

Results: Results are summarized in the table below, phosphocreatine and creatine kinase effectively remove the adenosine peak, **Table 17**, when compared to assays only containing one of the reagents or none. Separation between the peaks still needs to be improved as a small quantity of adenosine may still be produced and it is difficult to observe. If the peak is small enough it will merge into the cAMP peak, increasing AOC of cAMP.

When IBMX is removed from the assay there is a significant decrease in cAMP and an increase in the baseline, with several new peaks present, IBMX will continue to be included in all assay conditions. DTT had an insignificant effect on cAMP produced, it does not appear to have an effect on activity.

As the HPLC runs progressed there was a buildup of impurity at the 15-16-minute mark, this obscured the cAMP peak, preventing accurate reading of results of later samples. This made the

results of samples later in the run unusable. Due to the final sample volume being 150 μ L these samples cannot be rerun. This impurity has not been observed before, and the cause is currently undetermined main suspect is ATP or improper sample injection.

AC6	IBMX	DTT	PC	Ckin	[cAMP]	Impact on [cAMP]
+	+	+	+	+	++	-
+	+	-	+	+	++	DTT slight decrease
+	-	+	+	+	+	IBMX significant increase
+	+	+	-	+	+	Decrease
+	+	+	+	-	+	Decrease
+	+	+	-	-	+	Decrease

Table 17. Results from Assay Set eight. The presence of DTT decreases final cAMP concentration, while IBMX significantly increases it. Removing either component of the ATP regeneration system (PC and CKin) decreases cAMP production.

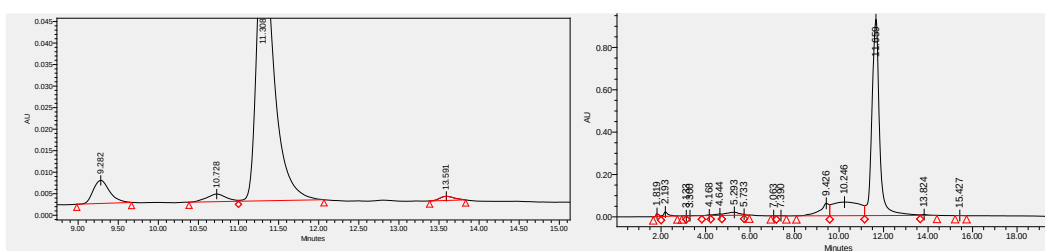


Figure 33. Chromatogram (A), a normal assay sample, ATP 11.3, cAMP 13.8. Chromatogram (B) Assay sample with large unknown peak at 10.2 mins, ATP shifts, causing it to overlap with the cAMP peak, preventing quantification.

Discussion: As this assay set used smaller protein volumes it was the largest set run so far. Issues that arose from this were mostly the increasing HPLC baseline, to prevent this from occurring again an additional blank injection will be repeated after ~5 assay samples. Alternatively, a wash method may be introduced and used repeatedly throughout a long assay HPLC run.

IBMX has been consistently included as a phosphodiesterase inhibitor, but the impact of it has yet to be tested, in this set of assays it is shown to be an essential component. When not included there is a significant decrease in the quantity of cAMP present at the end of each assay. By preventing the degradation of cAMP to AMP a more accurate result will be recorded, while decreasing inconsistency between samples.

DTT appears to have a negligible effect on production of cAMP, if the presence of other enzymes causes issues it may be reintroduced, but has been removed for the time being.

Creatine kinase regenerates ATP from ADP and AMP, this reduced the presence of adenosine in final samples, and both creatine kinase and phosphocreatine are required for this. Both will be included from this point onwards with any assays that are using unpurified protein, to counteract the effect of other enzymes present in solution.

The volume of each assay sample will be increased, this will decrease the effect of errors while still minimizing total protein used, this will also allow for rerunning of samples. This is important as sometime issues with the HPLC prevent accurate reading of results, also previous sample sets have been stopped due to power outages, and therefore being able to rerun samples is beneficial.

6.3.9 HEKAC Assay Set Nine

AC5HEK was used for this assay set.

Aim: The aim of this assay set was to increase volumes of assay to 200 μ L, and to test a range of $MgCl_2$ and ATP concentrations.

Conditions: Protein 20 μ L, $MgCl_2$ 10 mM, ATP 1 mM, Fsk 10 μ M, IBMX 1 mM, Phosphocreatine 2 mM, Creatine Kinase 5 units, Total volume 200 μ L (50 added post assay). Total volume of assay increased to 200 μ L. HPLC Method changed.

Results: Summarized in **Table 22**. Degradation of protein appears to have occurred.

HPLC Method: The method was altered to remove the Miliq water with acid modifier, this was an optimized method after adjustment of retention times, concentrations of buffer and pH of buffer. Increasing buffer concentration to 100 mM had no noticeable effect on retention, while decreasing concentration

Time (mins)	Flow rate (mL/min)	Solvent A (%)	Solvent C (%)
0.0	1.2	0	100
2.0	1.2	0	100
14.0	1.2	10	90
16.0	1.5	10	90
17.0	1.5	0	100
19.0	1.2	0	100
20.0	1.2	0	100

Table 18. Adjustments to the HPLC method to prevent impurity peak matching that of cAMP.

increased peak width, this while increasing the likelihood that precipitation of salts would occur. Increasing the pH from 6.0 to 6.5 improved separation of cAMP and adenosine. By altering the ratios of solvents separation of cAMP and adenosine was improved, however as separation improved both peaks started to broaden, to the point where the peak began to split. Buffer A: Methanol (100%). Buffer C: KH_2PO_4 (80 mM pH 6.5).

6.3.10 HEKAC Assay Set Ten

AC5HEK was used for this assay set.

Aim: Determine how varying ATP and creatine kinase concentrations affect turnover. An ATP concentration curve will be constructed from the results. An assay sample containing 50 μM forskolin (5 times standard) is also included to determine how that affects cAMP production.

Conditions: ATP concentrations between 0.5 mM and 1.5 mM were tested in duplicate, CK protein concentration ranged from 1 unit to 7 units. Other conditions unadjusted.

Results: Summarized in Table 22.

6.3.11 HEKAC Assay Set Eleven

AC6HEK was used for this set of assays.

Aim: The aim of these assays was to run an ATP concentration curve with reduced concentrations and a new HPLC method. This method achieves separation between ATP, ADP, AMP, adenosine and cAMP with standards, the method needs to be tested with assay samples.

Conditions: A variety of ATP concentrations were used from 0.01 mM to 0.75 mM, ideally higher concentrations of ATP would be used but this caused issues with HPLC column overload, so the ATP concentration is limited by practicality to 0.75 – 1.0 mM.

A new HPLC method was tested and is described below.

#	[ATP] mM	AOC #1	AOC #2
1	0.01	7611	0
2	0.05	26805	26104
3	0.1	44011	37573
4	0.25	47831	50036
5	0.5	53841	52969
6	0.75	49851	49995

Table 19. Assay results from HEK Assay Set Eleven. Examines cAMP production in the presence of various ATP concentrations.

Several matrix samples were also run, these samples contain only protein or protein and cAMP to show that the protein sample does not cause interference with detection of cAMP. Samples of this type will be run for multiple protein preparations as this is a requirement for validation of an assay.

Discussion: cAMP concentration does not increase greatly above a concentration of 0.25 mM ATP, with this method [ATP] at 1 mM or higher interferes with the cAMP peak, by the tailing of the ATP peak merging with the cAMP peak. The new HPLC method appears to be successful, cAMP is sufficiently separated from other peaks for quantification, column pressure remains acceptable

throughout run, and chromatograms are consistent, with little change in retention time. An attempt will be made to increase the separation

between cAMP and ATP, as this may

reduce issues if the ATP concentration needs to be increased or there is an increase in chromatogram variation.

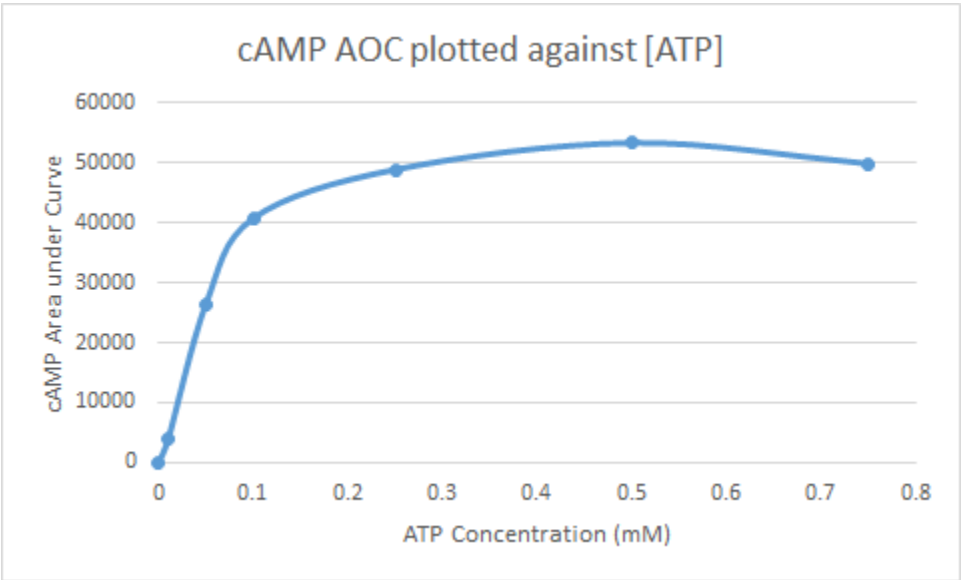


Figure 34. cAMP area under the curve plotted against ATP concentration to give a dose-response curve.

Time (mins)	A (%)	B (%)
0.0	100	0
3.0	70	30
7.5	20	80
10.0	0	100
13.0	0	100
14.0	100	0
15.0	100	0

Table 20. New HPLC method gradient throughout a run.

HPLC Method: The HPLC method was changed again due to increase in issues separating cAMP from adenosine, combined with the presence of the peak without a known source, observed in previous runs after multiple samples.

Buffer A: Potassium phosphate (65 mM, KH₂PO₄ 26 mM, K₂HPO₄ 39 mM TBAHS 4 mM, pH 6.0), Buffer B (65 mM, KH₂PO₄ 26 mM, K₂HPO₄ 39 mM MeOH 25%, pH 6.0). Flow rate set to 1 mL/min throughout the run. Gradient shown in **Table 20**.

Separation between cAMP and adenosine is large, no chance for interfering peaks, however cAMP is now much closer to the ATP peak in retention time. There is a chance for overlap between these peaks, especially when ATP concentration is very high and cAMP is low. The length of the run was increased to 30 minutes; however, separation did not increase in a meaningful way. The method was repeatedly altered and tested with cAMP, ATP and adenosine standards.

Time (mins)	A (%)	B (%)
0.0	100	0
3.0	70	30
7.5	20	80
15.0	5	95
16.0	5	95
18.0	100	0
20.0	100	0

Table 21. Finalized HPLC method gradient throughout a run.

A final method was decided on based on separation of cAMP and ATP, shown in **Table 21**, adjusting the total run time to be 20 minutes.

Separation could be further increased by increasing duration of the step switching the solvent ratios from 20:80 to 5:95, however this increased peak width, increasing separation time but increasing chance of peak tailing and overlap, decreasing the peak quality. This method was settled on for the time being, further adjustments will be made as issues arise.

6.3.12 HEKAC Assay Set Twelve

Aim: Repeat the ATP concentration curve, ranging from 0.001 mM to 0.75 mM, also to ensure that the HPLC method allows for consistent and accurate measurement of cAMP concentrations, as the retention times of standards are slightly different compared to assay samples.

Conditions: No adjustments made from previous runs except HPLC method. Separation of ATP and cAMP resolved, through cleaning of column and adjustment of separation method. ATP concentration curve run with a sample without regeneration of ATP and a sample without forskolin.

Results: No turnover of ATP to cAMP was observed. -80 °C freezer where protein samples were stored lost power and was disposed of, possibility of enzyme function loss due to that, one freeze thaw cycle removes activity.

At this point I decided to stop working on HEK AC assays, to focus on the bacterial assays. This decision was made after had a variety of issues with the HPLC methods and protein function, on several occasions observing no turnover of ATP to cAMP. This was on top of having access to limited quantities of protein due to impracticality of producing sufficient protein in a HEK cell system. The assays carried out with the BAC6 are more promising, with fewer issues arising and an HPLC method that consistently works. Finalized assay method can be found in Chapter 2.

#	Conditions Tested	Assay Results	Conclusions
1	Enzyme Functionality	No cAMP Produced	Enzyme Concentration too Low, Duration too short
2	Enzyme Functionality cAMP Degradation Scale down assay volume	Enzyme produces cAMP, cAMP degradation not cause of no cAMP in previous assay.	Adjust [protein], assay length
3	HEKAC6 vs WTHEK	AC6 overexpressed HEK produce significantly more cAMP than WTHEK	Current protein use acceptable, run duplicate experiments where possible
4	Various [ATP], consistency of duplicates	[cAMP] increases as [ATP] increases	Need to run full [ATP] curve to determine ideal [ATP]
5	NaF and PCR effect on [cAMP], AC6 vs HEK	NaF and PCR's role is filled by IBMX	NaF eliminated, PCR requires creatine kinase
6	AC6 ± Forskolin, time points	cAMP production increases until 60 minutes.	Adjust length of assay as necessary, 15 min works
7	AC6 vs HEK ± select fsk derivatives	NKH477 significantly increases cAMP production, negative control same [cAMP] as -fsk	AC6 functions as expected when different derivatives are tested
8	Regen System + IBMX + DTT, scale down volume	Regen system + IBMX significantly improve [cAMP] DTT decreases [cAMP]	Include regeneration system and IBMX in assays 100 µL too low volume
9	[MgCl ₂] [ATP] ranges, Scale up assay	No results recorded Baseline increase, disrupting cAMP peak,	HPLC method may need replacement if issues continue
10	Effect of [Ckinase], [Fsk], scale up assay	[cAMP] is increased at 3-4U of Ckinase compared to 1U or 8U.	Duplicates all within 5% except one, improved consistency. Ckinase can be reduced from 5U to 3U. 200 µL assay volume from now on
11	New HPLC Method [ATP] 0.01 mM -> 0.75 mM	[cAMP] hits maximum at 0.25 mM ATP, too high [ATP] causes peak interference in HPLC.	Decrease [ATP] to 0.25-0.35 mM to improve HPLC chromatogram without decreasing [cAMP]. Start validation experiments.
12	Use of assay solution, repeating [ATP] curve for publication	No cAMP produced.	Work on BAC6 assay, it is closer to completion and HPLC issues resolved. Better to get one assay working rather than two that almost work.

Table 22. Summary of key assay design experiments, from initial studies determining if the protein is functional to introducing the ATP regeneration system.

6.4 Conclusion

An assay has been adapted for HPLC analysis that can accurately and consistently measure adenylyl cyclase activity by quantification of cAMP production from ATP, once the regeneration system had been introduced assay results were generally within 5% of each other. This assay is suitable for both pure and impure adenylyl cyclase samples, as there is an ATP regeneration system to counter the presence of phosphodiesterase enzymes and IBMX which prevents the degradation of cAMP to AMP. If pure protein is to be used, these components can be eliminated from the assay.

This assay will not be worked on further for the time being, while this assay is sensitive it is not compatible with the quantity of protein produced by the HEK cell lines. Unlike a kit-based cAMP assay the HPLC requires a minimum assay volume of 150-200 μL , due to this it requires a much large quantity of protein to run equivalent assay, that is not practical currently.

When an appropriate amount of protein can be acquired the remaining validation experiments can be repeated or completed. This will be followed by activation assays with 10 μM of each forskolin derivative synthesized. Although this assay uses the sample protein as the HTRF-cAMP kit assay, they have been lysed and therefore cell permeability will not affect a molecules AC activity levels, examining a different condition.

This will allow for rapid comparison of forskolin derivative activity with the HEK produced AC6, when compared to other AC isoforms, this allows for screening of a larger quantity of molecules before further in-depth in vivo experiments are completed. These results can then be compared with the HEKAC assay kit used by our collaborators and computational ligand docking to determine if the derivative is selective for AC6.

Chapter Seven – Derivative Selection, Synthesis and Adenylyl Cyclase Isoform Specific Activation Assay Results

7.1 Designing a Library of Forskolin Derivatives for Specific Activation of AC6

To create a forskolin derivative that selectively activates adenylyl cyclase isoform 6 will require an initial set of compounds to find potential lead compounds. These compounds will have their activity tested which can then be compared with the computational results to validate those results. From previous studies it is predicted that the majority of derivatives synthesised will have no or significantly reduced activity, it may therefore take several attempts to find lead molecules, which will then need further refinement.

This library (**Table 3.**) was proposed at an early committee meeting as an initial library to test whether the molecular modelling and ligand docking studies carried out would be comparable to the in vitro enzyme activation assays. This had a focus on the C6 and C7

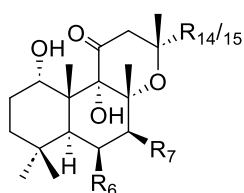


Figure 35. Structure of forskolin with relevant carbons labelled.

positions as these positions have been the most successful for

providing selectivity previously. Several of the molecules selected, such as **(26)**, **(37)** and **(54)**⁹², were picked due to high predicted binding, others were selected based on previous in vivo testing with AC5 such as **(39)**¹⁶⁵.

It also included several yet to be studied molecules **(44, 57, 58, 59)**, and

compounds **(47, 55, 56)** to examine the apparent pocket around the R₆ and R₇ in the forskolin binding site of AC6. Over time this target library of compounds changed significantly, to include more variation at different structural positions, to examine possibilities to introduce selectivity, and to increase the number of structures tested while reducing synthesis time. There was also

#	R6	R7	R14/15
26	OCO(CH ₂) ₂ NMe ₂	OCOCH ₃	C=C
39	OCO(CH ₂) ₂ NMe ₂	OCOCH ₃	C-C
37	OCO(CH ₂) ₂ NEt ₂	OCOCH ₃	C=C
53	OCO(CH ₂) ₂ NEt ₂	OCOCH ₃	C-C
54	OCO(CH ₂) ₂ NEt ₂	OH	C=C
47	OCO(CH ₂) ₈ NMe ₂	OCOCH ₃	C=C
55	OCO(CH ₂) ₈ NMe ₂	OCOCH ₃	C-C
56	OCO(CH ₂) ₈ NMe ₂	OH	C=C
44	NHCO(CH ₂) ₂ NMe ₂	OCOCH ₃	C=C
57	NHCO(CH ₂) ₂ NMe ₂	OCOCH ₃	C-C
58	NHCO(CH ₂) ₂ NMe ₂	OH	C=C
59	OCO(CH ₂) ₂ N(C ₅ H ₁₀)	OCOCH ₃	C=C
60	OCO(CH ₂) ₂ N(C ₅ H ₁₀)	OCOCH ₃	C-C
61	OCO(CH ₂) ₂ N(C ₅ H ₁₀)	OH	C=C

Table 23. The originally proposed list of forskolin derivatives for synthesis, with a focus on the C6 and C7 positions. For all of these compounds R₁ = OH, R₉ = OH, R₁₁ = =O.

less of a focus on molecules with slight changes in structure as they would be more appropriate in later stages of development.

In section **1.3.1** previously published forskolin derivatives and their activities were discussed, based on this a library of forskolin derivatives was designed. The aim of this library was to study how different derivatives would impact adenylyl cyclase activity and to continue the study of forskolin structural activity relationship with AC6, as this has yet to be completed.

7.2 Structure-Activity Relationship

While the structure-activity relationship of forskolin for adenylyl cyclase has been previously studied, this was not with AC6, so synthesis of similar derivatives will be useful for understanding how forskolin interacts with the binding site. Compounds that lack either the C1 or C9 alcohol groups will be generally avoided as they consistently produce no/low activity.

Both 1-TBDMS forskolin (**6**) and 1-TBDMS-7-deacylforskolin (**7**) will be tested for activity, these compounds have large steric bulk at the 1 position, preventing binding. This provides both a negative control for in vitro assays and ligand docking, as these molecules will not activate AC, providing binding scores of forskolin derivatives that cannot activate AC6. The acetyl group is predicated to be beneficial for AC6 activity, but previously synthesised 6-carbamate-7-deacetyl have produced higher levels of adenylyl cyclase activity¹⁶⁶, so both the 6-carbamate and 6-carbamate-7-deactyl derivatives of the carbamate series will be synthesized. Due to the previous anomalous results of the 1,9 carbonate/sulphonate the 1-carbamate derivatives will also be synthesized; these compounds are expected to perform poorly but they may also be the route to selectivity against other isoforms.

NKH477 (**26**) is predicted to have high AC6 activity, due to its selectivity of AC5 over AC2 and AC3, its activity in AC6 is currently unknown, this molecule would not be useful as a treatment due to elevated levels of AC5 activation. The 11 β -OH (**63**) derivative was synthesized as a potential route to introduce selectivity. The C14/15 epoxide (**64**) is of interest due to its poor activity in brain AC while retaining activity in heart AC, this offers an opportunity for selectivity from other isoforms and may be key in providing selectivity. A second epoxide was isolated (**65**),

presumably with the inverse stereochemistry as the only changes in HNMR shifts are protons attached to the C14 and C15 carbons, and the masses match exactly by LC-MS. As in literature the stereochemistry of the epoxide is unspecified there was a concern that the vastly different observed assay results are due to using two different compounds. However, both were synthesized with the same method and confirmed by NMR, any change in activity will be related to the isoform tested.

Due to being a terminal epoxide with few adjacent protons the stereochemistry was not determined. As these proton NMR peaks also overlap with other signals it is difficult to use NMR techniques such as NOESY to determine the product. This work will be completed once adenylyl cyclase assays have been complete and if either compound shows promising activity.

7.3 Library of Forskolin Derivatives

#	R1	R9	R11	R6	R7	R14/15
1	OH	OH	C=O	OH	OCOCH ₃	CH=CH ₂
3	OH	OH	C=O	OCOCH ₃	OH	CH=CH ₂
6	OSi(CH ₃) ₂ C(CH ₃) ₃	OH	C=O	OH	OCOCH ₃	CH=CH ₂
7	OSi(CH ₃) ₂ C(CH ₃) ₃	OH	C=O	OH	OH	CH=CH ₂
21	OH	OH	C=O	OH	OH	CH=CH ₂
26	OH	OH	C=O	OCO(CH ₂) ₂ N(CH ₃) ₂	OCOCH ₃	CH=CH ₂
62	OH	OH	C=O	OCOCH ₃	OCOCH ₃	CH=CH ₂
63	OH	OH	β-OH	OH	OCOCH ₃	CH=CH ₂
64	OH	OH	C=O	OH	OCOCH ₃	Epoxide
65	OH	OH	C=O	OH	OCOCH ₃	Epoxide
66	OH	OH	C=O	-OCOO-		CH=CH ₂
67	-OCOO-		C=O	OH	OCOCH ₃	CH=CH ₂
68	-OCOO-		C=O	OCOCH ₃	OH	CH=CH ₂
69	-OCOO-		C=O	-OCOO-		CH=CH ₂
70	β-Br	OH	C=O	OCOCH ₃	OH	CH=CH ₂
71	β-N3	OH	C=O	OH	OCOCH ₃	CH=CH ₂
72	β-NH ₂	OH	C=O	OCOCH ₃	OH	CH=CH ₂
73	OCN(C ₄ H ₈)	OH	C=O	OH	OCOCH ₃	CH=CH ₂
74	OCN(CH ₂) ₃ OH	OH	C=O	OH	OCOCH ₃	CH=CH ₂
75	OCN(CH ₂) ₂ C ₆ H ₅	OH	C=O	OH	OCOCH ₃	CH=CH ₂
76	OCNCH ₂ CH ₃	OH	C=O	OH	OCOCH ₃	CH=CH ₂
77	OH	OH	C=O	OH	OCN(C ₄ H ₈)	CH=CH ₂
78	OH	OH	C=O	OH	OCN(CH ₂) ₃ OH	CH=CH ₂
79	OH	OH	C=O	OH	OCN(CH ₂) ₂ C ₆ H ₅	CH=CH ₂
80	OH	OH	C=O	OCN(C ₄ H ₈)	OH	CH=CH ₂
81	OH	OH	C=O	OCN(CH ₂) ₃ OH	OH	CH=CH ₂
82	OH	OH	C=O	OCN(CH ₂) ₂ C ₆ H ₅	OH	CH=CH ₂
83	OH	OH	C=O	OCN(C ₄ H ₈)	OCOCH ₃	CH=CH ₂
84	OH	OH	C=O	OCN(CH ₂) ₃ OH	OCOCH ₃	CH=CH ₂
85	OH	OH	C=O	OCN(CH ₂) ₂ C ₆ H ₅	OCOCH ₃	CH=CH ₂
86	OCN(CH ₂) ₃ OH	OH	C=O	OCN(CH ₂) ₃ OH	OH	CH=CH ₂
87	OCNH ₂	OH	C=O	-OCOO-		CH=CH ₂
88	OH	OH	C=O	OCNH ₂	OH	CH=CH ₂
89	-OCH(N'N'(CH ₃) ₂)O-		C=O	OH	OCO-1,3-imidazole	CH=CH ₂
90	OCN(CH ₃) ₂	OH	C=O	OH	OH	CH=CH ₂
91	OH	OH	C=O	OH	OCN(CH ₃) ₂	CH=CH ₂
92	OH	OH	C=O	OH	OCO-2-pyridine	CH=CH ₂
93	OH	OH	C=O	OH	OCO(CH ₂) ₂ N(CH ₃) ₂	CH=CH ₂

Table 24. Complete list of derivatives synthesised for testing activity in various adenylyl cyclase isoforms. Derivatives features alterations made to the C1, C6, C7, C9, C11, C14 and C15 positions.

Compounds **(3, 21, 62)** are the 6-acetyl-7-deacetyl, 7-deacyl, 6-acetyl respectively and, how they will modify activity of AC6 is unknown, they were synthesised in part to assist with ligand docking, we can determine if the modelling is effective by comparison of that data with in vivo or in vitro assays. It will also contribute to determining if there is a consistent ideal structure that provides improved activity, eg 6-aminoacetyl vs 6-aminoacetyl-7-decetyl. From the literature it appears that one structure will not consistently be superior, it will depend on the length of chain and group attached as well as the groups position.

Compound **(6)** and **(7)** are the 1-OH protected forskolin and 1-OH protected-7-deacetyl forskolin respectively. They will act as negative controls in the assays, due to the removal of 1-OH bonding they would be expected to have no activity. As a tert-butyl group is used considerable steric bulk is also added, which is further expected to decrease activity. If these compounds have high activity or strong binding affinities in the ligand docking it would indicate the modelling is not representative of the adenylyl cyclase binding site, or the position of forskolin needs to be locked within the binding site to prevent conformations that would not occur.

Compounds **(63-69)**, are relate to previous studies of AC, reduction of the C11 position to the β -OH is expected to abolish or have almost no impact on AC6 activity. Introduction of the epoxide **(64, 65)** is expected to have little impact on the effect on AC6 activity, but potentially would abolish activity in group 1 and 2 ACs. The impact of introducing carbonates is expected to have little effect on activity in AC6, however this conflict with modelling data, in terms of important of C1 + C9 OH bonds. **(69)**, the 1,9-6,7-dicarbonate is expected to have poor activity.

Compounds **(70, 71 and 72)** are the first steps of converting the C1 position to an amino group. The identity of the bromide containing compound **(70)** is uncertain due to the LCMS mass not being the correct weight and a lack of the distinct bromine isotope pattern. The activity of the azide and the amine are unknown, it would be expected that the amine to perform well if the stereochemistry was unaltered, and also the lack of 7-acyl group will impact activity. Comparison to **(21)** 7-deacyl forskolin will be used to judge **(72)**.

Compounds **(73-89)** consist of the 1-,6-,7-carbamate derivatives. For the ring opening of carbonates to form carbamates three amines were selected. 3-aminopropan-1-ol, a polar chain

that introduces multiple hydrogen bond donors, phenylethylamine, introduces a short chain and a large hydrophilic structure that can contribute to π - π stacking and pyrrolidine, which forms a tertiary amine within a ring structure with no chain leading to it.

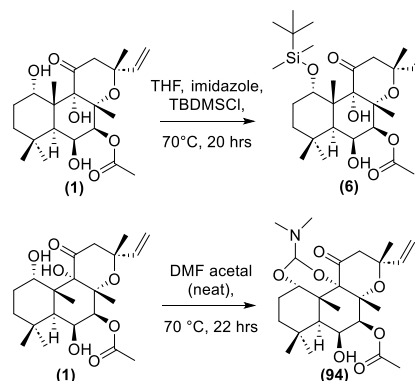
These amines were selected for their properties and introduced at the 1, 6, 7 positions, amines at the 6 positions were synthesized with the 7-acetyl group present and not.

The remaining compounds are **(26, 90, 91, 92 and 93)**. This set of molecules includes the derivatives with aminoacyl chains. These molecules examine the C6/C7 binding region to determine if selectivity for AC6 can be found with this route. I suspect that it will be possible to create new derivatives selective towards AC Type III (AC5 and AC6) but not specifically AC6. Due to this only a limited number of these compounds were made.

7.4 Synthesis Results and Discussion

The synthesis of forskolin derivatives will be focused around derivatization of the C6 and C7 positions, the C7 position is relatively easy to derivatize. After removal of the acetyl group the alcohol can undergo a number of reactions to produce appropriate compounds, protecting the C1 alcohol is necessary to prevent unwanted product formation. The C6 position is more difficult to modify, direct derivatization is not possible under the conditions studied so far, however it can be indirectly derivatized through two different mechanisms, an acyl transfer and the formation of a carbonate ring.

There are two published methods for the protection of the 1-OH, the first uses a tert-butyldimethylsilyl protecting group **(6)**, added with imidazole (1.5 eq) in THF at 70 °C. The second uses dimethyl formamide acetal, to form an additional ring between the 1-OH and 9-OH **(94)**. This

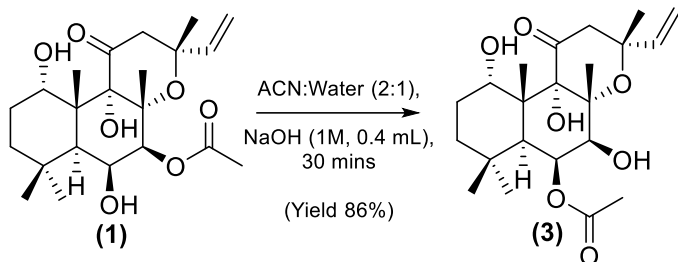


Scheme 2. Protection reactions of the 1-OH position with TBDMSCl and the formation of a 1,9-acetal product.

reaction is carried out in DMF acetal at 70 °C for 22 hrs (**Scheme 2**). The 6-OH is sufficiently

sterically hindered that it does not react even under harsh conditions. As this is one of the sites of interest for derivatization, methods are required that do not directly modify the site.

Fortunately, it is possible to migrate acyl groups from the C7 position to the C6 position under mild conditions (**Scheme 3**). This reaction does produce 7-deacetylforskolin as a side product. This reaction is effective with similar yields on a variety of acyl chains, not just an acetyl group.



Scheme 3. Acyl migration from the C7 to the C6 position, this reaction appears to be non-reversible. The deacyl product is also formed.

Through this method several forskolin derivatives have been synthesized, first by deacetylation of the 7-position, followed by introduction of another acyl chain, at this point the compound can be deprotected to form a final product or a migration reaction can be carried out to shift the chain to the 6-

position (**Scheme 1**). The acetyl group can then be reintroduced to the 7-position and the protecting group removed to form a final product.

An alternate method to introduce new groups to the 6 position is through carbonate ring opening reactions. First the 7-acetyl group is hydrolysed then carbonyl diimidazole is used to form a carbonate ring at the 6/7 positions (**Figure 36**). This ring can then be opened with various primary and secondary amines, this reaction is selective for the 6-carbamate. As with previous reactions if desired the acetyl group can be reintroduced at the 7-position.

Carbonyl diimidazole can also be used to synthesis 7-carbamates. If no base is added to the reaction a carbonyl imidazole intermediate (**100**) (**Figure 36**) forms at the 7-position, this can be displaced with a primary or secondary amine, selectively forming the 7-carbamate.

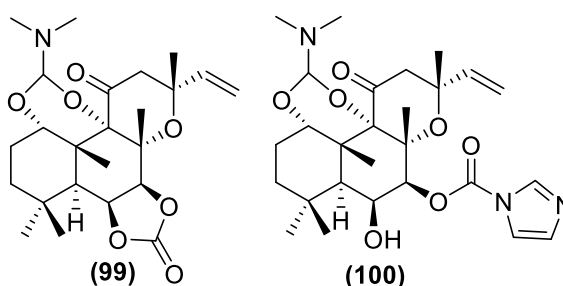


Figure 36. Protected carbonate used for the synthesis of 6-carbamates, carbonylimidazole intermediate used for the synthesis of 7-carbamates.

These compounds were successfully synthesized and used to make the corresponding carbamates (**Figure 37**). Three amines were selected for their properties and used to synthesize the 1-carbamate, 6-carbamate-7-deacetyl, 6-carbamate and 7-deacetyl-7-carbamate derivatives (**Figure 38**).

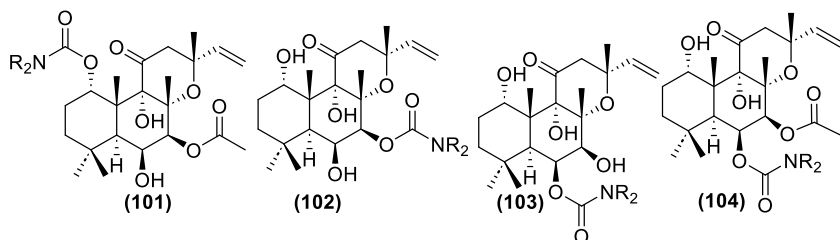


Figure 37. 1-carbamate, 7-deacetyl-7-carbamate, 6-carbamate-7-deacetyl and 6-carbamate derivatives respectively.

These two synthetic routes will be used to synthesize most of the compounds tested.

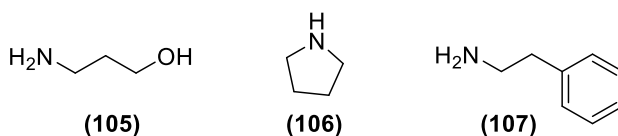
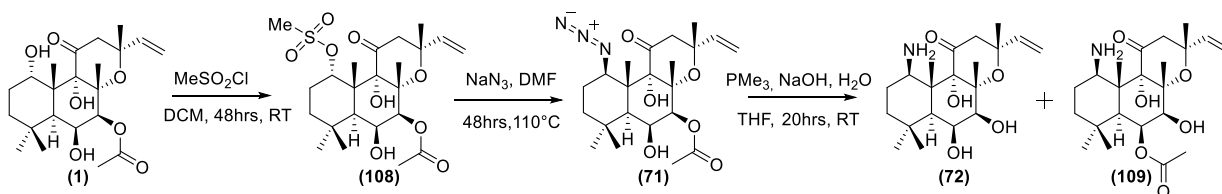


Figure 38. Amines selected for derivatization; 3-aminopropan-1-ol, pyrrolidine and phenylethylamine.

A derivatization route not explored in the literature is the replacement of alcohol groups with amines. This project was carried out by an honors' project student in the Sorensen Group. As the 1-OH is essential for reactivity we are more interested in derivatization at the C6 or C7 positions, however the C1 position requires fewer steps and reduced side product possibilities so was decided to be a more appropriate initial target.

This began with the mesylation of the 1-OH to improve its ability to act as a leaving group, the mesyl was then displaced with sodium azide to form the β -azide. To reduce the azide to an amine a Staudinger reaction using PPh_3 , regardless of the temperature or reaction length no product formed. This reaction was then repeated using PMe_3 to replace PPh_3 which successfully formed



Scheme 4. Synthetic route to form an amine at the C1 position with inverted stereochemistry (β).

the β -amine. As the reduction reaction is carried out in basic conditions only the 7-deacyl (**72**) and 7-deacyl-6-acyl product were formed (**109**) (**Scheme 4**).

While this compound was of interest, due to changing the stereochemistry at the 1 position, it is possible this derivative will display poor activity as essential hydrogen bonds are no longer able to form to the appropriate residues in the AC binding site. To synthesize the α -amine another route was proposed, this involved displacement of the 1-OH with a bromine atom, which could then be eliminated using sodium azide, the reaction would invert the stereochemistry twice to form a product with the desired stereochemistry. Unfortunately, the displacement with bromine led to the formation of a number of side products. This route was repeated but instead of forskolin as the starting material the mesylated version (**108**) was used, this allowed for milder conditions and specific displacement of the 1-OH with bromine. When sodium azide was used to displace the bromine atom much of the starting material was unreacted and a number of side products formed. At this point there was no time for further experimentation before the honors' project student graduated. This has shown however that amine containing forskolin derivatives can be synthesized at the one position, and the C1 position is protected the same reactions could be carried out at the C7 position, allowing for examination of different moieties and stereochemistry at this position. Further experimentation is needed. Due to the C6 positions low reactivity it is likely not be possible to carry out these reactions at that position.

Other derivatives synthesized in one or two steps such as the 7-deacyl forskolin (**21**) and 14,15-epoxyforskolin (**64/65**), synthetic methods can be found in the Appendix, Chapter 9.

Approximately 35 forskolin derivatives have been synthesized, characterized by NMR and LCMS, then studied with adenylyl cyclase activation assays, this value is then compared to forskolin activity to give a % activation. This is done as even with the same quantity of protein each AC isoform produces a varying quantity of cAMP, and it is difficult to compare the values directly.

7.5 Characterization

To characterize the molecules synthesized NMR and LCMS were carried out. Generally, ^1H NMR, ^{13}C NMR, Hetero Single Bond Quantum Correlation (HSQC), Homo Multiple Bond Correlation (HMBC) and COSY were the experiments completed.

Confirming the identity of several molecules such as the product of the ketone reduction, may require additional experiments such as NOESY, this is due to a limited number of adjacent protons increasing the difficulty of stereochemical assignment.

During acylation reactions each of the alcohol groups has the potential to react and form a product, fortunately the proton NMR shifts of these peaks are characteristic and can be further confirmed through proton splitting patterns and COSY experiments.

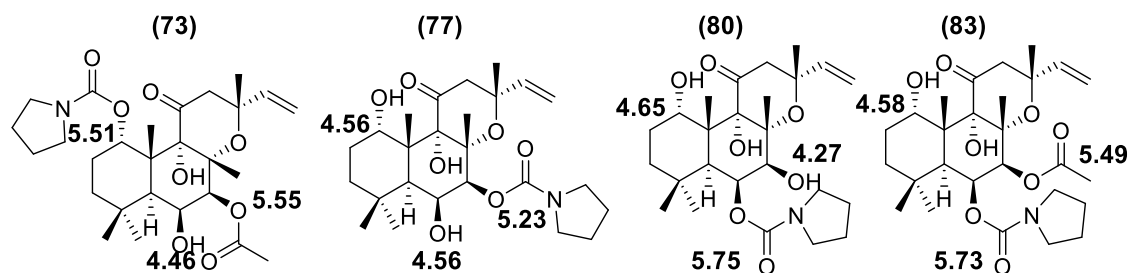


Figure 39. Structures of the pyrrolidine carbamates labelled with HNMR shifts of the protons at the C1, C6 and C7 positions. The C1 proton is a multiplet, the C6 is a triplet and C7 is a doublet, allowing for quick identification of which alcohol has been functionalized.

In the deacyl product the shifts are as follows C1 - 4.50, C6 - 4.64 and C7 - 4.14 ppm. In products where the C6 or C7 position are derivatized the C1 position only shifts slightly, appearing in the 4.40-4.60 ppm range. When C6 is acylated it appears in the 5.50-5.80 ppm region while C7 is consistently between 5.30-5.50 ppm. The synthetic methods used are mostly regiospecific, but confirming the correct product has formed is essential. COSY can then be used to confirm these results as the C7 α -proton will only correlate to the C6 α -proton, while the C6 α -proton will

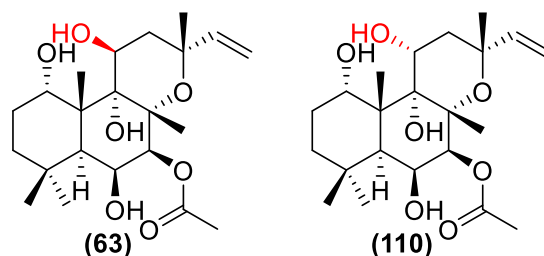


Figure 40. Structures of the two potential products when reducing the C11 ketone to an alcohol with NaBH_4 .

correlate to the C5 α -proton which appears in the 2.10-2.15 ppm range. For the majority of reactions, the C1 OH was protected, however, if it was not protected it would be clear if an acylation occurred at that position as there would be a shift of the C1 β -proton from 4.45 ppm, structures labelled with characteristic shifts of C6 and C7 α protons in **Figure 39**.

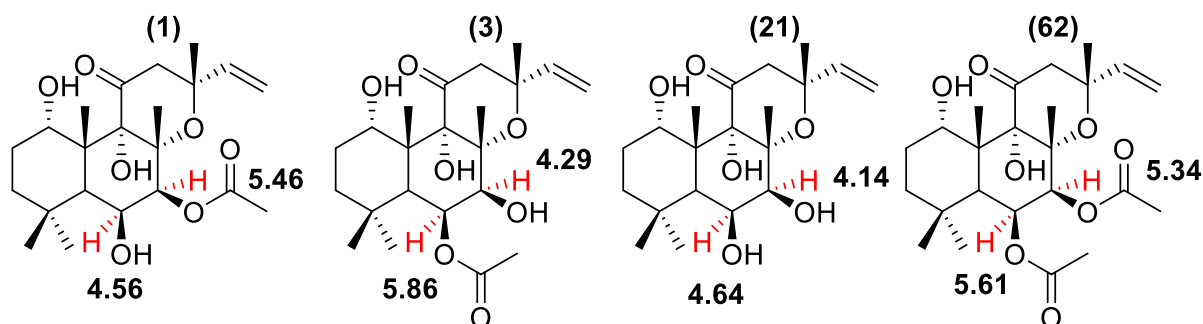


Figure 41. From left to right: forskolin (1), 6-acyl-7-deacyl forskolin (3), 7-deacyl forskolin (21) and 6-acyl forskolin (62), with ^1H NMR shifts for the C1, C6 and C7 positions. While these values will change depending on the acyl substituent they are representative of the ppm shifts of synthesized compounds. Protons of interest are highlighted in red.

These characteristic chemical shifts are also observed in the carbamate products that alongside the splitting patterns of the proton can be used to confirm which position has been derivatized. When reducing a ketone with NaBH_4 two different products could potentially form, the α or β alcohol **Figure 40**. The product formed is the β alcohol, this was determined using Nuclear Overhauser Effect NMR (NOE) experiments, this technique can be used to identify which stereoisomer was synthesized by observing through space (oppose to through bond) proton-proton interactions. The C11 proton (4.04 ppm) correlates to both protons at the C12 carbon (2.42, 1.60 ppm), this is expected as they are on the adjacent carbon. It also correlates to C10 β -CH₃ (1.66 ppm), C1 β -H (4.40 ppm), C2 α -H (2.20 ppm) and C2 β -H (1.43 ppm).

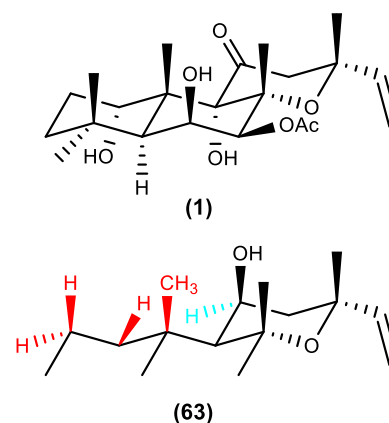


Figure 42. Forskolin shown in its chair conformation. Below is (63) in a chair conformation with groups not displayed to improve clarity. Protons that the C11 proton (teal) interact with observed through NOESY NMR experiments are highlighted in red.

Protons that interact with the C11 proton are highlighted in red in **Figure 42**. This indicates that the C11 proton is in the α position, as if it was in the β position it would correlate to the C8 β ,

C10 β and C13 β -CH₃ groups that are all above the plane of the ring. Therefore, the alcohol is in the β position, giving **(63)**.

LCMS was used to confirm the mass of each compound, with the experimental mass matching the predicted mass. Compound **(70)**, which contains a bromine atom, did not show a characteristic isotope pattern (1:1), which indicates that there is not a bromine atom present. The mass did also not match the expected mass or any common adducts, the identity of these compounds is currently unknown. This occurred in both (-) and (+) ion mode. A similar issue was observed with the azide, the mass did not match the expected mass, or that of possible adducts. It does appear to be the azide by ¹HNMR, but that provides little direct information about the azide group. The 1 β -NH₂ product has been synthesized which confirms the identity of the azide. I currently expect that this is an issue related to the LCMS, potentially an unknown adduct has formed, however repeated LCMS runs with different conditions have not altered the results. The NMR spectra appear as it would be predicted to be, however, this does not provide sufficient information to confirm the structure of these molecules. They will therefore not be published until further investigation has been completed.

7.6 Protecting Groups

While it is possible to selectively derivatize the C7-OH without protecting the C1-OH¹⁶⁷, protecting groups were introduced at the 1 position to prevent formation of C1 acyl derivatives. TBDMS appeared to be the superior protecting group to DMF acetal, as it is cheaper and the protecting reaction itself had higher yield of protected product. However, with the DMF acetal it is possible to protect and deacylate the C7 position in a single reaction.

For synthesis of the carbamates both the TBDMS and DMF acetal protecting groups were successfully used. The carbonate ring opening reaction to form 6-carbamate derivatives worked with no protecting group present.

7.7 Assay Results

The forskolin derivatives were examined by adenylyl cyclase assay using the CisBio HTRF-based cAMP kit, methods described in Chapter 2. Results are given as total cAMP produced (nM) within fifteen minutes. This value is then compared to cAMP produced when forskolin is present to give a % activation relative to forskolin, alongside the absolute nM value. Included in the table is the relative activity for wild-type HEK (WTHEK), AC3, AC5, AC6, AC7 and AC9 for all compounds that have been tested. Highlighted in green are all activity levels above 90% of that of forskolin. All highlighted in red are below 25% of forskolin activity. Colourless represent compounds between 25 and 90% activity.

Thesis #	HEK	AC3	AC5	AC6	AC7	AC9
1	100%	100%	100%	100%	100%	100%
3	11%	21%	30%	43%	26%	17%
6	1%	1%	2%	4%	5%	2%
7	1%	0%	1%	1%	4%	2%
21	5%	3%	8%	13%	9%	6%
26	126%	21%	135%	191%	39%	15%
62	1%	0%	1%	1%	5%	5%
63	36%	4%	65%	90%	18%	7%
64	1%	0%	1%	2%	6%	1%
65	0%	0%	1%	1%	7%	1%
66	1%	1%	2%	5%	9%	1%
67	12%	7%	25%	153%	22%	6%
68	1%	0%	1%	2%	4%	2%
69	1%	0%	1%	1%	4%	1%
70	0%	0%	0%	1%	3%	1%
71	10%	3%	10%	13%	4%	14%
72	11%	5%	10%	29%	13%	4%
73	0%	0%	0%	1%	8%	1%
74	0%	0%	0%	1%	9%	1%
75	1%	0%	1%	1%	4%	5%
76	1%	0%	0%	1%	2%	2%
77	200%	57%	83%	82%	234%	194%
78	120%	42%	39%	60%	47%	87%
79	27%	9%	14%	27%	13%	22%
80	74%	4%	24%	48%	25%	30%
81	2%	0%	3%	4%	6%	2%
82	5%	1%	7%	10%	11%	3%
83	172%	19%	62%	95%	142%	220%
84	75%	6%	35%	64%	25%	49%
85	72%	14%	43%	130%	40%	37%
86	1%	1%	0%	1%	4%	1%
87	1%	1%	1%	1%	1%	1%
88	3%	2%	3%	4%	2%	2%
89	0%	1%	1%	2%	2%	1%
91	39%	15%	71%	108%	53%	36%
92	109%	96%	106%	137%	60%	38%
93	41%	12%	59%	97%	14%	8%
Basal	0%	0%	0%	0%	3%	0%

Table 25. Forskolin derivative assay results from an HTRF-based cAMP kit. Data from each final molecule is shown with AC3, AC5, AC6, AC7, AC9 and HEKWT cell lines. Results are shown as a % of forskolin activity, this is due to high variance in the quantity of cAMP produced by each isoform. Red = below 25%, White = 25-90%, green = 95%+.

7.8 Discussion

The derivatives synthesised to examine the structural activity relationship between forskolin and AC6 generally performed as expected, with a few notable exceptions, key results shown in **Figure 43**. A full list of structures can be found in the supporting information, page 135.

(3) 6-acyl-7-deacyl forskolin has reduced activity across all isoforms, this supports the acetyl group at the C7 position being beneficial for binding. The same is observed in compound **(21)** 7-deacetylforskolin, with consistently lower activities than the 6-acetyl equivalent. Compound **(62)** the 6, 7-diacetyl abolished activity across isoforms. This compound was predicted to score highly in one round of ligand docking studies, it is however, incredibly insoluble, which may affect its ability to cross the cell membrane. This highlights the benefits of screening forskolin derivatives in more than one system, with the HPLC assay solubility and membrane permeability would not impact activity. Although this may prevent the exact molecule tested from being a useful drug molecule, it can be modified to, in this case, increase water solubility while having the C6 and C7 positions acylated.

Compounds **(6)** and **(7)** contain a bulky TBDMS protecting group at the C1 position and are used as negative controls, these compounds had some of the lowest activity levels across all isoforms.

(63) 11- β hydroxyforskolin is a compound of interest as AC6 activity was reduced to 90% of that of forskolin, however across all other isoforms was significantly lower between 4 and 65%. This molecule is selective for group 3 adenylyl cyclase isoforms.

Both epoxide stereoisomers **(64, 65)** performed very poorly with the highest activity across all isoforms being 7%. This was unexpected as previous results suggest that one stereoisomer would retain some degree of activity in AC6.

The addition of a carbonate group caused a significant loss in activity across derivatives and isoforms, with the exception of **(67)** the 1, 9-carbonate, which has increased activity (150%) in AC6 while the highest activity in another isoform is 25%. This agrees with previous literature, the removal of the 1-OH causes a significant decrease in binding to AC, however the 1,9-carboante and sulphonate, have shown unexpectedly high activity in AC heart isoforms¹⁶⁸. This

is a key structural difference in AC isoforms that can be exploited to create selective AC6 derivatives.

These initial trials suggest that **(67)** is highly selective for AC6 and is a primary candidate for further development.

The 1 β -azide and 1 β -amino-7-deacyl performed poorly across isoforms, **(71)** this is expected for the azide, while it is difficult to judge **(72)** due to the removal of the 7-acyl group. Comparison between **(72)** and **(21)** the 7-deacyl derivative shows higher activity in the β -amino derivative. This is worth investigating further as there is a degree of selectivity for AC6, with addition of the C7-acetyl group it could potentially outperform forskolin, while the change in stereochemistry at the C-1 position would influence isoform selectivity.

Compounds **(73) – (88)** consist of the various carbamate synthesized. Across all isoforms the 1-carbamate derivatives performed poorly, this was expected due to the bulk added to the C-1 position and removal of a hydrogen bond donor.

The 7-pyrrolidine carbamate **(77)** showed selectivity for AC7 and AC9, with slightly decrease activity across other isoforms, the 7-(3-aminopropanol) **(78)** carbamate had reduced activity across all isoforms, between 40 and 60% of forskolin activity. The 7-phenylethylamine carbamate **(79)** had poor activity across all isoforms.

Next the 6-carbamate-7-deacyl derivatives, all performed poorly, the introduction of a carbamate group at the C6 position does not make up for the loss of the 7-acetyl group.

In the 6-carbamate derivative series two compounds were of interest. **(83)**, the pyrrolidine derivative, which maintained activity in AC6, had high activity in AC7 and 9 whilst being selective away from AC3 and 5. This molecule has potential as a general AC activator, for conditions where activation of specifically AC3 must be avoided or as a specific activator of AC9.

Another candidate for development is compound **(85)**, the 6-phenylethyl carbamate, which has increased activity in AC6, with significantly decreased activity across all other isoforms.

The remaining carbamates had poor activity across all isoforms.

The remaining derivatives (**26**, **91**, **92**, and **93**) consist of 7-amino acyl derivatives, NKH477 (**26**) is a strong, selective activator of AC5, this suggests, as observed, that it will maintain strong activity in AC6 as well. This was observed with 135% and 191% activity compared to forskolin respectively. While these compounds are of interest, with increased selectivity for AC6, they maintain high levels of activity in AC5, making them poor choices for further development for treatment of PPHN.

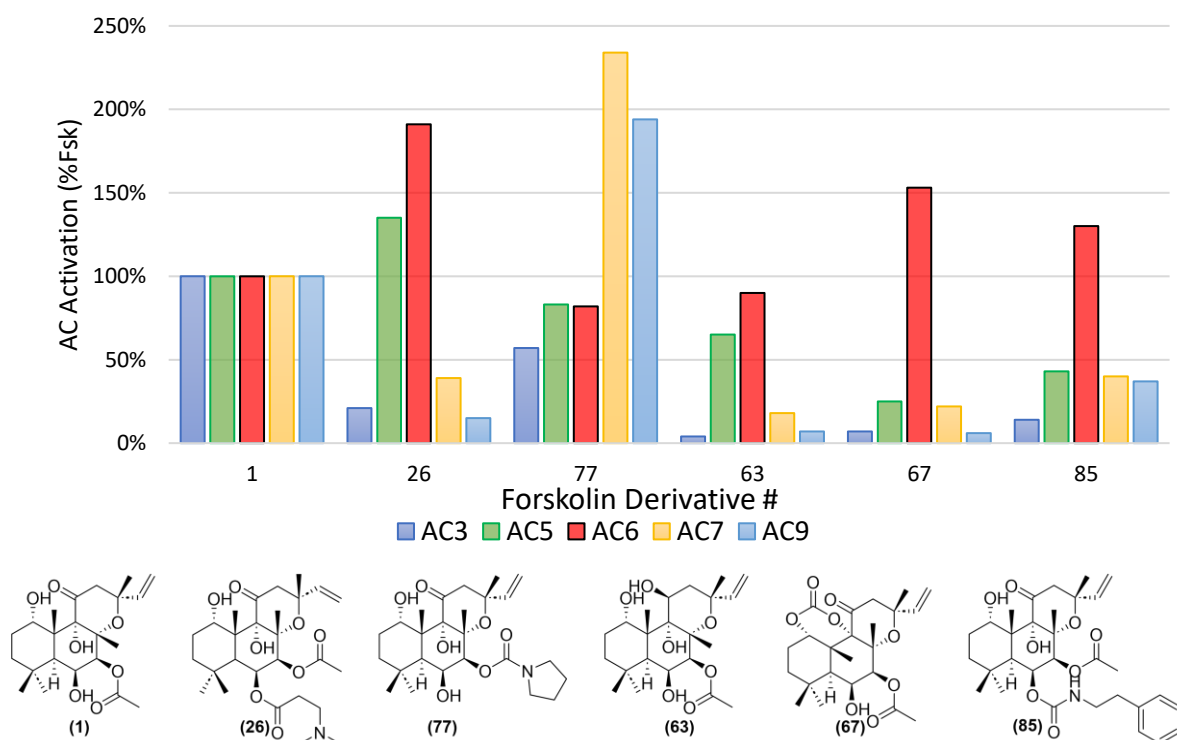


Figure 43. AC isoform activity of compounds of interest and associated structures.

Relating this experimental work back to earlier computational work, NKH477 (**26**) consistently performed well in the computational studies, and as expected acted as a strong activator of AC5 and AC6. As this compound performed worse in the other isoforms, introduction of an amino containing chain at the C6 position appears to favour AC5/AC6 activation, other derivatives such as related compounds that performed well in computational studies (**37,38, 45 and 46**) could be synthesized as group 3 (AC5/6) specific activators. For the activation of AC6 compounds without the 7-acyl group should be avoided, compounds such as **40-42, 51** and **52** contain amino chains at the 6 position but no acyl group at the 7, so would be expected

to perform poorly based off of the experimental results, as observed with compounds **80-82** vs **83-85**. From these initial results the computational predications appear to undervalue the importance of the C7 carbonyl group, known to be involved with the forskolin binding site. Once modelling of these derivatives has been finished a more complete comparison can be made between computational and experimental results. Testing of derivative AC activity in other systems would also help, to determine if other factors such as membrane permeability have impacted the activity of each compound.

Chapter Eight

8.1 Concluding Statement

A number of forskolin derivatives have been synthesised and tested for their ability to activate adenylyl cyclase isoforms. Several molecules of interest (**67**, **85**) were found that could act as lead molecules for the specific activation of AC6 with the intention to use these molecules as a treatment for PPHN. (**26**), (**63**) may be used for the specific of activation of group 3 AC (AC5 and AC6) as they have reduced activity in other isoforms, compound (**77**) is a strong activator of AC7 (Type 2) and AC9 (Type 4), with reduced activity in other isoforms.

An HPLC method has been established for assaying adenylyl cyclase activity, this compliments the other currently available AC assays that our collaboration has access too. This assay allows for quick analysis of activity and can be used with purified AC protein (bacterial) or impure lysates (HEK) with low overall AC content. This assay avoids issues such as cell permeability and eliminates background cAMP production, so compliments the HTRF cAMP kit assays well.

These assays will be finalized and published by two different students taking over the projects. This will also allow for examination of the already synthesized forskolin derivatives and the study of new experimental conditions.

As expected most derivatives synthesized lost all activity when undergoing a small structural change. Derivatives that maintained activity in AC6 or other isoforms will be studied further.

8.2 Future Hurdles

Major potential obstacles not yet explored or discussed is drug delivery and tissue selectivity. As AC6 is present in other tissues such as the liver, kidney and brain⁸², if specific activation of AC6 can be achieved there is still the risk of increased side effects due to activation of AC6 in other body parts/regions. Ideally a drug molecule could be delivered primarily to the vascular system, due to the requirement of activating AC6 in the heart/lung region. This would still cause activation of AC6 in other areas.

The effects of this are currently unknown and not well explored. This may be a hurdle that appears later in this project. There has previously been a study on humans with idiopathic congestive cardiomyopathy treated with forskolin and no major side effects were observed; this study did only include 12 people, however and included a note that longer-term studies would need to be completed to give a more accurate picture¹⁶⁹.

Forskolin has poor water solubility, which is not ideal for a drug molecule, as it affects cell permeability and drug delivery. The initial library of molecules often included nitrogen atoms to attempt to increase the water solubility, but the two compounds of most interest contain groups that would reduce water solubility.

As they were successful in a whole cell assay this shows that they can cross the cell membrane, a promising result. If necessary later in the design process this issue can be addressed, and many future recommendations of compounds to synthesize will include nitrogen atoms.

8.3 Future Work

Recommendations for future work:

8.3.1 Further testing of forskolin derivatives that perform well

The initial screening of forskolin derivatives showed promising activity, before the publication of this data, compounds of interest will have dose-response curves completed to complement the other data collected, this acts as validation and improves our understanding of the drug activity.

Once published, I will contact groups that may be interested in the specific derivatives published, offering to send them samples for analysis. AC6 is currently being studied in relation to many health conditions such as inflammation¹⁷⁰, cardiac contractions¹⁷¹ and bone formation¹⁷², access to specific AC6 activators may improve our understanding of these conditions as AC6 can be targeted, and activity promoted without impacting other AC isoforms that may make interpretation of results impossible.

(67) should also be tested for inhibitory effects on other AC isoforms, as it is expected to fit within and bind to the allosteric binding site, this could be tested against both forskolin and G α s mediated activity.

The 7-amino acyl derivatives maintained or increased activity in AC6, selective away from the other isoforms, except AC5, which also maintains moderate activity. This was expected as these molecules are selective towards group 2 AC isoforms, and is in part why these structures were not explored further in this study. However, compound **(85)** showed that selectivity away from AC5 can be achieved through modification of the C6 position. It would be best to examine this through computational modelling as it is difficult to establish a pattern from the assay results so far. I would encourage further computational modelling with validation against the currently tested molecules, if the results correlate well then, an extensive library of molecules can be tested before further synthesis work begins. This is especially worth while with this project in particular due to how easily the forskolin derivatives lose activity and the high similarity between AC5 and AC6's allosteric binding site.

8.3.2 Computational modelling

Now that we have some initial experimental data more in depth computational studies can be completed. Firstly, all molecules synthesized and others will be tested in silico for AC6 binding energies, these results can then be compared with the experimental data to determine how well the model functions. Other factors such as cell permeability should be taken into consideration when analysing these results.

If determined to be an acceptable model I would then create a model for AC3 and AC5, and test all derivatives against these models for binding energies. Again, comparison to the experimental results can be completed to determine how well the model works.

A large library of molecules can then be made and tested against all AC models to attempt to find other specific activators or to refine the structures of those molecules selected as lead compounds. This will then direct further synthetic work.

8.3.3 Synthesis of forskolin derivatives

I've created a list of molecules, split into two, one set that I believe will produce AC6 specific activators, and other molecules that explore new synthetic options. Several molecules that explore new synthetic options are shown in **Figure 44**, all structures are modified versions of the 1,9-carbonate. An example of this is compounds **(112, 113)** which contains an amine instead of hydroxyl group before formation of the carbonate, adjusting the selectivity and biochemical properties of the molecules. **(111)** contains the reduced 11- β hydroxyl, combining two AC6 selective elements to determine if that improves selectivity. Molecules containing multiple structural changes may see a decrease in overall cAMP production, however this is a fair trade off for increase selectivity, as with increasing selectivity the useable dosage would increase assuming it does not lead to toxicity issues.

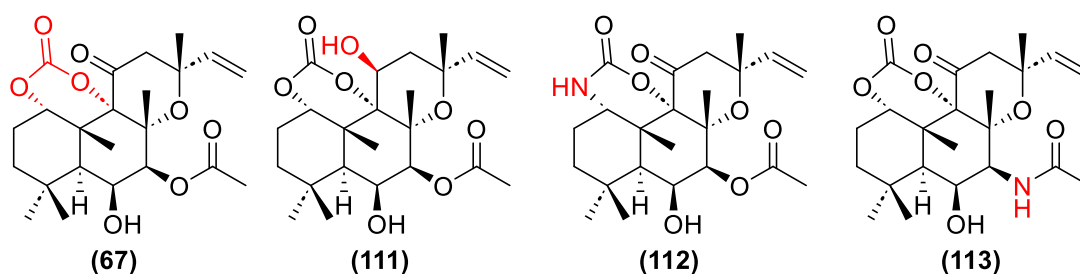


Figure 44. Structures of future forskolin derivatives for synthesis, experimental compounds.

Shown in

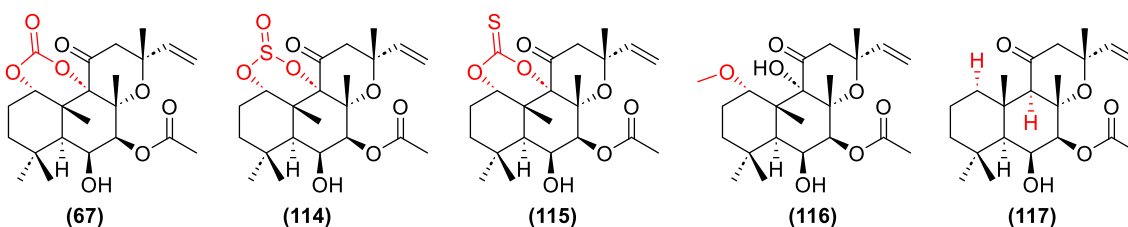


Figure 45 several molecules are shown with high potential for being selective activators, better than the carbonate due to previous results in the literature, **(114)** showed strong activity in heart AC, **(115)** is currently untested but may be promising given its similarity to **(67)**. The 1-O methyl **(116)** is a compound of interest for specific AC activation, this molecule would still be expected to bind to the forskolin binding site, but not cause a change in conformation in isoforms other than AC6. This molecule has a potential inhibitor effect, reducing forskolin and

G α s activity, by preventing the appropriate conformation shift. If this occurs while also activating AC6 this would be an excellent molecule for the study of AC6. 1,9-dideoxy forskolin **(117)** may act as an AC6 activator due to the apparent lack of need for the 1-OH for activation, this compound is commercially available, and would be expected to perform poorly in other AC isoforms.

The remaining suggestions are isosteres of the 1,9-carbonate, the changes made do not affect the number of valence electrons present, these molecules will have similar chemical and physical properties. Both molecules containing amines will have increased water solubility, and will influence adenyl cyclase activity.

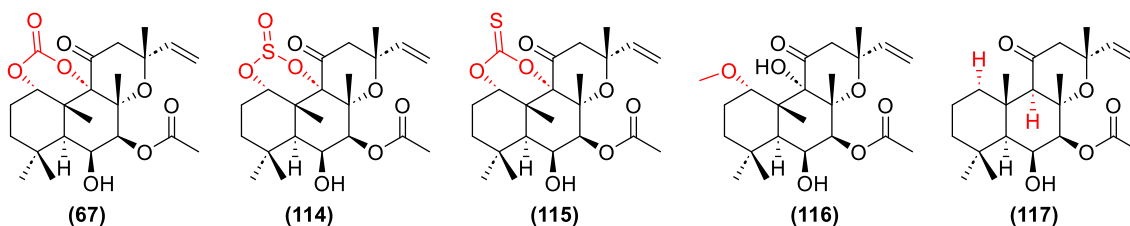


Figure 45. Suggested forskolin derivatives for future synthesis.

I think the 1-amino derivative has potential as specific activators of AC, this would need to be explored in more depth. Initial steps have been taken that suggest the synthesis would be possible, which was previously not certain. These molecules have not yet been explored in the literature, so the effects of these changes are unknown and difficult to predict, I think that it would be a promising derivative selective towards or against AC6 as it appears to be a difference in the binding pocket in that area compared to all other AC isoforms.

8.3.4 HPLC Assays

Unfortunately, I did not have time to finalize the HPLC assays, for the bacterial assay, new protein is currently being produced following more modern methods, once this protein is functional validation experiments can be completed and the assay published. Initially only AC6 protein will be available, if tested against all derivatives this would validate the other experimental assay results and check for issues relating to cell permeability.

For the HEK assay a different method will be used to allow for larger quantities of AC6 protein to be produced, if successful sufficient quantities could be produced for HPLC assays. A graduate student will take over this project in time. Once protein production is complete the impure lysate can be used for AC6 activation assays, again once validated this would allow for validation of the kit-based AC assay results.

How these assays are published will depend heavily on the timing of completion, and may be combined with assay results for improved impact.

8.3.5 Other potential experiments

Pre-COVID an LCMS proteomics project was discussed as to explore the forskolin binding site on AC6. This study would use purified AC6, fragment the protein and compare that with AC6 fragmented in the presence of forskolin, this will indicate which residues forskolin interacts with in the binding pocket. This could then be used to determine which areas of forskolin may be adapted and optimize that for AC6. It could also be compared with other isoforms of AC to determine where isoform specificity could be introduced. Although residue-ligand interactions can be studied using alternative methods this is an elegant method that could provide new data.

Chapter Nine - Appendix

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Supporting Information -

Chemical Structure and Numbering Table

1		2		3		4		5	
6		7		8		9		10	
11		12		13		14		15	
16		17		18		19		20	
21		22		23		24		25	
26		27		28		29		30	
31		32		33		34		35	
36		37		38		39		40	
41		42		43		44		45	

46		47		48		49		50	
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56		57		58		59		60	
61		62		63		64		65	
66		67		68		69		70	
71		72		73		74		75	
76		77		78		79		80	
81		82		83		84		85	
86		87		88		89		90	
91		92		93		94		95	-

96	-	97	-	98	-	99		100	
101		102		103		104		105	H ₂ N-CH ₂ -CH ₂ -OH
106		107	H ₂ N-CH ₂ -CH ₂ -OH	108		109		110	
111		112		113			-		-

Bacterial AC6 Plasmids:

Original AC6 plasmid with a Flag-tag.

Full length protein: > U971YED230-1 (AC6(1-151aa, 306-673aa, 914-1168aa)) NdeI--ATG --AC6(1-151aa, 306-673aa, 914-1168aa) --Flag tag --Stop codon--HindIII

Protein Length=782 MW=89168.7 Predicted pI=7.95 vector: pET30a

Protein Length = 782 AA.

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GEKEEMEELQAYNRRLLHNILPKDVAAHFLARERRNDELYQSCECVAVMFASIANFSEFYVELEANNEGVECLRLNE
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DNA sequence: 2361bp

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AC6 truncated version with His-tag.

Gas with His-tag.

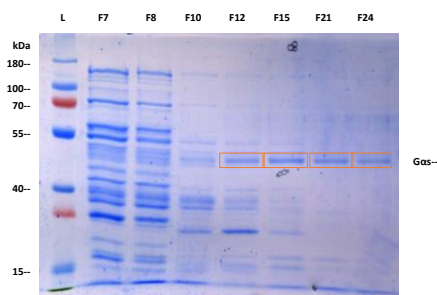
Full length protein: > U971YED230-8 (Gas) NdeI--ATG--His tag--Gas--Stop codon--HindIII Protein

Length=400 MW=46487.1 Predicted pI=6.33 vector: pET30a

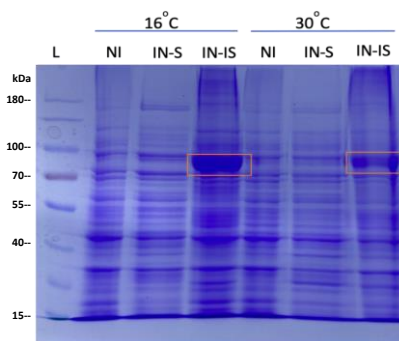
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DNA sequence: 1215bp

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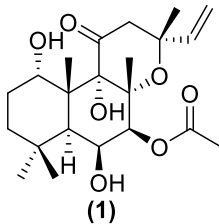


SFigure 47. SDS-Page Gel of fractions from purification of Gas by Co-NTA resin. DNA ladder is the left lane, remaining labels are labelled by fraction number. Gas presence confirmed by Western Blot.



SFigure 46. SDS-Page Gel of AC6 expression trials at 16 and 30 °C. From left to right, DNA Ladder, Not Induced, Induced Soluble Fraction, Induced Insoluble Fraction. AC6 is highlighted. Produced by Harjasmin. Presence of AC6 confirmed by Western Blot

Synthesis of Forskolin Derivatives



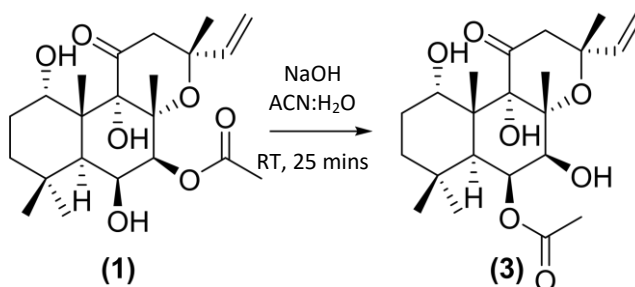
Preparation of forskolin (**1**)

Forskolin was purified from a weight loss supplement as previously described¹. 180 capsules were opened by separating the two halves of the outer gel cap by hand and emptying the contents into an Erlenmeyer flask. The extract was then washed with approximately 1 L of ethyl acetate, until the residue had an off-white sandy appearance. Solvent was removed by rotary evaporation, a brown tar like substance (17.6 g) remains.

A flash chromatography column was carried out using hexane-EtOAc (7:3) isocratically. The fractions were examined by TLC using hexane-EtOAc (7:3) as eluent. The fractions that appeared to contain forskolin (**1**) (rf= 0.44) were combined and the solvent removed by rotary evaporation to give 12.2 g of a residue that appeared to be forskolin (**1**) with >90 % purity as observed by NMR.

A mixed-solvent recrystallization was carried out on the full amount (12.2 g) of the crude material. The solid was first fully dissolved in ethyl acetate, then hot hexane was added until precipitation occurred. At this point hot ethyl acetate is added dropwise until the cloudiness dissipates and the solution was then allowed to cool to room temperature followed by one hour at 0 °C (ice bath). Hexane was then used to wash the crystals under vacuum filtration; 3.1 g of material was recovered. Further recrystallization of mother liquid gave 1.2 g of product. This process was repeated to yield an additional 0.8 g respectively providing a total of 5.1 g of forskolin (**1**) with a purity that appears to be > 95 % by NMR. Further recrystallization provided a sample that was >98% purity by NMR.

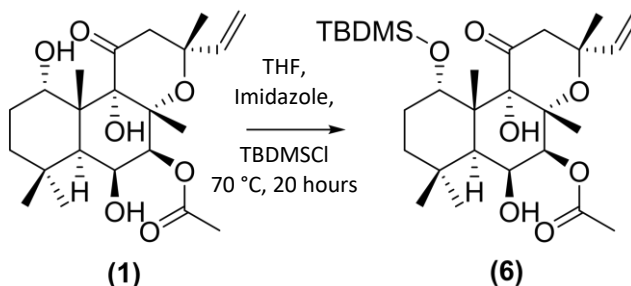
¹H NMR (500 MHz, CDCl₃): δ 6.05 (bs, 1H), 5.93 (dd, *J* = 10.5, 17.8, 1H), 5.46 (dd, *J* = 4.0 Hz, 1H), 5.29 (dd, *J* = 1.1, 17.1, 1H), 4.97 (dd, *J* = 1.1, 10.6, 1H), 4.56 (m, 1H), 4.45 (m, 1H), 3.18 (d, *J* = 17.2 Hz, 1H), 3.04 (d, *J* = 2.2 Hz, 1H), 2.47 (d, *J* = 17.2 Hz, 1H), 2.19 (s, 1H), 2.18 (m, 1H), 2.16 (s, 3H), 1.81 (s, 1H), 1.77 (m, 1H), 1.70 (s, 3H), 1.41 (m, 1H), 1.43 (s, 3H), 1.33 (s, 3H), 1.25 (s, 3H), 1.09 (m, 1H), 1.03 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 205.6, 169.6, 146.3, 110.8, 82.7, 81.4, 76.5, 75.0, 74.4, 70.0, 48.7, 43.0, 42.8, 36.1, 34.4, 33.0, 31.6, 26.6, 24.3, 23.6, 21.8, 19.8. **HRMS (ESI-TOF) m/z:** [M-H]⁺ calcd for C₂₂H₃₃O₇ 409.222; Found 409.223



Preparation of Compound (3)

Forskolin **(1)** (102 mg, 0.25 mM) was dissolved in ACN:H₂O (40:20 mL), NaOH (1 M, 5 mL) was then added, stirred at room temperature for 25 minutes. ACN was removed by rotary evaporation, leaving water and an off-white solid, ethyl acetate was used to dissolve and extract twice. The organic layers were combined then washed twice with water, followed by one wash with brine. The solution was dried with sodium sulphate, then filtered and the solvent removed by rotary evaporation. 118 mg white solid collected. The sample was purified by column chromatography, using EtOAc:hexane (10:90) as a solvent system. 102 mg of product collected, yield 86%. Recrystallized with EtOAc:hexane, 24 mg of pure product **(3)** isolated.

¹H NMR (500 MHz, CDCl₃): δ 6.37 (s, 1H), 6.12 (dd, *J* = 10.8, 17.4 Hz, 1H), 5.86 (dd, *J* = 2.9, 4.4 Hz, 1H), 5.19 (d, *J* = 17.5 Hz, 1H), 5.00 (d, *J* = 11.0 Hz, 1H), 4.67 (m, 1H), 4.29 (m, 1H), 3.18 (d, *J* = 17.3, 1H), 2.64 (d, *J* = 3.0, 1H), 2.54 (d, *J* = 17.3, 1H), 2.32 (d, *J* = 2.6 Hz, 1H), 2.16 (m, 1H), 2.11 (s, 3H), 2.06 (d, *J* = 3.0 Hz, 1H), 1.77 (m, 1H), 1.62 (s, 3H), 1.45 (m, 1H), 1.42 (s, 3H), 1.41 (s, 3H), 1.12 (m, 1H), 1.07 (s, 3H), 1.00 (s, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 205.4, 170.8, 146.4, 110.6, 82.11, 82.10, 75.2, 74.6, 73.7, 71.2, 48.7, 43.2, 42.0, 36.3, 34.0, 32.8, 30.9, 26.7, 23.3, 22.6, 21.6, 19.7. HRMS (ESI-TOF) *m/z*: [M-H]⁺ calcd for C₂₂H₃₃O₇ 409.223; Found 409.230

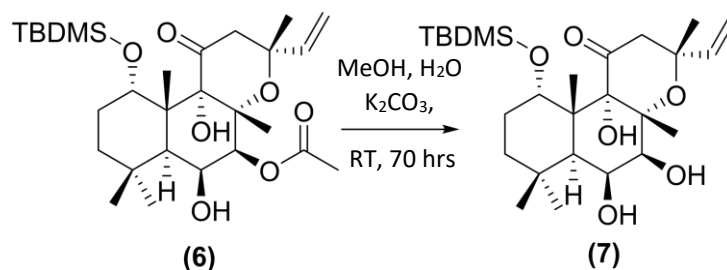


Preparation of Compound (6)

Forskolin (2.10 g, 5.1 mM) **(1)** was dissolved in THF (4 mL) and heated to 70 °C, then imidazole (1.20g, 17.6 mM) and tert-butyldimethylsilyl chloride (1.65 g, 11.0 mM) were added. The reaction was stirred for 20 hours. The solution was then extracted with ethyl acetate, washed with H₂O twice, brine and dried then with NaSO₄.

Purified by column chromatography with 7:3 hexane:ethyl acetate, 2.53 g (yield 94%), of product **(6)** was collected.

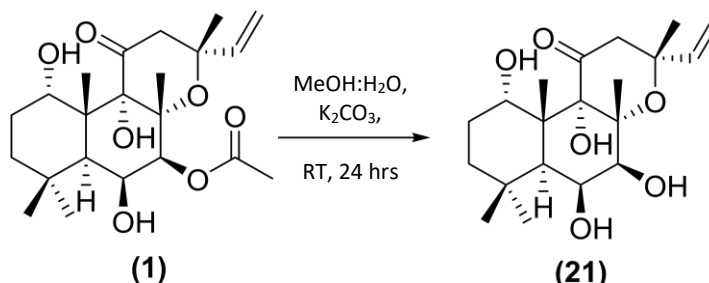
¹H NMR (500 MHz, CDCl₃): δ 6.83 (s, 1H), 6.00 (dd, *J* = 16.0, 10.5, 1H), 5.54 (d, *J* = 4.2 Hz, 1H), 5.14 (dd, *J* = 16.1, 1.0 Hz, 1H), 4.88 (dd, *J* = 10.6, 1.0 Hz, 1H), 4.58 (m, 1H), 4.56 (m, 1H), 3.25 (d, *J* = 16.1 Hz, 1H), 2.33 (d, *J* = 16.0 Hz, 1H), 2.21 (m, 1H), 2.15 (m, 4H), 1.79 (s, 1H), 1.67 (m, 2H), 1.64 (s, 3H), 1.45 (s, 3H), 1.41 (m, 1H), 1.32 (s, 3H), 1.24 (m, 4H), 1.07 (m, 1H), 1.02 (s, 3H), 0.88 (s, 9H), 0.13 (s, 3H), 0.00 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 207.3, 169.7, 146.6, 109.8, 82.1, 81.4, 77.2, 76.9, 75.5, 69.8, 49.5, 43.4, 43.3, 36.3, 34.3, 33.0, 31.0, 25.9, 25.8, 24.1, 24.0, 21.2, 19.9, 18.1, -4.8, -4.7. **HRMS (ESI-TOF) *m/z*:** [M-H+Na₂]⁺ calcd for C₂₈H₄₇O₇Si 569.288; Only forskolin, 7-deacyl forskolin, 7-deacyl 6-acyl forskolin is observed.



Preparation of Compound (7)

1-TBDMS forskolin (**6**) (4.1g, 78 mM) was dissolved in MeOH (200 mL), then water (50 mL) and potassium carbonate (24 g) were added sequentially, the solution was stirred at room temperature for 70 hours. As much solvent as possible removed by rotary evaporation then residue was dissolved in ethyl acetate, washed with water twice and brine once. The solution was dried with sodium sulfate. The solvent was removed by rotary evaporation, 3.8g residue recovered. The product was purified by biotage column, 25g Snap Cartridge, ACN:DCM 99->90%. 3.4g of pure product (**7**) collected. 90% yield.

¹H NMR (500 MHz, CDCl₃): δ 6.98 (bs, 1H), 6.16 (dd, *J* = 10.7, 17.4 Hz, 1H), 5.11 (d, *J* = 17.2 Hz, 1H), 4.94 (d, *J* = 10.7 Hz, 1H), 4.62 (m, 1H), 4.48 (m, 1H), 4.17 (d, *J* = 4.3 Hz, 1H), 3.20 (d, *J* = 16.7 Hz, 1H), 2.41 (d, *J* = 16.7 Hz, 1H), 2.27 (bs, 1H), 2.17 (m, 1H), 2.11 (d, *J* = 2.7 Hz, 1H), 1.67 (m, 1H), 1.60 (s, 3H), 1.43 (m, 1H), 1.42 (s, 3H), 1.40 (s, 3H), 1.26 (s, 3H), 1.08 (m, 1H), 1.05 (s, 3H), 0.87 (s, 9H), 0.14 (s, 3H), 0.03 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 206.5, 146.7, 110.0, 82.3, 82.0, 77.1, 75.4, 74.9, 70.4, 49.1, 43.4, 43.4, 36.2, 34.2, 33.1, 30.5, 25.9, 25.8, 24.1, 23.6, 19.9, 18.1, -4.6, -4.8. **HRMS (ESI-TOF) *m/z*:** [M-H]⁺ calcd for C₂₄H₃₅O₈ 481.299; Only deacyl forskolin observed 367.2197.



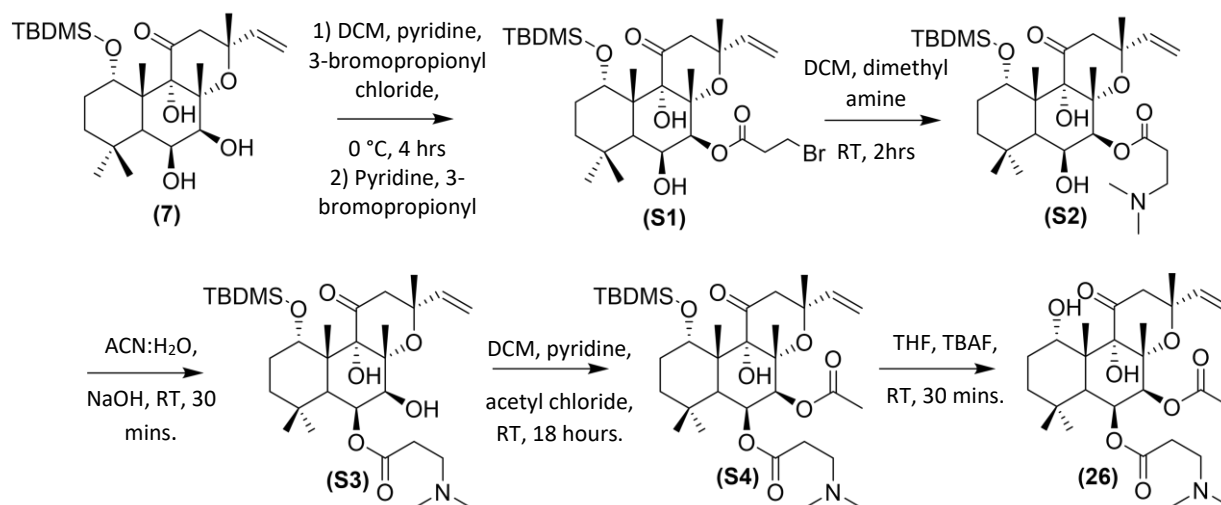
Preparation of Compound (21)

Forskolin (**1**) (1.10 g, 2.7 mM) was dissolved in methanol (95 mL) and water (40 mL), potassium carbonate (6.1 g) was then added. The reaction was stirred at room temperature for 24 hours.

Methanol was removed by rotary evaporation, leaving water and an off-white solid, ethyl acetate was used to dissolve and extract twice. The organic layers were combined then washed twice with water, followed by one wash with brine. The solution was dried with sodium sulphate, then filtered and the solvent removed by rotary evaporation.

0.98g of white solid was collected. Sample was purified by biotage column chromatography from 1:99 DCM:MeOH to 6:94 DCM:MeOH, 0.85g of product (**21**) was collected. 87% yield.

¹H NMR (500 MHz, CDCl₃): δ 6.40 (bs, 1H), 6.12 (dd, *J* = Hz, 1H), 5.20 (d, *J* = Hz, 1H), 5.00 (d, *J* = Hz, 1H), 4.64 (m, 1H), 4.50 (m, 1H), 4.14 (m, 1H), 3.18 (d, *J* = Hz, 1H), 2.52 (d, *J* = 17.2 Hz, 1H), 2.45 (d, *J* = 3.2 Hz, 1H), 2.37 (bs, 1H), 2.27 (m, 1H), 2.23 (m, 1H), 2.11 (d, *J* = 2.4 Hz, 1H), 1.73 (dt, *J* = 13.5, 3.2 Hz, 1H), 1.66 (s, 3H), 1.56 (H₂O), 1.42 (s, 3H), 1.42 (s, 3H), 1.40 (m, 1H), 1.27 (s, 3H), 1.13 (m, 1H), 1.07 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 205.6, 146.5, 110.4, 82.3, 82.2, 75.2, 74.8, 74.7, 70.5, 48.8, 43.0, 42.8, 36.1, 34.3, 33.0, 30.9, 26.9, 24.2, 23.4, 20.0. **HRMS (ESI-TOF) *m/z*:** [M-H]⁺ calcd for C₂₀H₃₁O₆ 367.212; Found 367.2232



Preparation of Compound (26)

1-TBDMS-7-deacylforskolin (**7**) (330 mg, 0.68 mM) was dissolved in DCM (4 mL) the solution was then cooled to 0 °C under argon. Then pyridine (75 µL, 1 mM) and 3-bromopropionyl chloride (70 µL, 0.70 mM) were added dropwise. The solution was stirred for four hours at 0 °C, then pyridine (75 µL, 1 mM) and 3-bromopropionyl chloride (70 µL, 0.70 mM) were added dropwise. The solution was allowed to reach room temperature and was stirred overnight. DCM (5 mL) was added to the reaction mixture, it was then washed with 0.1 M HCl once, water twice and brine once. It was then dried with NaSO₄ and the solvent was removed by rotary evaporation. 371 mg of clear oily residue (**S1**) collected. Product not purified before derivatization.

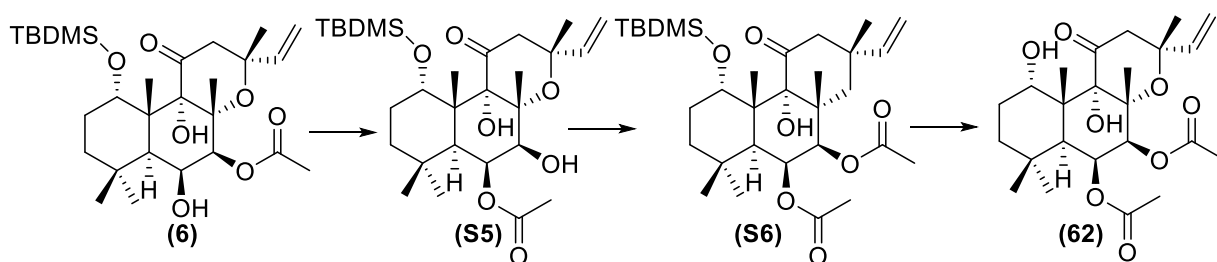
(**S1**) (340 mg, 0.55 mM) was dissolved in DCM (5 mL) then dimethyl amine (40% in H₂O, 0.5 mL) was added to the solution. Stirred for two hours at room temperature. DCM (5 mL) added, organic layer washed twice with water then once with brine. Solution dried with NaSO₄, then removed by rotary evaporation. 321 mg recovered. 125 mg purified by column chromatography using a biotage isolera, 10g KP-Sil column, DCM:MeOH. 98 mg product (**S2**) recovered, 80% yield.

(S2) (418 mg, 0.72 mM) was dissolved in ACN (22.5 mL), water was added until the solution began to turn cloudy (approximately 15 mL), NaOH (1 M, 2.0 mL) was then added. Precipitation began to form, ACN (3 mL) was added, solution was then stirred at RT for 30 minutes. Solvent removed by rotary evaporation, the water and white solid were extracted with EtOAc which was then washed with water twice and brine once. The solution was then dried with NaSO₄ and the solvent was removed by rotary evaporation. 480mg of residue recovered, starting material and deacyl product also observed in NMR. 300 mg purified by column with ACN (100%) (TLC 90:10 DCM:MeOH), 192 mg product **(S3)** isolated. 45% yield.

(S3) (186 mg, 0.30 mM) was dissolved in DCM (20 mL), then acetyl chloride (200 μ L, 2.82 mM) and pyridine (240 μ L, 2.98 mM) were added dropwise in four equal portions (50 μ L and 60 μ L respectively) once an hour for four hours. The solution was then stirred overnight for a total of 18 hours. The reaction mixture was washed twice with water, once with brine. The solution was then dried with magnesium sulfate, the solvent was removed by rotary evaporation to give a 280 mg of residue. The residue was purified by column chromatography, 5:95 DCM:MeOH to 80:20 DCM:MeOH. 112mg product **(S4)** purified. 56% yield.

(S4) (70 mg, 0.11 mM) was dissolved in THF, then TBAF (1 M in THF, 16 μ L) was added. The reaction was stirred at room temperature for thirty minutes, EtOAc (10 mL) added to the reaction, the solution was then washed twice with water, once with brine. The solution was dried with sodium sulfate, the solvent was then removed by rotary evaporation. Column 5:95 MeOH:DCM to 10:90, 15 mg of pure product **(26)** collected. 26% yield. 7% overall yield.

¹H NMR (500 MHz, CDCl₃): δ 6.08 (s, 1H), 5.95 (dd, J = 10.6, 17.0, 1H), 5.84 (m, 1H), 5.52 (d, J = 4.6, 1H), 5.26 (dd, J = 0.7, 17.0 Hz, 1H), 4.98 (dd, J = 0.7, 10.6, 1H), 4.61 (m, 1H), 3.21 (d, J = 17.0 Hz, 1H), 2.64 (m, 2H), 2.50 (m, 2H), 2.49 (d, J = 17.0 Hz, 1H) 2.40 (d, J = 2.7 Hz, 1H), 2.24 (s, 6H), 2.12 (m, 1H), 2.02 (s, 3H), 1.79 (m, 1H), 1.66 (s, 3H), 1.44 (s, 3H), 1.44 (m, 1H), 1.35 (s, 3H), 1.10 (m, 1H), 1.03 (s, 3H), 0.97 (s, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 205.3, 171.4, 169.8, 146.1, 110.8, 82.3, 80.9, 75.2, 74.2, 74.0, 69.7, 54.4, 48.8, 45.1, 45.1, 43.1, 41.9, 36.2, 34.0, 33.6, 32.8, 31.3, 26.5, 23.5, 23.1, 20.8, 19.6. HRMS (ESI-TOF) m/z : [M-H]⁺ calcd for C₂₇H₄₂NO₈ 508.291; Found 508.3021.



Preparation of Compound (62)

(6) (117 mg, 0.22 mM) was dissolved in mix of ACN:H₂O (15:8 mL), NaOH (1 M, 0.4 mL) was added, the solution was stirred for thirty minutes. The solvent was reduced by rotary evaporation. The remaining solid/aqueous layer was extracted with ethyl acetate, washed twice

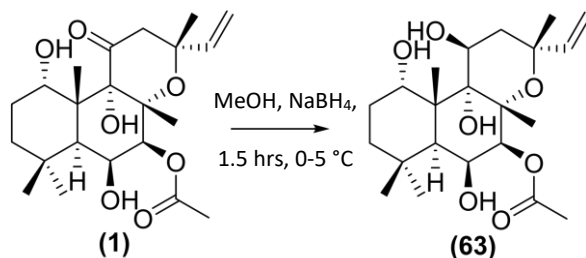
with water, once with brine. The solution was dried with sodium sulphate, the ethyl acetate was then removed by rotary evaporation to give a residue. 168 mg recovered.

The recovered residue (**S5**) (164 mg) was dissolved in DCM (10 mL), then pyridine (150 μ L, 0.13 mM) and CH_3COCl (125 μ L, 1.75 mM) were added, acetyl chloride was then added dropwise. Conversion appeared complete by TLC after two hours. The solution was washed with water twice followed by brine once, and dried with sodium sulfate, solvent removed with rotary evaporation.

(**S6**) (125 mg, 0.24 mM) was dissolved in THF (2 mL), TBAF (1 M in THF, 60 μ L, 0.06 mM) was then added. The reaction was stirred at room temperature for 30 minutes, after which the solution was diluted with ethyl acetate (10 mL), washed twice with water and once with brine. The solution was dried with sodium sulfate, the solvent was then removed by rotary evaporation to give a white solid. 108 mg product recovered.

Column chromatography with hexane:EtOAc 80:20 to 70:30, 26mg (**62**) recovered. 26% yield. Further purified by recrystallization with methanol.

^1H NMR (500 MHz, DMSO d_6): δ 6.34 (bs, 1H), 6.00 (dd, J = 10.6, 17.5 Hz, 1H), 5.61 (m, 1H), 5.34 (d, J = 4.5 Hz, 1H), 5.07 (dd, J = 1.3, 17.5, 1H), 4.83 (dd, J = 1.3, 10.6 Hz, 1H), 4.43 (s, 1H), 3.31 (H₂O), 3.29 (HDO), 3.10 (d, J = 16.6 Hz, 1H), 2.46 (d, J = 16.5 Hz, 1H), 2.24 (d, J = 2.9 Hz, 1H), 2.07 (s, 3H), 2.05 (m, 1H), 1.92 (s, 3H), 1.70 (m, 1H), 1.56 (s, 3H), 1.35 (s, 3H), 1.33 (m, 1H), 1.27 (s, 3H). ^{13}C NMR (125 MHz, DMSO d_6): δ 206.3, 170.4, 170.0, 147.3, 110.2, 82.0, 75.4, 74.7, 73.0, 69.3, 49.1, 42.5, 42.1, 40.3, 36.5, 34.0, 32.9, 30.9, 23.5, 23.4, 21.6, 21.1, 19.6. HRMS (ESI-TOF) m/z : [M-H]⁺ calcd for $\text{C}_{24}\text{H}_{35}\text{O}_8$ 451.233; Found 451.2448



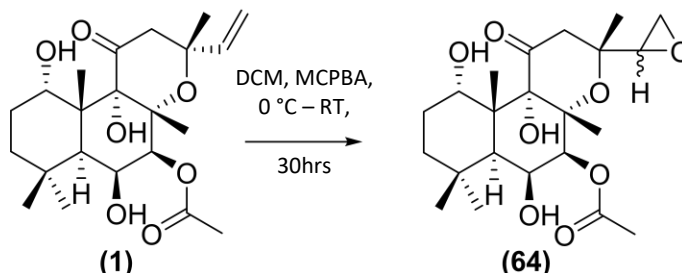
Preparation of Compound (63)

Forskolin (**1**) (402 mg, 0.98 mM) was dissolved in methanol (20 mL), placed in an ice bath until 0-5°C was reached, then NaBH_4 (275 mg, 7.3 mM) was added to the reaction vessel. Stirred for 90 minutes. Solvent was removed by rotary evaporation. A work up was carried out by dissolving the residue in ethyl acetate, washed twice with H_2O , washed with brine once and dried with Na_2SO_4 . 394 mg of product collected.

Column chromatography using a biotage isolera was carried out on 202mg of recovered material. A 25g Snap Cartridge was used, EtOAc:hexane 12-80%. 80mg product collected, 39% yield. Further purification by recrystallization with DCM, 30mg of product (**63**) recovered.

^1H NMR (500 MHz, MeOD): δ 5.89 (dd, J = 14.0, 10.6, 1H), 5.17 (d, J = 4.4, 1H), 5.06 (dd, J = 17.6, 1.5, 1H), 4.82 (dd, J = 10.6, 1.5, 1H), 4.48 (m, 1H), 4.40 (m, 1H), 4.04 (m, 1H), 2.42 (dd, J = 14.3, 4.8, 1H), 2.23 (d, J = 3.2, 1H), 2.21 (m, 1H), 2.11 (s, 3H), 2.08 (s, 3H), 1.80 (m, 1H), 1.67 (s, 3H), 1.60 (dd, J = 14.3, 2.8, 1H), 1.53 (s, 3H), 1.44 (m, 1H, C-12 β), 1.32 (s, 3H), 1.04 (m, 1H), 1.02 (s, 3H). ^{13}C NMR (125MHz, MeOD): δ 173.2, 149.5, 109.8, 80.4, 79.6,

78.6, 75.0, 73.4, 71.2, 69.8, 45.9, 44.6, 41.9, 37.9, 35.3, 33.7, 29.8, 27.7, 25.0, 23.6, 21.4, 20.1. **HRMS (ESI-TOF)** **m/z**: [M-H]⁺ calcd for C₂₂H₃₅O₇ 411.238; Found 411.2726, 457.2526 [M-H+Na]

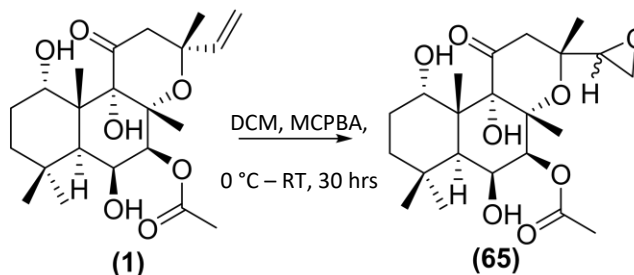


Preparation of Compound (64)

Forskolin (**1**) (310 mg, 0.76 mM) was dissolved in DCM (6 mL), the solution was then cooled to 0-5 °C, MCPBA added (212 mg, 1.2 mM), and stirred for 30 hours. A work up was carried out by dissolving adding DCM (10 mL) the solution was then washed twice with H₂O, washed with brine once and dried with Na₂SO₄. Solvent removed by rotary evaporation. 487 mg of white solid was collected. 250 mg purified by column (30:70 EtOAc:hexane), one additional product observed, initially believed to be the ring opened product, but is the epoxide with opposite stereochemistry. 102 mg recovered, 60% yield. Final purification by recrystallization with DCM/pentane.

This epoxide matches NMR of published epoxide².

¹H NMR (500 MHz, CDCl₃): δ 6.48 (s, 1H), 5.31 (bs, 1H), 5.28 (d, *J* = 4.0 Hz, 1H, C7), 4.51 (m, 1H, C1), 4.40 (m, 1H, C6), 3.22 (dd, *J* = 4.8, 2.8 Hz, 1H), 3.18 (d, *J* = 19.0 Hz, 1H), 2.92 (dd, *J* = 2.8, 4.0 Hz, 1H), 2.80 (t, *J* = 4.4 Hz, 1H), 2.67 (d, *J* = 19.0 Hz, 1H), 2.18 (m, 1H, C5), 2.15 (s, 3H), 1.96 (m, 2H), 1.82 (bs, 1H), 1.71 (s, 3H), 1.51 (m, 1H), 1.38 (s, 3H), 1.36 (s, 3H), 1.25 (s, 3H), 1.02 (s, 3H), 1.02 (m, 1H). **¹³C NMR (125 MHz, CDCl₃):** δ 200.9, 169.4, 82.8, 80.9, 75.1 (C7), 73.7 (C1), 70.4, 70.1 (C6), 60.7, 46.0, 45.9, 43.1, 42.4, 36.1, 34.6, 33.1, 28.3, 25.7, 24.8, 22.7, 21.1, 19.8. **HRMS (ESI-TOF) m/z**: [M-H]⁺ calcd for C₂₂H₃₃O₈ 425.218; Found 471.2632 [M-H+Na₂].



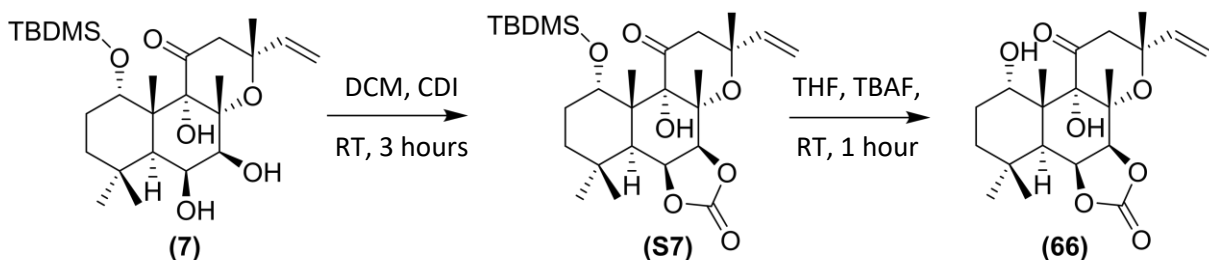
Preparation of Compound (65)

Forskolin (**1**) (310 mg, 0.76 mM), was dissolved in DCM then secured in an ice bath, the solution was left to cool to 0 °C. MCPBA (212 mg, 1.25 mM) was then added to the cooled solution, and it was stirred for 30 hours, 0 °C -> RT. A mixture of the epoxide and ring open product formed.

DCM (10 mL) was added to the reaction mixture, which was then washed with water twice and brine once. The solution was then dried with sodium sulfate, followed by rotary evaporation to remove the remaining solvent. 450 mg residue collected.

200 mg of the residue was purified by column chromatography, using the biotage isolera with a 10g sfär cartridge, with EtOAc:hexane with a gradient from 5 to 100% ethyl acetate. 62 mg of product **(65)** collected and recrystallized with DCM/pentane.

¹H NMR (500 MHz, CD₃OD): δ 5.23 (d, *J* = 3.8 Hz, 1H), 4.43 (t, *J* = 3.8 Hz, 1H), 4.32 (m, 1H), 4.16 (t, *J* = 4.6 Hz, 1H), 3.72 (dd, *J* = 3.6, 11.9 Hz, 1H), 3.55 (dd, *J* = 5.5, 11.9 Hz, 1H), 2.50 (bs, 1H), 2.14 (s, 3H), 2.13 (m, 1H), 2.08 (m, 1H), 1.87 (td, *J* = 3.4, 13.4 Hz, 1H), 1.54 (s, 3H), 1.52 (s, 3H), 1.41 (m, 1H), 1.30 (s, 1H), 1.16 (s, 3H), 1.03 (s, 3H), 1.00 (m, 1H). **¹³C NMR (125 MHz, CD₃OD):** δ 202.0, 171.2, 84.6, 81.5, 79.4, 77.8, 73.3, 71.7, 67.8, 60.6, 46.1, 43.7, 42.6, 35.8, 34.0, 32.1, 25.3, 23.9, 23.6, 20.4, 193.7, 18.6. **HRMS (ESI-TOF) *m/z*:** [M-H]⁺ calcd for C₂₂H₃₅O₉ 443.228; Found 471.22 [M-H+Na₂].

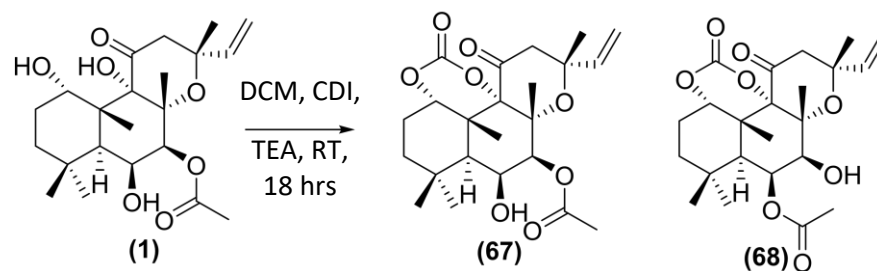


Preparation of Compound (66)

1-TBDMS forskolin **(7)** (120mg, 0.24 mM) was dissolved in DCM (3 mL), then carbonyl diimidazole (96 mg, 0.6 mM) was added, the solution was stirred at room temperature for 3 hours. The solution was then diluted with DCM (10 mL), which was washed with water twice followed by brine and dried with NaSO₄. 190 mg collected. No purification carried out.

130 mg product dissolved in THF (2 mL), the protecting group removed by addition of TBAF (1m in THF, 35 µL), the reaction was then stirred at room temperature for one hour. Once completed the solution was diluted with EtOAc, which was washed with water twice followed by brine and dried with NaSO₄. 119 mg product recovered. Purified by recrystallization (hexane:EtOAc) 18 mg of pure product **(66)** collected. 19% yield.

¹H NMR (500 MHz, CDCl₃): δ 6.30 (s, 1H), 6.05 (dd, *J* = 10.8, 17.3 Hz, 1H), 5.22 (d, *J* = 17.3, 1H), 5.14 (dd, *J* = 3.7, 7.0 Hz, 1H), 5.01 (d, *J* = 10.8 Hz, 1H), 4.99 (d, *J* = 7.0 Hz, 1H), 4.62 (m, 1H), 3.43 (d, *J* = 15.3 Hz, 1H), 2.53 (d, *J* = 3.9 Hz, 1H), 2.36 (d, *J* = 15.3 Hz, 1H), 2.27 (m, 2H), 1.74 (td, *J* = 3.7, 13.9 Hz, 1H), 1.49 (s, 3H), 1.45 (dq, *J* = 3.1, 6.4, 15.3, 1H), 1.41 (s, 3H), 1.32 (s, 3H), 1.24 (m, 1H), 1.20 (s, 3H), 1.12 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 207.3, 155.3, 145.7, 110.7, 84.2, 82.1, 81.8, 78.5, 77.1, 73.5, 49.2, 41.9, 41.4, 35.8, 34.3, 32.3, 30.1, 26.8, 24.4, 23.1, 20.0. **HRMS (ESI-TOF) *m/z*:** [M-H]⁺ calcd for C₂₁H₂₉O₇ 393.191; Found 393.2022

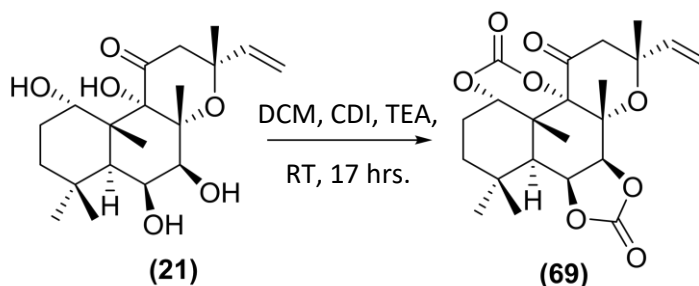


Preparation of Compound (67) and (68)

Forskolin (**1**) (205 mg, 0.5 mM) was dissolved in DCM (5 mL), 1,1-carbonyldiimidazole (81 mg, 0.5 mM) and triethylamine (69 μ L, 0.5 mM) were added. Solution stirred at room temperature for 18 hours. After 18 hours the solution was diluted with DCM (10 mL) then washed with water twice, followed by brine once then dried with NaSO_4 . 230 mg of product were isolated. Purification was complete by column chromatography (20:80 EtOAc:hexane), 192 mg of product (**67**) recovered, 89% yield followed by recrystallization with DCM/pentane. 18 mg of another product (**68**) was isolated, as a side product during production of 1,9-carbonateforskolin.

(**67**) $^1\text{H NMR}$ (500 MHz, CDCl_3): δ 5.95 (dd, J = 10.6, 17.1 Hz, 1H), 5.36 (d, J = 4.0, 1H), 5.29 (d, 17.1, 1H), 5.02 (d, J = 10.6 Hz, 1H), 4.89 (m, 1H), 4.48 (m, 1H), 3.12 (d, J = 16.5, 1H), 2.54 (d, J = 16.5, 1H), 2.17 (s, 3H), 2.11 (m, 1H), 1.90 (s, 1H), 1.82 (m, 1H), 1.73 (s, 3H), 1.65 (s, 1H), 1.58 (s, 1H), 1.55 (m, 1H), 1.54 (m, 1H), 1.39 (s, 3H), 1.26 (s, 3H), 1.25 (m, 1H), 1.04 (s, 3H). $^{13}\text{C NMR}$ (125 MHz, CDCl_3): δ 201.2, 169.2, 147.2, 144.8, 111.6, 87.2, 81.3, 80.1, 76.4, 75.8, 69.0, 49.9, 43.4, 36.5, 35.9, 33.9, 32.5, 30.4, 23.3, 23.05, 23.00, 21.1, 17.6. HRMS (ESI-TOF) m/z : $[\text{M}-\text{H}]^+$ calcd for $\text{C}_{23}\text{H}_{31}\text{O}_8$ 435.202; Found 481.2194 $[\text{M}-\text{H}+\text{Na}_2]$.

(**68**) $^1\text{H NMR}$ (500 MHz, CDCl_3): δ 6.12 (dd, 10.6, 17.4 Hz, 1H), 5.87 (m, 1H), 5.24 (d, J = 17.4 Hz, 1H), 5.08 (d, J = 10.6 Hz, 1H), 4.95 (m, 1H), 4.21 (m, 1H), 3.15 (d, J = 16.8 Hz, 1H), 2.59 (d, J = 16.8 Hz, 1H), 2.20 (d, J = 3.2 Hz, 1H), 2.13 (s, 3H), 2.07 (m, 1H), 1.84 (m, 1H), 1.65 (s, 1H), 1.64 (s, 3H), 1.61 (s, 3H), 1.57 (H_2O), 1.56 (m, 1H), 1.47 (s, 3H), 1.26 (m, 1H), 1.06 (s, 3H), 1.02 (s, 3H). $^{13}\text{C NMR}$ (125 MHz, CDCl_3): δ 200.8, 170.1, 147.1, 145.0, 111.8, 86.9, 81.1, 81.1, 76.6, 73.4, 69.8, 49.8, 42.8, 36.8, 36.1, 33.6, 32.5, 29.8, 22.9, 22.5, 21.8, 21.4, 19.4. HRMS (ESI-TOF) m/z : $[\text{M}-\text{H}]^+$ calcd for $\text{C}_{23}\text{H}_{31}\text{O}_8$ 435.202; Found 435.1960, 409.21 $[\text{M}-\text{acetyl}]$.

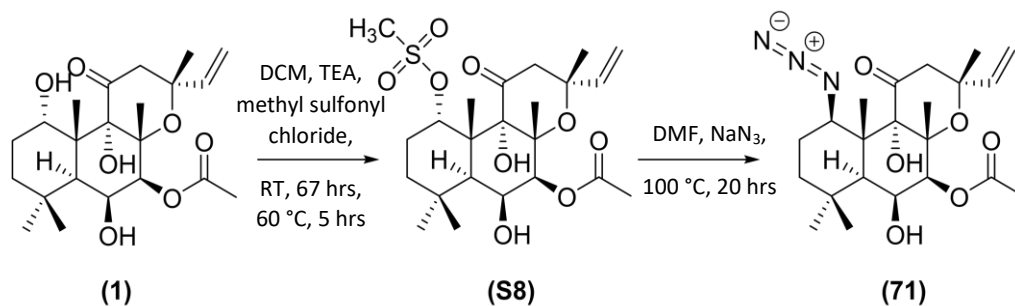


Preparation of Compound (69)

(**21**) (1.12 g, 2.8 mM) was dissolved in DCM (6 mL), then carbonyl diimidazole (0.9 g, 5.5 mM), triethyl amine (800 μ L, 5.7 mM) were added to the solution, which was then stirred at room temperature for 17 hours. The reaction was diluted with DCM (10 mL), then washed with water

twice, brine once follow. 1.34g residue recovered. The product was purified by biotage column DCM:EtOAc 98->80%, 10g sfär. 720 mg product recovered, 70% yield. As a side product 104 mg of 1,9-6,7-dicarbonate collected. Purified by biotage column, 10g sfär cartridge used. 2->20% EtOAc:DCM. 18mg of pure product recovered.

¹H NMR (500 MHz, CDCl₃): δ 6.07 (dd, *J* = 10.6, 17.1 Hz, 1H), 5.28 (d, *J* = 17.6 Hz, 1H), 5.11 (m, 1H), 5.11 (d, *J* = 10.6 Hz, 1H), 4.96 (m, 1H), 4.95 (m, 1H), 3.20 (d, *J* = 16.0 Hz, 1H), 2.57 (d, *J* = 16.0 Hz, 1H), 2.15 (m, 1H), 1.92 (m, 1H), 1.79 (d, *J* = 3.2 Hz, 1H), 1.60 (m, 1H), 1.56 (s, 3H), 1.53 (s, 3H), 1.39 (m, 1H), 1.25 (s, 3H), 1.12 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 200.5, 154.4, 146.6, 144.4, 112.1, 86.2, 82.7, 80.9, 80.3, 77.8, 76.5, 50.1, 41.9, 35.7, 33.8, 32.1, 29.5, 23.5, 22.8, 22.7, 17.6. **HRMS (ESI-TOF) *m/z*:** [M-H]⁺ calcd for C₂₂H₂₈O₈ 419.171; Found 419.1775

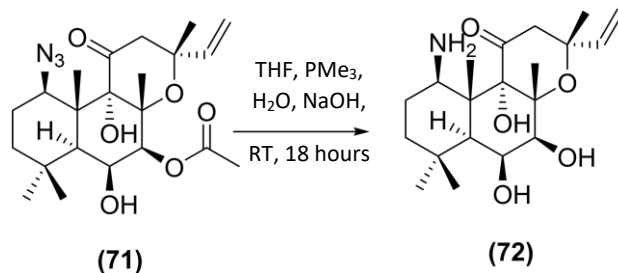


Preparation of Compound (71)

To a solution of **(1)** (117.4 mg, 0.29 mmol) in 4 mL of DCM, 126 mg (1.14 mmol) of triethylamine was added and the reaction mixture was cooled to 0 °C. Then, methanesulfonyl chloride (77 μL, 1.0 mmol) in 1 mL of DCM was added dropwise and reaction mixture was stirred at room temperature for 67 hours, by TLC no reaction appeared to have occurred. It was then stirred for 5 hours at 60 °C before being stopped and allowed to cool down to room temperature. The reaction mixture was diluted with 5 mL DCM and washed with water once, and brine three times then another 10 mL of water. The organic layer was dried with Na₂SO₄ and filtered before all solvent was evaporated with a rotary evaporator to leave behind 162.7 mg of crude product.

To a solution of 162.7 mg of crude **1-mesyl forskolin (S8)** dissolved in dimethylformamide (5 mL) sodium azide (49.5 mg, 0.762 mmol) was added. The reaction mixture was refluxed at 100 °C for 20 hours, then the solution was cooled to room temperature and diluted with water (20 mL). This solution was extracted with DCM twice. The organic layers were combined and washed with water and brine once before being dried with Na₂SO₄, filtered, and the solvent was removed by rotary evaporation to leave 85.2 mg of crude product. The crude product was purified via column chromatography using a 5g Sfär Biotage column to yield 19.2mg of **(71)** (15% over 2 steps).

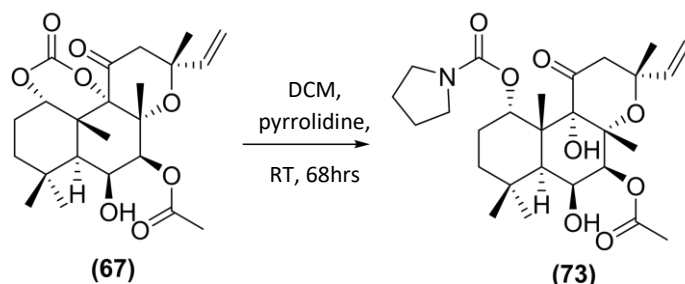
¹H NMR (CDCl₃, 500 MHz): 5.88 (dd, 1H, *J* = 11 Hz, 17.5 Hz), 5.38 (d, 1H, *J* = 4 Hz), 5.23 (dd, 1H, *J* = 1, 17.5 Hz), 5.11 (m, 1H), 4.99 (dd, 1H, *J* = 1, 11 Hz), 4.48 (m, 1H), 2.70 (d, 1H, *J* = 18.5 Hz), 2.38 (d, 1H, *J* = 2.5 Hz), 2.31 (d, 1H, *J* = 18.5 Hz), 2.19 (m, 1H), 2.15 (s, 3H), 1.83 (m, 1H), 1.73 (s, 3H), 1.69 (m, 1H), 1.53 (s, 3H), 1.35 (s, 3H), 1.28 (s, 3H), 1.16 (m, 1H), 1.08 (s, 3H). **¹³C NMR (CDCl₃, 125 MHz):** 201.7, 169.2, 145.4, 111.4, 88.3, 80.5, 75.7, 74.8, 69.4, 49.1, 42.5, 42.3, 35.4, 34.6, 32.8, 32.1, 24.7, 23.9, 23.1, 21.2, 17.1. **HRMS (ESI-TOF) *m/z*:** [M-H]⁺ calcd for C₂₂H₃₃N₃O₈ 434.229; Found 455.17 + 501.284 [M-H+Na₂]⁺



Preparation of Compound (72)

To a solution of **(71)** (195 mg) dissolved in 5 mL THF, PMe_3 (0.23 μL , 0.23 mM) in THF was added dropwise followed by 50 μL of distilled water. The reaction was stirred for 10 minutes, then NaOH (1.0 mL, 2M) was added. The solution was stirred for 18 hours at room temperature. The reaction mixture was then diluted with distilled water (10 mL) and HCl (1M) was added dropwise until the pH reached ~ 2 . This mixture was then washed with DCM (2x10 mL). NaOH (2 M) was added dropwise to the aqueous layer until the pH reached ~ 14 , then it was extracted with ethyl acetate (2x10 mL). The organic layers were then combined and was then washed with 10 mL brine, then dried with Na_2SO_4 , filtered, and the solvent was removed by rotary evaporation. The crude product was then separated via column chromatography using a 5g Sfar Biotage column to yield 32.4 mg of **(72)** (16% over 3 steps).

^1H NMR (CDCl_3 , 500 MHz): 6.12 (dd, $J=10, 17.5$ Hz, 1H), 5.20 (d, $J=17.5$ Hz, 1H), 4.99 (d, $J=10.5$ Hz, 1H), 4.64 (m, 1H), 4.50 (m, 1H), 4.14 (m, 1H), 2.52 (d, $J=17$ Hz, 1H), 2.37 (broad m, 1H), 2.22 (m, 1H), 2.18 (d, 1H, $J=17$ Hz), 2.11 (d, 1H), 1.73 (m, 1H), 1.66 (s, 3H), 1.45-1.39 (m, 7H), 1.28 (s, 3H), 1.12 (m, 1H), 1.07 (s, 3H). **^{13}C NMR (CDCl_3 , 125 MHz):** 205.6, 146.6, 110.4, 82.3, 82.2, 75.1, 74.9, 74.7, 70.5, 48.8, 43.0, 42.8, 36.1, 34.4, 33.0, 30.9, 26.9, 24.2, 23.4, 20.0. Mass Spec: Mass spec does not match as expected, further investigation needed.

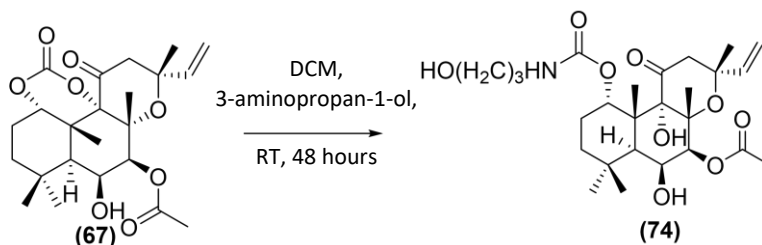


Preparation of Compound (73)

(67) (42mg, 0.096 mM) was dissolved in DCM (3 mL), then pyrrolidine (0.1 mL) was added, the solution was stirred at room temperature for 68 hours. DCM (10 mL) was added to the reaction mixture, then washed with water twice, brine once, then dried over NaSO₄, solvent removed by rotary evaporation.

Once dry the product was purified by flash chromatography with 1:1 ethyl acetate:hexane. 20mg of pure product **(73)** was recovered, a white solid. 42% yield.

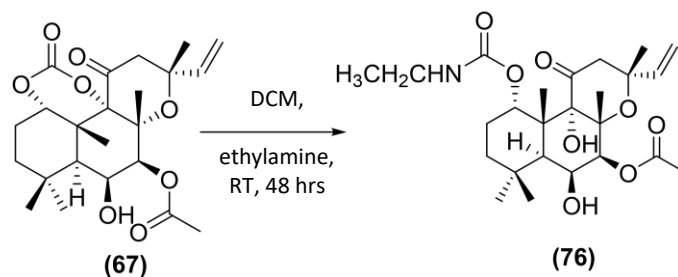
¹H NMR (500 MHz, CDCl₃): δ 5.98 (dd, *J* = 17.2, 10.5 Hz, 1H), 5.55 (d, *J* = 4.4 Hz, 1H), 5.51 (m, 1H), 5.18 (dd, *J* = 17.2, 0.9 Hz, 1H), 5.12 (s, 1H), 4.90 (dd, *J* = 5.6, 0.9 Hz, 1H), 4.46 (t, *J* = 3.2 Hz, 1H), 3.37 (m, 3H), 3.24 (m, 1H), 3.21 (d, *J* = 16.2 Hz, 1H), 2.38 (d, *J* = 16.2 Hz, 1H), 2.20 (d, *J* = 2.8 Hz, 1H), 2.17 (s, 3H), 2.14 (m, 1H), 1.86 (m, 5H), 1.71 (m, 1H), 1.69 (s, 3H), 1.55 (s, 3H), 1.54 (m, 1H), 1.33 (s, 3H), 1.28 (s, 3H), 1.16 (m, 1H), 1.04 (s, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 205.7, 169.7, 152.2, 146.3, 110.0, 82.0, 81.7, 78.1, 76.7, 75.6, 69.8, 48.9, 46.4, 45.9, 44.2, 43.3, 37.0, 34.2, 33.0, 31.0, 25.7, 24.7, 24.1, 24.0, 23.2, 21.2, 20.0. HRMS (ESI-TOF) *m/z*: [M-H]⁺ calcd for C₂₇H₄₀NO₈ 506.275; Found 552.2938 [M-H+Na₂].



Preparation of Compound (74)

(67) (110 mg, 0.25 mM) was dissolved in DCM (3 mL), 5 eq (94 μL) of a 3-aminopropan-1-ol was added to the mixture. This was stirred at room temperature for 48 hours, then washed with 5 mL of water. The water was back extracted with 5 mL of DCM, then the organic layers were combined and washed water twice and brine once before being dried with Na₂SO₄, filtered, and the solvent was removed by rotary evaporation. The crude product was purified via column chromatography using a 5g Sfär Biotage column (1:1 EtOAc:hexane) to yield 68 mg **(74)** of the final product.

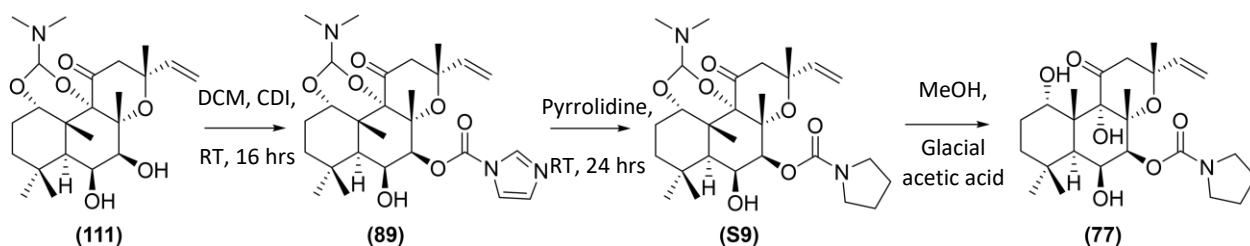
¹H NMR (CDCl₃, 500 MHz): δ 5.89 (dd, *J* = 16.5, 10.5 Hz, 1H), 5.51 (m, 1H), 5.47 (m, 1H), 5.24 (apparent d, *J* = 10.5 Hz, 1H), 5.14 (m, 1H), 4.91 (d, *J* = 16.5 Hz, 1H), 4.44 (m, 1H), 3.61 (m, 2H), 3.30 (m, 2H), 3.17 (d, *J*



Preparation of Compound (76)

(67) (110 mg, 0.25 mM) was dissolved in DCM (3 mL), 5 eq (82 μ L) of ethylamine was added to the mixture. This was stirred at room temperature for 48 hours, then washed with 5 mL of water. The water was back extracted with 5 mL of DCM, then the organic layers were combined and washed with water twice and brine once before being dried with Na_2SO_4 , filtered, and the solvent was removed by rotary evaporation. The crude product was purified via column chromatography using a 5g Sfär Biotage column to yield the final product. A total of 52.7 mg of (76) was obtained (41% yield).

$^1\text{H NMR}$ (CDCl_3 , 500 MHz): 5.89 (dd, $J = 17.1, 10.5$ Hz, 1H), 5.50 (d, $J = 4.2$ Hz, 1H), 5.48 (m, 1H), 5.23 (dd, $J = 17.1, 1.0$ Hz, 1H), 4.92 (dd, $J = 10.5, 1.0$ Hz, 1H), 4.80 (bs, 1H), 4.44 (t, $J = 3.3$, 1H), 3.62 (t, $J = 5.6$ Hz, 1H), 3.30 (m, 1H), 3.17 (d, $J = 16.5$ Hz, 1H), 2.41 (d, $J = 16.5$, 1H), 2.16 (s, 3H), 2.15 (m, 1H), 2.14 (m, 1H), 1.69 (s, 3H), 1.67-1.56 (m, 4H), 1.32 (s, 3H), 1.26 (s, 3H), 1.15 (m, 1H), 1.02 (s, 3H). $^{13}\text{C NMR}$ (CDCl_3 , 125 MHz): 205.1, 169.8, 146.2, 110.1, 82.0, 81.8, 77.6, 77.0, 76.4, 75.5, 70.0, 48.6, 43.9, 43.2, 36.8, 34.2, 32.9, 32.4, 31.3, 24.2, 23.7, 23.6, 21.2, 19.8. HRMS (ESI-TOF) m/z : $[\text{M}-\text{H}]^+$ calcd for $\text{C}_{25}\text{H}_{38}\text{NO}_8$ 480.268; Found 480.2886, 526.2686 $[\text{M}-\text{H}+\text{Na}_2]^+$.

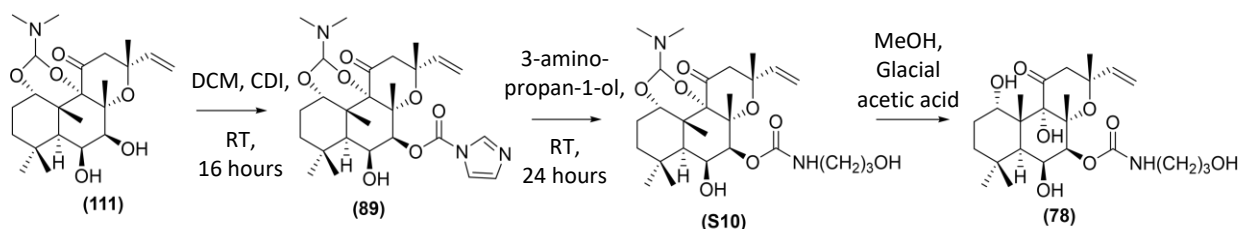


Preparation of Compound (77)

(111) (98 mg, 0.23 mM) was dissolved in DCM (4 mL), then carbonyldiimidazole (46 mg, 0.28 mM) was added, the solution was stirred at RT for 16 hours. Pyrrolidine (100 μ L, 1.2 mM) was then added to solution and stirred at RT for 24 hours. When finished the reaction was washed with water, which was then back extracted with DCM, the organic layers were then combined, washed with water twice and brine once. The solution was dried with sodium sulphate and the solvent removed by rotary evaporation.

To remove the protecting group MeOH (3.4 mL) and glacial acetic acid (1.2 mL) were added to the residue, stirred for 18 hours at room temperature. Saturated sodium carbonate was added to the reaction, then extracted three times with DCM. The organic layers were combined then dried with brine and sodium sulphate. 105 mg residue was recovered. This was then purified by column chromatography, 80:20 DCM:EtOAc -> 50:50. 32 mg pure product (**77**) collected. Yield 30%.

¹H NMR (500 MHz, CDCl₃): δ 5.97 (dd, *J* = 10.6, 17.2, Hz, 1H), 5.81 (s, 1H), 5.33 (d, *J* = 17.2 Hz, 1H), 5.23 (d, *J* = 4.0 Hz, 1H), 4.99 (d, *J* = 10.6 Hz, 1H), 4.56 (m, 1H), 4.56 (m, 1H), 3.44 (m, 4H), 3.22 (s, 1H), 3.17 (d, *J* = 17.2 Hz, 1H), 2.50 (d, *J* = 17.2 Hz, 1H), 2.20 (d, *J* = 2.2 Hz, 1H), 2.15 (m, 1H), 1.92 (m, 4H), 1.83 (s, 1H), 1.80 (m, 1H), 1.73 (s, 3H), 1.44 (m, 4H), 1.36 (s, 3H), 1.26 (m, 4H), 1.09 (m, 1H), 1.04 (s, 1H). **¹³C NMR (125 MHz, CDCl₃):** δ 205.0, 154.0, 146.3, 110.9, 82.7, 81.5, 77.6, 74.8, 74.4, 70.0, 48.6, 46.3, 45.9, 43.0, 42.5, 36.1, 34.5, 33.0, 31.5, 26.5, 25.7, 24.9, 24.4, 23.7, 19.8. **HRMS (ESI-TOF) *m/z*:** [M-H]⁺ calcd for C₂₅H₈₅NO₇ 464.264; Found 510.2667 [M-H+Na₂]⁺.



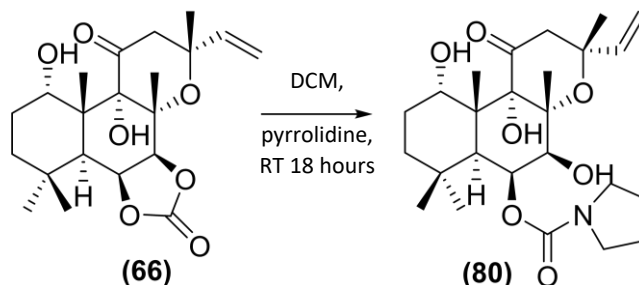
Preparation of Compound (78)

(**111**) (98mg, 0.22 mM) was dissolved in DCM (4 mL), then 1-1' carbonyldiimidazole (46 mg, 0.28 mM) was added, the reaction was stirred at room temperature for 19 hours. 3-amino propanol (90 μL, 1.2 mM) was then added, and the solution was stirred for a further 24 hours. When finished the reaction was washed with water, which was then back extracted with DCM twice, the organic layers were then combined, washed with water twice and brine once. The solution was dried with sodium sulphate and the solvent removed by rotary evaporation.

The dried sample was dissolved in MeOH (3.4 mL), then glacial acetic acid (1.2 mL) was added. The solution was stirred at room temperature for 18 hours, then a saturated solution of Na₂CO₃ (10 mL) was added. The reaction mixture was then extracted with DCM three times. DCM layers were combined and dried with NaSO₄, then the solvent was removed by rotary evaporation.

Purified by flash chromatography with 1:1 ethyl acetate:hexane. 62 mg product (**78**) recovered. 57% yield.

¹H NMR (500 MHz, CDCl₃): δ 6.04 (s, 1H), 5.97 (dd, *J* = 10.7, 17.2 Hz, 1H), 5.34 (d, *J* = 17.2 Hz, 1H), 5.23 (m, 1H), 5.00 (d, *J* = 10.7 Hz, 1H), 4.56 (m, 1H), 4.52 (m, 1H), 3.67 (m, 2H), 3.45 (m, 1H), 3.29 (m, 1H), 3.20 (d, *J* = 17.2 Hz, 1H), 3.08 (s, 1H), 2.69 (bs, 1H), 2.47 (d, *J* = 17.2 Hz, 1H), 2.19 (m, 1H), 2.17 (m, 1H), 1.92 (bs, 1H), 1.76 (m, 3H), 1.69 (s, 3H), 1.63 (s, 1H), 1.43 (s, 3H), 1.41 (m, 1H), 1.35 (s, 3H), 1.26 (m, 3H), 1.01 (m, 1H), 1.04 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 205.3, 156.9, 146.2, 111.0, 82.7, 81.9, 77.8, 75.2, 74.4, 70.3, 59.3, 48.8, 43.0, 42.7, 37.7, 36.1, 34.4, 33.0, 32.7, 31.5, 26.6, 24.4, 23.6, 19.8. **HRMS (ESI-TOF) *m/z*:** [M-H]⁺ calcd for C₂₄H₃₈NO₈ 468.260; Found 514.26 [M-H+Na₂]⁺.

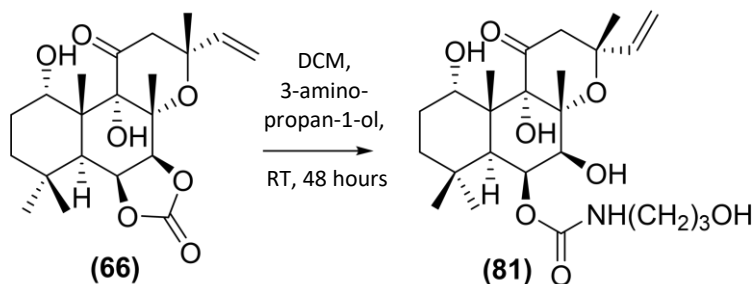


Preparation of Compound (80)

7-deactyl-6,7-carbonate forskolin (**66**) (25 mg, 0.05 mM) was dissolved in DCM (2 mL), then pyrrolidine (0.1 mL) was added. The reaction was stirred at room temperature for 18 hours. DCM (10 mL) was added to the reaction mixture, then washed with water twice, HCl (0.1 M) once, brine once, then dried over NaSO₄, solvent was removed by rotary evaporation. 23 mg of residue recovered.

The product was purified by column chromatography with 20% then 50% isocratically EtOAc:hexane. 12 mg of product (**80**) was recovered. Yield 41%.

¹H NMR (500 MHz, CDCl₃): δ 6.15 (bs, 1H), 6.11 (dd, *J* = 10.6, 17.4 Hz, 1H), 5.75 (m, 1H), 5.22 (d, *J* = 17.4 Hz, 1H), 5.01 (d, *J* 10.6 = Hz, 1H), 4.65 (m, 1H), 4.27 (d, *J* = 4.4, 1H), 3.47 (m, 2H), 3.37 (m, 2H), 3.18 (d, *J* = 17.4 Hz, 1H), 2.98 (bs, 1H), 2.54 (d, *J* = 17.4 Hz, 1H), 2.34 (d, *J* = 2.5 Hz, 1H), 2.12 (m, 1H), 1.88 (m, 4H), 1.81 (m, 1H), 1.64 (s, 3H), 1.44 (m, 1H), 1.42 (s, 3H), 1.41 (s, 3H), 1.11 (m, 1H), 1.09 (s, 3H), 1.01 (s, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 205.2, 155.4, 146.4, 110.7, 82.3, 82.1, 75.1, 74.4, 73.9, 72.3, 48.6, 46.6, 45.7, 43.1, 42.1, 36.2, 34.1, 33.0, 31.0, 26.5, 25.8, 24.8, 23.8, 22.7, 19.5. HRMS (ESI-TOF) *m/z*: [M-H]⁺ calcd for C₂₅H₃₈NO₇ 464.265; Found 464.2584

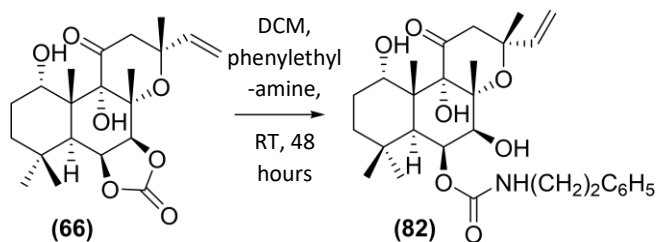


Preparation of Compound (81)

(**66**) (70 mg, 0.18 mM) was dissolved in DCM (3 mL), then 3-amino-propan-1-ol (80 μL, mM) was added, and then stirred at room temperature for 48 hours. DCM (10 mL) was added to the reaction mixture, then washed with water twice, HCl (0.1 M) once, brine once, then dried over NaSO₄, solvent was removed by rotary evaporation. Column run biotage 5 g sfär. 41 mg of product (**81**) recovered, yield 49%. Recrystallized with DCM:pentane.

¹H NMR (500 MHz, CDCl₃): δ 6.21 (bs, 1H), 6.11 (dd, *J* = 10.7, 17.4 Hz, 1H), 5.69 (m, 1H), 5.21 (d, *J* = 17.4 Hz, 1H), 5.00 (d, *J* 10.6 = Hz, 1H), 4.90 (m, 1H), 4.64 (m, 1H), 4.26 (m, 1H), 4.15 (t, *J* = 6.1 Hz, 2H), 3.30 (m, 2H), 3.18 (d, *J* = 17.4 Hz, 1H), 2.79 (m, 1H), 2.53 (d, *J* 17.4 = Hz, 1H), 2.35 (s, 1H), 2.30 (d, *J* = 2.3 Hz, 1H), 2.14 (m, 1H), 2.05 (s, 3H),

1.86 (m, 2H), 1.79 (m, 1H), 1.61 (s, 3H), 1.43 (m, 2H), 1.42 (s, 3H), 1.12 (m, 1H), 1.09 (s, 3H), 1.02 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 205.3, 171.2, 156.6, 146.4, 110.6, 82.2, 82.2, 75.2, 74.5, 73.9, 72.6, 61.7, 48.7, 43.2, 42.1, 38.0, 36.2, 34.0, 32.9, 26.6, 23.6, 22.7, 20.9, 19.8. **HRMS (ESI-TOF) m/z:** [M-H]⁺ calcd for C₂₄H₃₈NO₈ 468.260

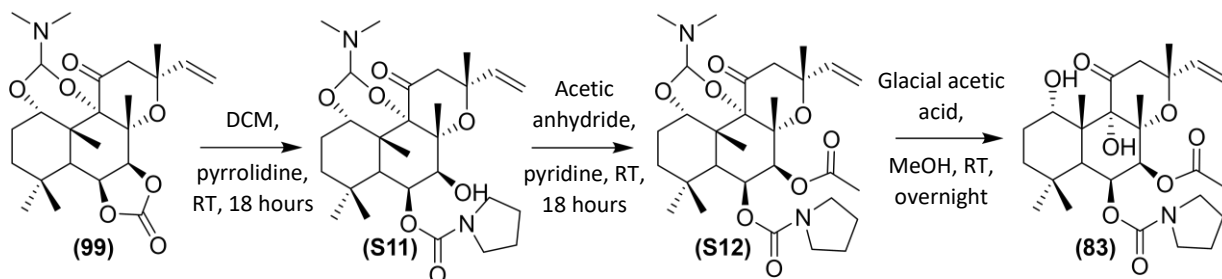


Preparation of Compound (82)

(66) (70 mg, 0.18 mM) was dissolved in 3 mL of DCM, then phenylethylamine (120 μL, 1 mM), was added and the mixture was stirred at RT for 48 hours. DCM (10 mL) was added to the reaction mixture, then washed with water twice, HCl (0.1 M) once, brine once, then dried over NaSO₄, solvent was removed by rotary evaporation. 62 mg recovered. The product was purified by column chromatography with hexane:EtOAc 7:3. 50 mg product (82) collected. 55% yield.

¹H NMR (500 MHz, CDCl₃): δ 7.29 (m, 2H), 7.21 (m, 3H), 6.25 (bs, 1H), 6.11 (dd, *J* = 10.7, 17.2 Hz, 1H), 5.69 (m, 1H), 5.21 (d, *J* = 71.2 Hz, 1H), 5.00 (d, *J* = 10.7 Hz, 1H), 4.66 (m, 1H), 4.62 (s, 1H), 4.27 (m, 1H), 3.46 (m, 2H), 3.18 (d, *J* = 17.1 Hz, 1H), 2.95 (s, 1H), 2.83 (m, 2H), 2.52 (d, *J* = 17.1 Hz, 1H), 2.44 (m, 1H), 2.30 (bs, 1H), 2.11 (m, 1H), 1.78 (t, *J* = Hz, 1H), 1.65 (s, 1H), 1.57 (s, 3H), 1.43 (s, 3H), 1.42 (m, 1H), 1.30 (s, 3H), 1.11 (m, 1H), 1.09 (s, 3H), 0.98 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 205.4, 156.6, 146.4, 138.8, 129.0, 129.0, 128.5, 128.5, 126.4, 110.7, 82.2, 75.2, 74.4, 74.0, 72.6, 48.7, 43.1, 42.5, 42.1, 36.2, 36.1, 34.0, 32.9, 31.0, 26.5, 23.7, 22.8, 19.7. **HRMS (ESI-TOF) m/z:** [M-H]⁺ calcd for C₂₉H₄₀NO₇ 514.280; Found 514.2731.

Preparation of Compound (83)



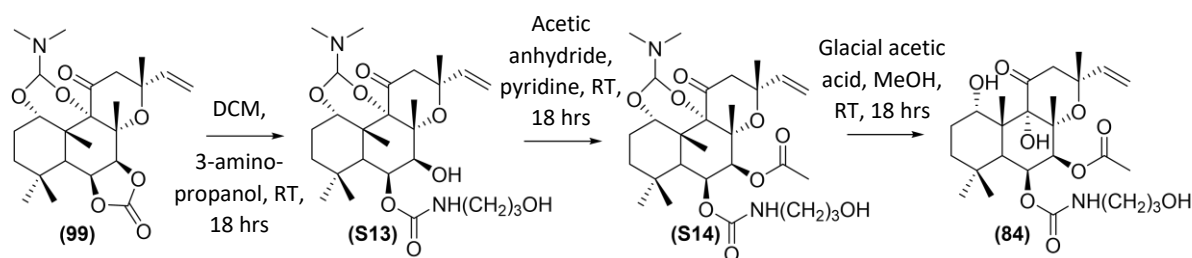
(99) (70 mg, 0.14 mM) was dissolved in DCM (3 mL), then pyrrolidine (0.1 mL) was added, the solution was stirred at room temperature for 18 hours. DCM (10 mL) was added, then the solution was washed with H₂O twice and brine once, the solvent was then removed by rotary evaporation.

Pyridine (0.75 mL), acetic anhydride (0.75 mL) added to residue, then stirred for 18 hours. Once complete the reaction was diluted with DCM (10 mL), washed with HCl (0.1 M), water twice and brine once, the remaining solvent removed by rotary evaporation.

MeOH (3.0 mL), glacial acetic acid (1.5 mL) was added to residue, stirred overnight at room temperature, saturated sodium carbonate was then added. This was then extracted with DCM three times. The organic layers were combined, and dried with NaSO₄. Solvent was removed by rotary evaporation. 106 mg residue collected.

The product was purified by column chromatography, with 5->25% EtOAc:DCM. 16 mg of product (**83**) was collected, yield 21%.

¹H NMR (500 MHz, CDCl₃): δ 6.03 (bs, 1H), 5.95 (dd, *J* = 10.9, 17.1, 1H), 5.73 (m 1H), 5.49 (d, *J* = 4.7 Hz, 1H), 5.28 (d, *J* = 17.1, 1H), 4.98 (d, *J* = 10.9 Hz, 1H), 4.58 (m, 1H), 3.41 (m, 4H), 3.22 (d, *J* = 17.1, 1H), 3.20 (bs, 1H), 2.47 (d, *J* = 10.9 Hz, 1H), 2.38 (d, *J* = 2.8 Hz, 1H), 2.10 (m, 1H), 2.03 (s, 3H), 1.89 (m, 4H), 1.80 (m, 1H), 1.68 (s, 3H), 1.66 (m, 1H), 1.44 (s, 3H), 1.44 (m, 1H), 1.35 (s, 3H), 1.08 (m, 1H), 1.06 (s, 3H), 0.98 (s, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 205.5, 169.9, 154.2, 146.1, 110.8, 82.5, 80.9, 75.1, 74.1, 74.09, 70.2, 48.8, 46.5, 45.6, 45.6, 43.0, 42.1, 36.2, 34.2, 31.3, 26.4, 25.8, 24.8, 23.7, 23.1, 21.0, 19.4. HRMS (ESI-TOF) *m/z*: [M-H]⁺ calcd for C₂₇H₄₀NO₈ 506.275; Found 552.286 [M-H+Na]⁺.



Preparation of Compound (84)

(**99**) (Protected Carbonate) (53 mg, 0.1 mM) was dissolved in DCM (3 mL), then 3-amino-1-propanol (0.1 mL) was added, and stirred overnight at room temperature. DCM (10 mL) was added, the solution was washed with H₂O twice and brine once. The solution was dried with NaSO₄, then the solvent was removed by rot evaporation.

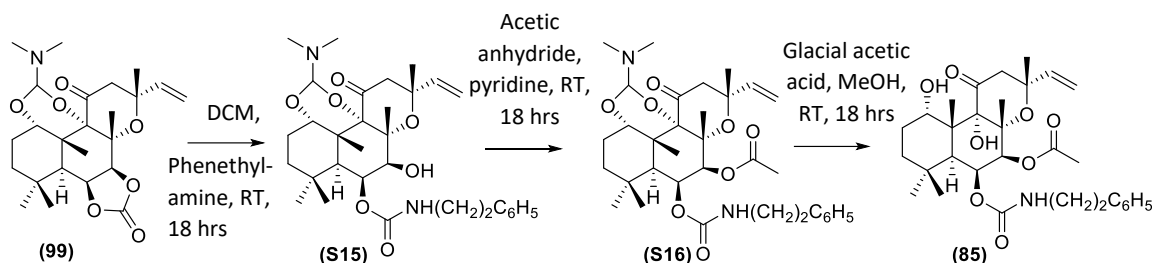
Pyridine (0.8 mL), acetic anhydride (0.8 mL) were added to the residue, the reaction was stirred for 18 hours. Then the reaction was diluted with DCM (10 mL), then washed with HCl (0.1 M) and water twice, the remaining solvent was removed by rotary evaporation.

MeOH (1.5 mL), glacial acetic acid (1 mL) were added to the residue, stirred overnight at room temperature, then saturated sodium carbonate (10 mL) was added, and extracted with DCM three times. The organic layers were combined, then dried with NaSO₄. The solvent was removed by rotary evaporation. 67 mg of residue was recovered.

The product was purified by biotage isolera, with a 10 g Sfär cartridge. DCM:EtOAc 98->80%. 15 mg of pure product (**84**) recovered. 32% yield.

¹H NMR (500 MHz, CDCl₃): δ 5.98 (bs, 1H), 5.95 (dd, *J* = 10.6, 17.1, 1H), 5.66 (m, 1H), 5.49 (d, *J* = 4.2, 1H), 5.27 (d, *J* = 17.1 Hz, 1H), 4.98 (d, *J* = 10.6 Hz, 1H), 4.59 (m, 1H), 4.15 (m, 2H), 3.28 (m, 2H), 3.21 (d, *J* = 17.1 Hz, 1H), 3.00 (bs, 1H), 2.48 (d, *J* = 17.1 Hz, 1H), 2.36 (d, *J* = 2.0 Hz, 1H), 2.11 (m, 1H), 2.06 (s, 3H), 2.03 (s, 3H), 1.86 (m, 2H), 1.80 (m, 1H), 1.65 (s, 3H), 1.44 (m, 1H), 1.39 (s, 3H), 1.35 (s, 3H), 1.25 (m, 1H), 1.10 (m, 1H), 1.05 (s, 3H), 0.99 (s, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 205.2, 169.8, 146.1, 110.8, 82.4, 81.0, 75.1, 74.3, 74.1, 70.5, 61.6, 48.8, 43.1, 41.9, 37.9,

36.1, 34.1, 32.8, 31.3, 29.7, 29.1, 26.4, 23.7, 23.1, 20.9, 19.7 **HRMS (ESI-TOF) m/z:** [M-H]⁺ calcd for C₂₆H₄₀NO₉ 510.270; Found 510.2633



Preparation of Compound (85)

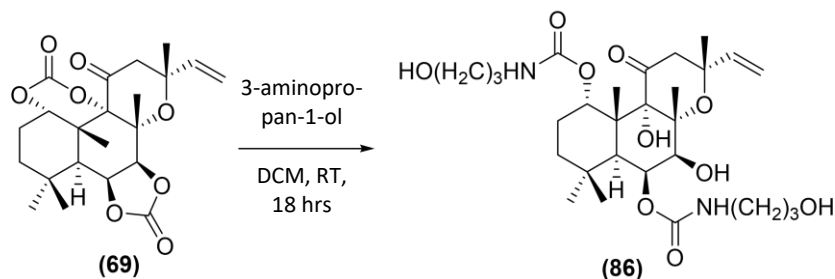
(99) (Protected Carbonate) (70 mg, 0.14 mM) was dissolved in DCM (3 mL), then phenethylamine (0.1 mL) was added, the solution was stirred for 40 hours at room temperature. DCM (10 mL) was then added, and washed with H₂O twice and brine once, the solvent was then removed by rotary evaporation.

Pyridine (0.75 mL), acetic anhydride (0.75 mL) were added to the residue, stirred overnight. Reaction mixture was then diluted with DCM (10 mL), washed with HCl (0.1 M), remaining solvent removed by rotary evaporation.

MeOH (0.75 mL), glacial acetic acid (0.5 mL) were added to the residue, and stirred for 20 hours at room temperature. Saturated sodium carbonate (10 mL) was added, then extracted with DCM three times. Organic layers were combined, then dried with NaSO₄. Solvent removed by rotary evaporation.

The product was then purified by column chromatography, biotage 10g Sfär, DCM:EtOAc, 95->70%. 32 mg of product **(85)** recovered. Yield 38%.

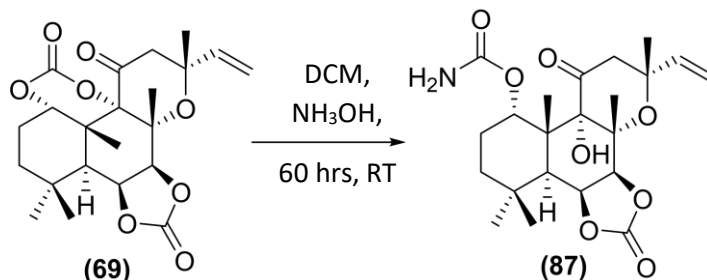
¹H NMR (500 MHz, CDCl₃): δ 7.30 (m, 2H), 7.22 (m, 3H), 6.03 (bs, 1H), 5.96 (dd, *J* = 10.6, 17.3 Hz, 1H), 5.68 (m, 1H), 5.51 (d, *J* = 4.3, 1H), 5.28 (d, *J* = 17.3 Hz, 1H), 4.99 (d, *J* = 10.6 Hz, 1H), 4.64 (m, 1H), 4.57 (bs, 1H), 3.72 (q, *J* = 6.9, 13.8, 1H), 3.52 (m, 1H), 3.39 (m, 1H), 3.22 (d, *J* = 16.9, 1H), 3.07 (bs, 1H), 2.82 (t, *J* = 6.9 Hz, 1H), 2.48 (d, *J* = 16.9 Hz, 1H), 2.36 (m, 1H), 2.10 (m, 2H), 2.06 (s, 3H), 1.80 (m, 1H), 1.63 (s, 3H), 1.58 (s, H₂O), 1.45 (m, 2H), 1.37 (s, 3H), 1.34 (s, 2.75H), 1.10 (m, 1H), 1.06 (s, 3H), 0.97 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 205.4, 169.9, 155.4, 146.1, 138.8, 128.9, 128.6, 126.5, 110.8, 82.4, 81.0, 75.2, 74.4, 74.1, 70.4, 58.5, 48.8, 43.1, 42.4, 41.9, 36.3, 32.1, 34.1, 32.8, 31.3, 26.4, 23.7, 23.2, 21.0, 19.7, 18.4. **HRMS (ESI-TOF) m/z:** [M-H]⁺ calcd for C₃₁H₄₂NO₈ 556.291; Found 556.296



Preparation of Compound (86)

1,9-6,7-dicarbonate (**32**) (40 mg, 0.12 mM), was dissolved in DCM (3 mL), then 3-aminopropan-1-ol (0.1 mL) was added. The reaction was stirred at room temperature for 20 hours. DCM (10 mL) was then added, and washed with H₂O twice and brine once, followed by drying with NaSO₄. The solvent was then removed by rotary evaporation. 55 mg of off-white solid collected. TLC 70:30 EtOAc:DCM. Biotage column carried out, 15-100% EtOAc with DCM. Product elutes in 100% EtOAc.

¹H NMR (500 MHz, CDCl₃): δ 6.07 (dd, *J* = 10.7, 17.3 Hz, 1H), 5.71 (s, 1H), 5.55 (s, 1H), 5.17 (d, *J* = 17.3 Hz, 1H), 5.02 (m, 1H), 4.97 (d, *J* = 10.7 Hz, 1H), 4.96 (s, 1H), 4.84 (s, 1H), 4.29 (m, 1H), 3.71 (m, 2H), 3.64 (m, 2H), 3.42 (m, 2H), 3.33 (m, 2H), 3.17 (d, *J* = 16.7 Hz, 1H), 2.66 (bs, 1H), 2.49 (d, *J* = 16.7 Hz, 1H), 2.40 (m, 2H), 2.26 (bs, 1H), 2.10 (m, 1H), 1.67 (m, 8H), 1.60 (s, 3H), 1.45 (s, 3H), 1.40 (s, 3H), 1.17 (m, 1H), 1.1 (s, 3H), 1.03 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 204.8, 157.6, 146.3, 110.2, 82.5, 81.6, 77.7, 75.7, 73.9, 72.4, 59.7, 59.5, 48.6, 43.4, 43.2, 38.0, 37.0, 33.8, 32.9, 32.7, 32.3, 30.8, 23.6, 23.5, 23.0, 19.8. **HRMS (ESI-TOF) *m/z*:** [M-H]⁺ calcd for C₂₈H₄₅N₂O₁₀ [M-H]⁺ 569.307 found 615.316, [M-H+Na₂]⁺.

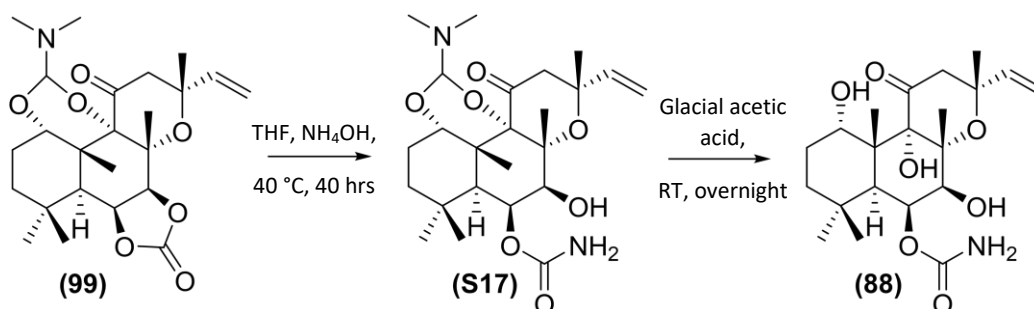


Preparation of Compound (87)

(**69**) (40 mg, 0.9 mM) was dissolved in DCM (3 mL), then ammonium hydroxide (0.1 mL) was added. The solution was stirred at room temperature for 60 hours. DCM (10 mL) was then added, and washed with H₂O twice and brine once, followed by drying with NaSO₄. The solvent was then removed by rotary evaporation. 34 mg residue recovered. Purification by column chromatography (biotage, 5 g, Sfar) with 5-20% EtOAc:DCM. 18 mg of product (**87**) isolated (46% yield).

¹H NMR (500 MHz, CDCl₃): δ 6.01 (dd, *J* = Hz, 1H), 5.51 (m, 1H), 5.23 (d, *J* = Hz, 1H), 5.14 (m, 1H), 5.02 (d, *J* = Hz, 1H), 4.98 (d, *J* = Hz, 1H), 4.62 (bs, 1H), 4.62 (s, 1H), 4.53 (s, 1H), 3.32 (d, *J* = Hz, 1H), 2.46 (d, *J* = Hz, 1H), 2.42 (d, *J* = Hz, 1H), 2.19 (m, 1H), 1.78 (m, 1H), 1.58 (m, 1H), 1.55 (H₂O), 1.53 (s, 3H), 1.42 (s, 3H), 1.41 (s, 3H), 1.30 (m, 1H), 1.23 (s,

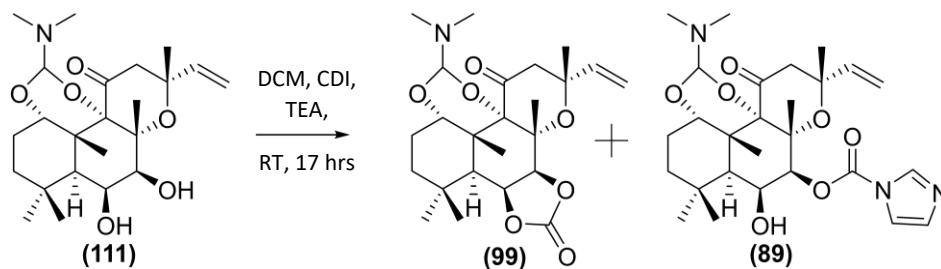
3H), 1.14 (s, 3H). **¹³C NMR (125 MHz, CDCl₃)**: δ 205.5, 154.9, 153.2, 145.6, 110.7, 83.9, 82.3, 81.5, 78.0, 77.1, 49.2, 42.7, 42.3, 36.5, 34.2, 32.3, 30.1, 24.4, 23.0, 23.0, 20.0. **HRMS (ESI-TOF) m/z**: [M-H]⁺ calcd for C₂₂H₃₀NO₈ [M-H]⁺ 436.197 found 482.208 [M-H+Na₂]⁺.



(99) (Protected Carbonate) (55 mg, 0.14 mM) was dissolved in THF (2 mL), then ammonium hydroxide (0.1 mL) was added, and stirred for 40 hours at 40 °C. The reaction was quenched with H₂O (5 mL), the reaction was then extracted with DCM three times, the organic layers were combined and washed with brine, solvent removed by rot evaporation. 50 mg white solid collected.

MeOH (0.75 mL), then glacial acetic acid (0.5 mL) were added to the residue, which was stirred overnight at room temperature, then saturated sodium carbonate (10 mL) was added, and extracted with DCM x3. The organic layers were combined, and dried with NaSO₄. Solvent removed by rotary evaporation. The product was purified by column chromatography with DCM:EtOAc 20:80, 22 mg recovered, 42% yield. Final purification by recrystallization with DCM:pentane, 3 mg of product **(88)** recovered.

¹H NMR (500 MHz, (CD₃)₂CO): δ 7.34 (m, 1H), 6.17 (dd, *J* = 10.6, 17.3, 1H), 5.63 (dd, *J* = 3.7, 4.0 Hz, 1H), 5.15 (dd, *J* = 1.1, 17.3 Hz, 1H), 5.11 (m, 1H), 4.84 (dd, *J* = 1.1, 106 Hz, 1H), 4.61 (m, 1H), 4.28 (m, 1H), 3.47 (m, 1H), 3.15 (dd, *J* = 1.6, 16.4, 1H), 2.82 (H₂O), 2.79 (HDO) 2.40 (dd, *J* = 1.6, 16.4 Hz, 1H), 2.19 (m, 1H), 1.86 (m, 1H), 1.60 (s, 3H), 1.44 (m, 1H), 1.41 (s, 3H), 1.35 (s, 3H), 1.11 (s, 3H), 1.09 (m, 1H), 1.08 (s, 3H). **¹³C NMR (125 MHz, (CD₃)₂CO)**: δ 206.2, 163.4, 147.9, 108.7, 82.3, 75.3, 73.7, 73.4, 72.7 72.0, 49.1, 42.5, 36.6, 33.7, 32.3, 30.0, 29.5, 26.3, 22.9, 22.6, 19.2. **HRMS (ESI-TOF) m/z**: [M-H]⁺ calcd for C₂₁H₃₂NO₇ 410.218; Found 410.2245.

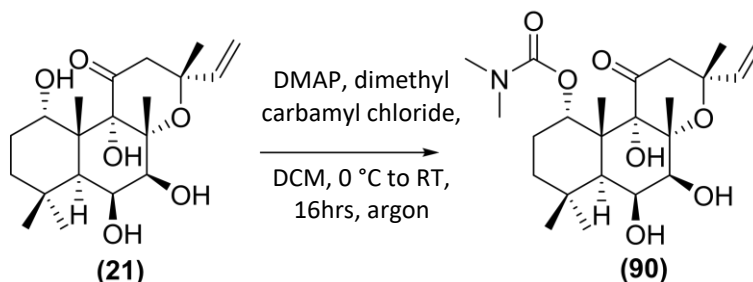


Preparation of Compound (89)

(111) (1.12 g, 2.8 mM) was dissolved in DCM (6 mL), carbonyl diimidazole (0.90 g, 5.5 mM) and triethyl amine (800 μ L, 5.8 mM) were added, the reaction was stirred at room temperature for 17 hours. The reaction was diluted with DCM (10 mL), then washed with water twice, followed by brine once. The organic layer was dried with NaSO_4 , A rotary evaporator was used to remove remaining solvent. 1.34 g residue recovered.

Purified by column chromatography with DCM:EtOAc 80:20%. 38 mg/ 100 mg collected when column run on fractions from another column.

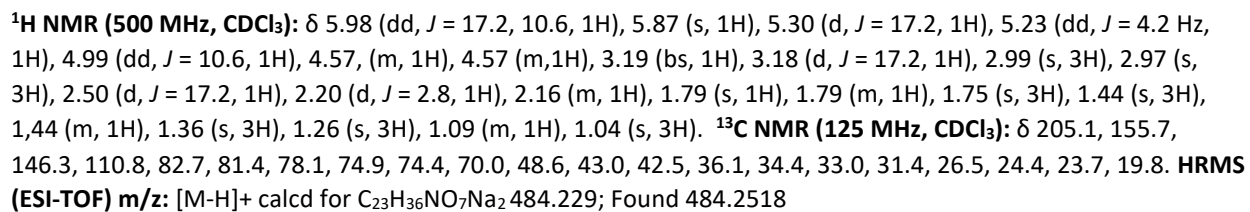
$^1\text{H NMR}$ (500 MHz, CDCl_3): δ 8.02 (s, 1H), 7.38 (s, 1H), 7.00 (s, 1H), 5.82 (dd, J = 10.7, 17.1 Hz, 1H), 5.41 (d, J = 3.9, 1H), 5.23 (d, J = 17.1 Hz, 1H), 4.93 (d, J = 10.7, 1H), 4.77 (s, 1H), 4.74 (d, J = 2.6 Hz, 1H), 4.18 (m, 1H), 3.66 (s, 1H), 2.89 (d, J = 16.4, 1H), 2.45 (s, 6H), 2.45 (m, 1H), 2.09 (m, 1H), 1.86 (s, 3H), 1.82 (m, 1H), 1.64 (m, 1H), 1.60 (s, 3H), 1.34 (s, 3H), 1.32 (s, 3H), 1.14 (m, 1H), 1.09 (s, 3H). $^{13}\text{C NMR}$ (125 MHz, CDCl_3): δ 205.3, 147.7, 145.5, 136.8, 130.2, 117.4, 110.8, 104.7, 84.3, 81.4, 80.8, 75.7, 75.6, 68.8, 50.0, 43.1, 38.6, 37.4, 37.4, 36.5, 34.4, 33.2, 31.2, 24.5, 23.6, 23.5, 17.0. **HRMS (ESI-TOF) m/z** : $[\text{M}-\text{H}]^+$ calcd for $\text{C}_{27}\text{H}_{38}\text{N}_3\text{O}_7$ 516.271; Found Not LCMS Stable

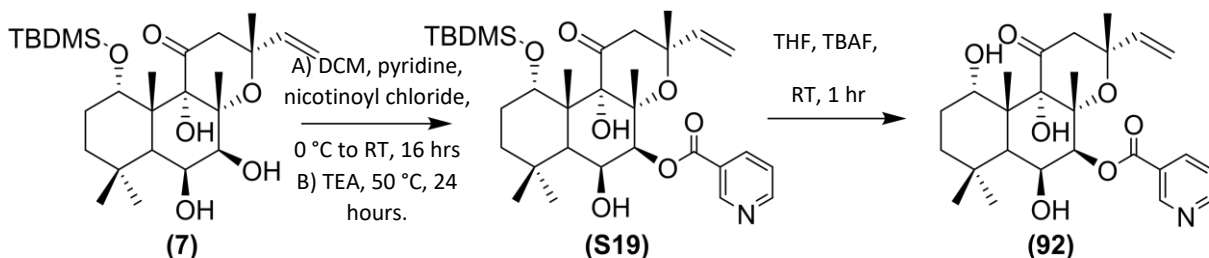


Preparation of Compound (90)

Deacyl forskolin (**(21)**) (48 mg, 0.13 mM) was dissolved in DCM (3 mL), then DMAP (35 mg, 0.29 mM) followed by dimethyl carbamyl chloride (20 μ L, 0.22 mM) were added once the solution had reached 0 $^\circ\text{C}$, and the vessel had been purged with argon. The solution was then stirred for 16 hours, with the temperature being allowed to come to room temperature. The reaction mixed was diluted with DCM (10 mL) then washed twice with water and once with brine. Solution was then dried with sodium sulfate and the DCM was removed by rotary evaporation.

¹H NMR (500 MHz, CDCl₃): δ 6.11, (dd, *J* = 17.4, 10.6, 1H), 5.58 (m, 1H, C1-βH), 5.15 (d, *J* = 17.4, 1H), 5.05 (s, 1H), 4.96 (d, *J* = 10.6, 1H), 4.50 (m, 1H, C6-αH), 4.17 (m, 1H, C7-αH), 3.16 (d, *J* = 17.2 Hz, 1H), 2.90 (s, 6H) 2.47 (d, *J* = 17.2 Hz, 1H), 2.41 (bs, 1H), 2.33 (m, 1H), 2.15 (m, 1H), 2.09 (m, 1H) 1.66 (m, 4H), 1.51 (m, 4H), 1.41 (s, 3H), 1.29 (s, 3H), 1.18 (m, 1H), 1.07 (s, 3H), 1.02 (s, 3H). **¹³C NMR (125 MHz, CDCl₃):** δ 205.0, 154.0, 146.5, 110.2, 82.4, 81.8, 78.9, 75.5, 74.7, 70.3, 48.7, 44.2, 43.4, 37.0, 34.2, 33.1, 30.5, 24.1, 23.8, 23.2, 20.1. **HRMS (ESI-TOF) m/z:** [M-H]⁺ calcd for C₂₃H₃₆NO₇ 438.249; Found 438.2675





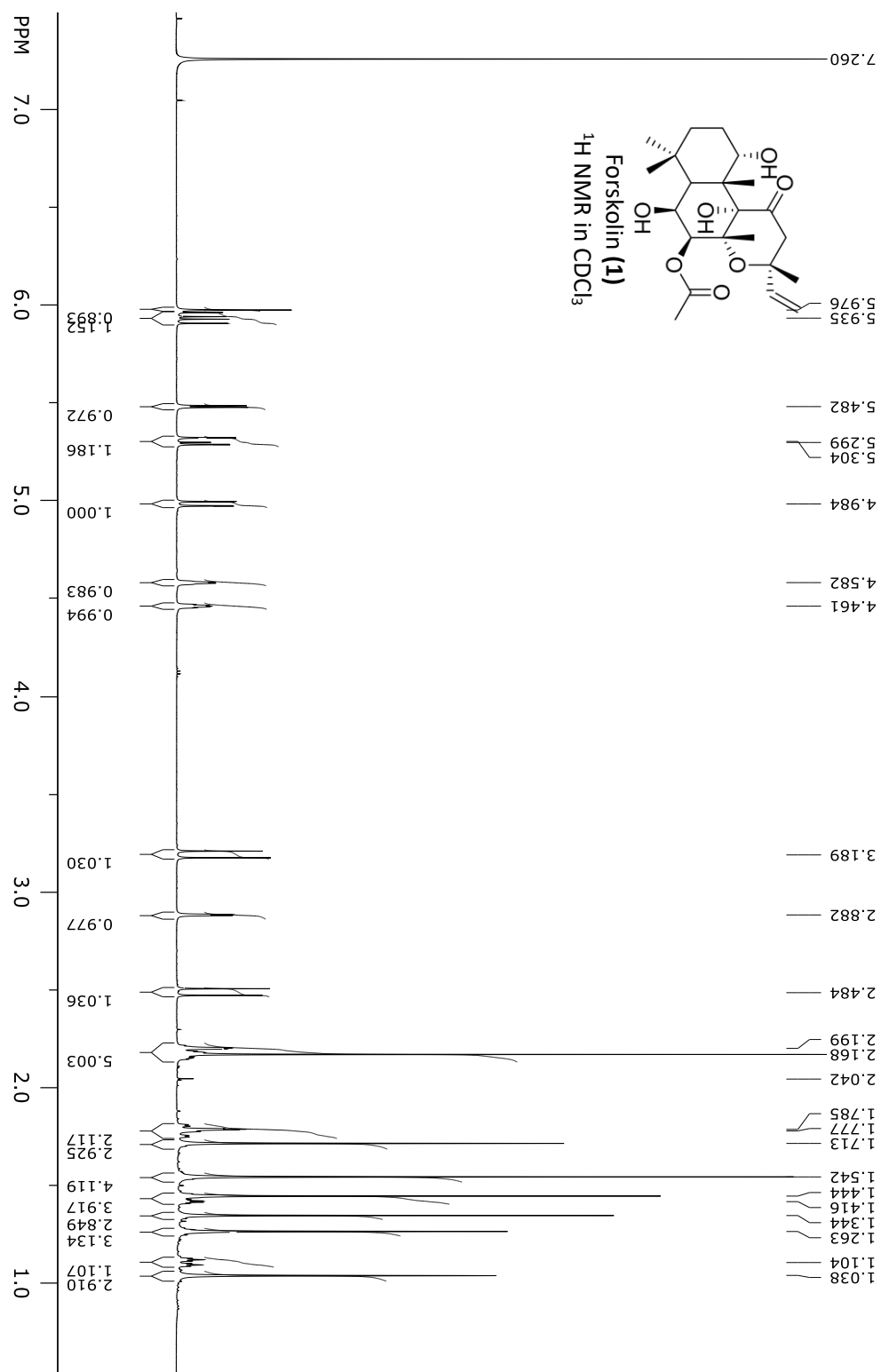
Preparation of Compound (92)

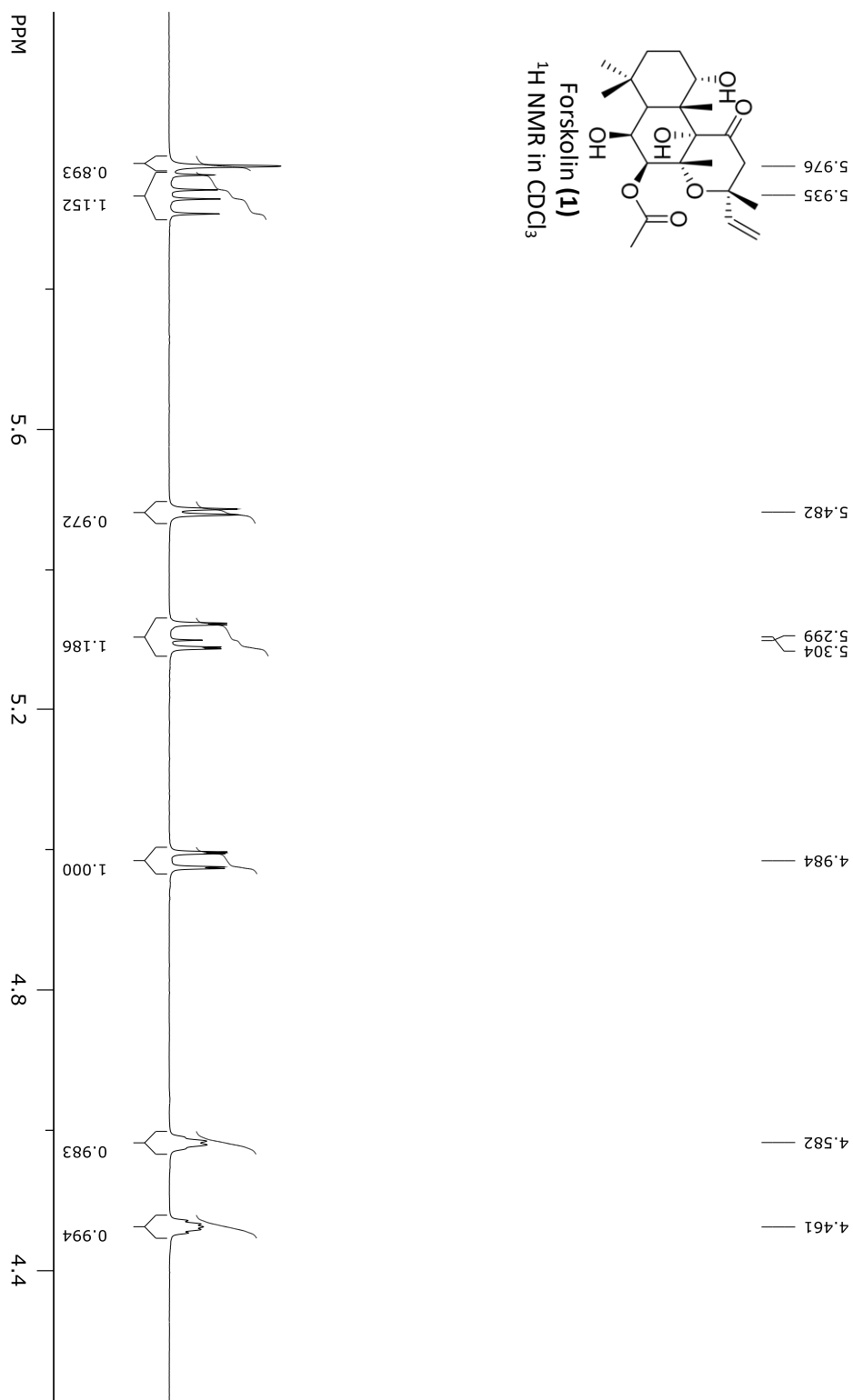
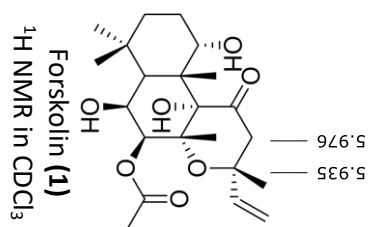
Under argon and on ice **(7)** (106 mg, 0.22 mM) was dissolved in DCM (2 mL) and allowed to reach 0-5 °C, pyridine (1 mL) and nicotinoyl chloride (85 mg, 0.48 mM) were then added. The reaction was allowed to come to room temperature, while being stirred for 16 hours. No reaction occurred by TLC, triethylamine (50 μ L) was then added, the reaction temperature was increased to 50 °C, and stirred for an additional 24 hours. The reaction was dried with a rotary evaporator, EtOAc (10 mL) was added, and the solution was then washed with water twice, HCl (0.1 M) once and brine once, followed by drying with sodium sulfate. Solvent was then removed by rotary evaporation and purified by biotage isolera column chromatography (10g KP-SIL cartridge) with a gradient from 10 to 80% EtOAc with hexane. 63 mg of product collected. 48% yield.

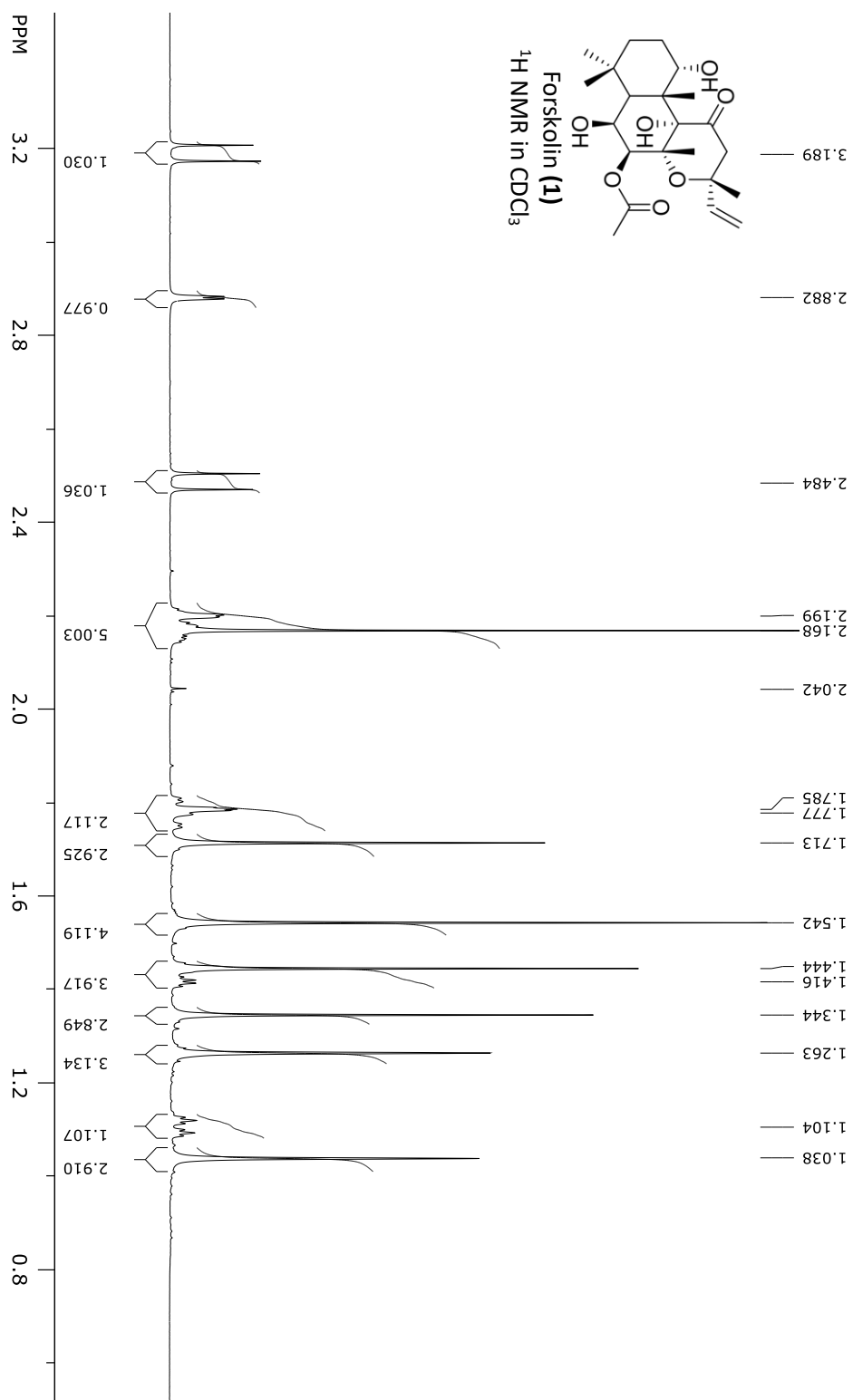
AP-3-121-2-1 (protected product) (60 mg, 0.1 mM) was dissolved in THF (1 mL), TBAF (1 M in THF, 120 μ L) was then added, stirred at room temperature for 1 hour. Standard work up (EtOAc, NaSO₄) carried out. Purified by column chromatography (30% EtOAc/hexane), total of 39 mg of product **(92)** purified, yield 68%. Recrystallized with DCM:pentane.

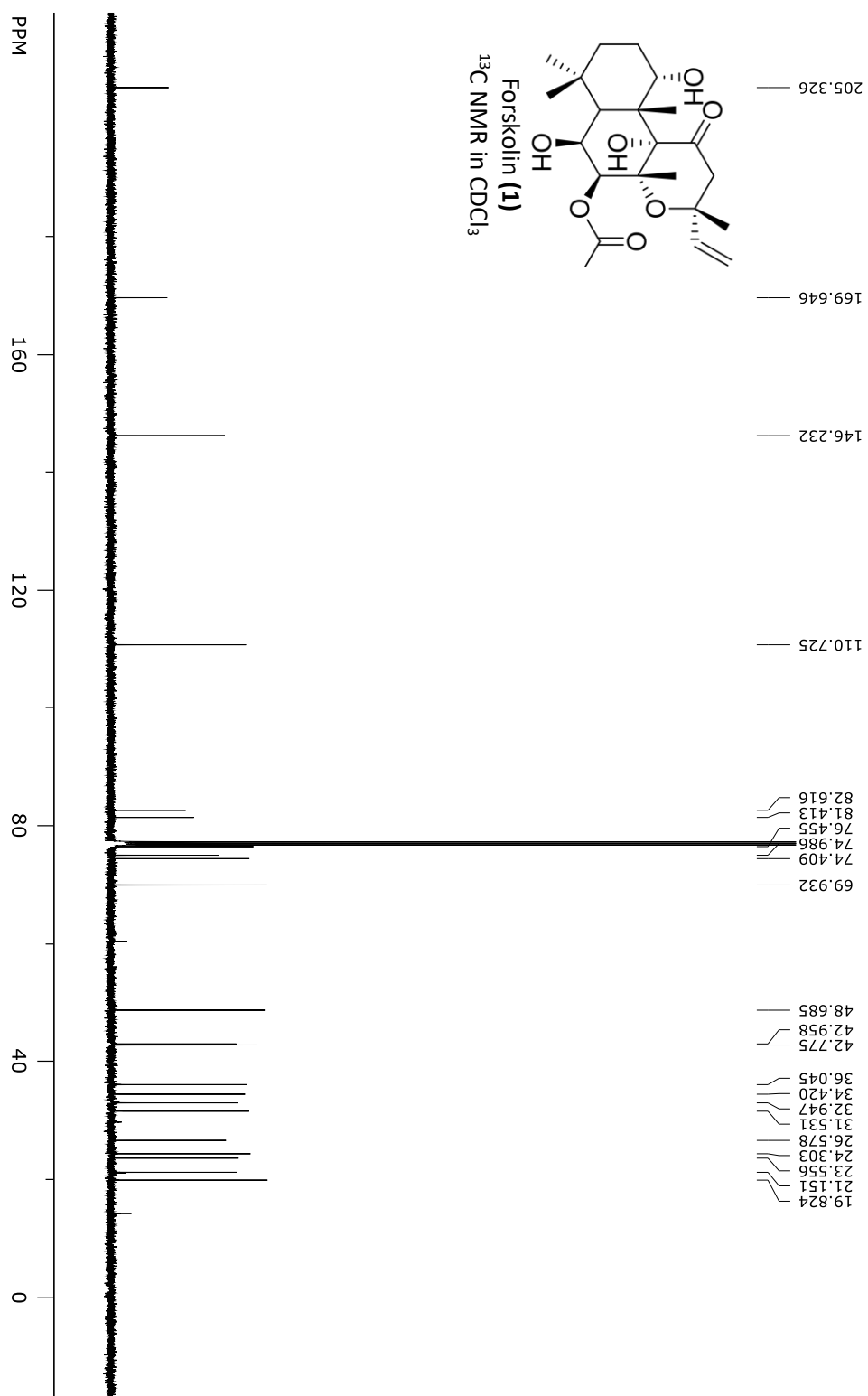
¹H NMR (125 MHz, CDCl₃): δ 9.27 (s, 1H), 8.77 (dd, J = 1.4, 4.8 Hz, 1H), 8.32 (m, 1H), 7.42 (dd, J = 4.8, 8.0, 1H), 6.32 (s, 1H), , 5.95 (dd, J = 10.7, 17.1 Hz, 1H), 5.71 (d, J = 4.1 Hz, 1H), 5.25 (d, J = 17.1 Hz, 1H), 4.95 (d, J = 10.7 Hz, 1H), 4.67 (m, 1H), 4.63 (m, 1H), 3.21 (d, J = 17.1 Hz, 1H), 2.96 (s, 1H), 2.50 (d, J = 17.1, 1H), 2.29 (s, 1H), 2.29 (s, 1H), 2.22 (m, 1H), 1.89 (s, 3H), 1.80 (m, 1H), 1.50 (s, 3H), 1.45 (m, 1H), 1.31 (s, 3H), 1.29 (s, 3H), 1.25 (s, 1H), 1.14 (m, 1H). ¹³C NMR (125 MHz, CDCl₃): δ 205.6, 164.1, 153.4, 150.8, 146.3, 137.4, 126.5, 123.5, 110.6, 82.6, 81.5, 78.2, 75.2, 74.6, 69.7, 48.8, 43.1, 42.9, 36.1, 34.5, 33.0, 31.4, 26.8, 24.3, 23.9, 19.9. HRMS (ESI-TOF) m/z : [M-H]⁺ calcd for C₂₆H₃₄NO₇ 472.233; Found 472.2463

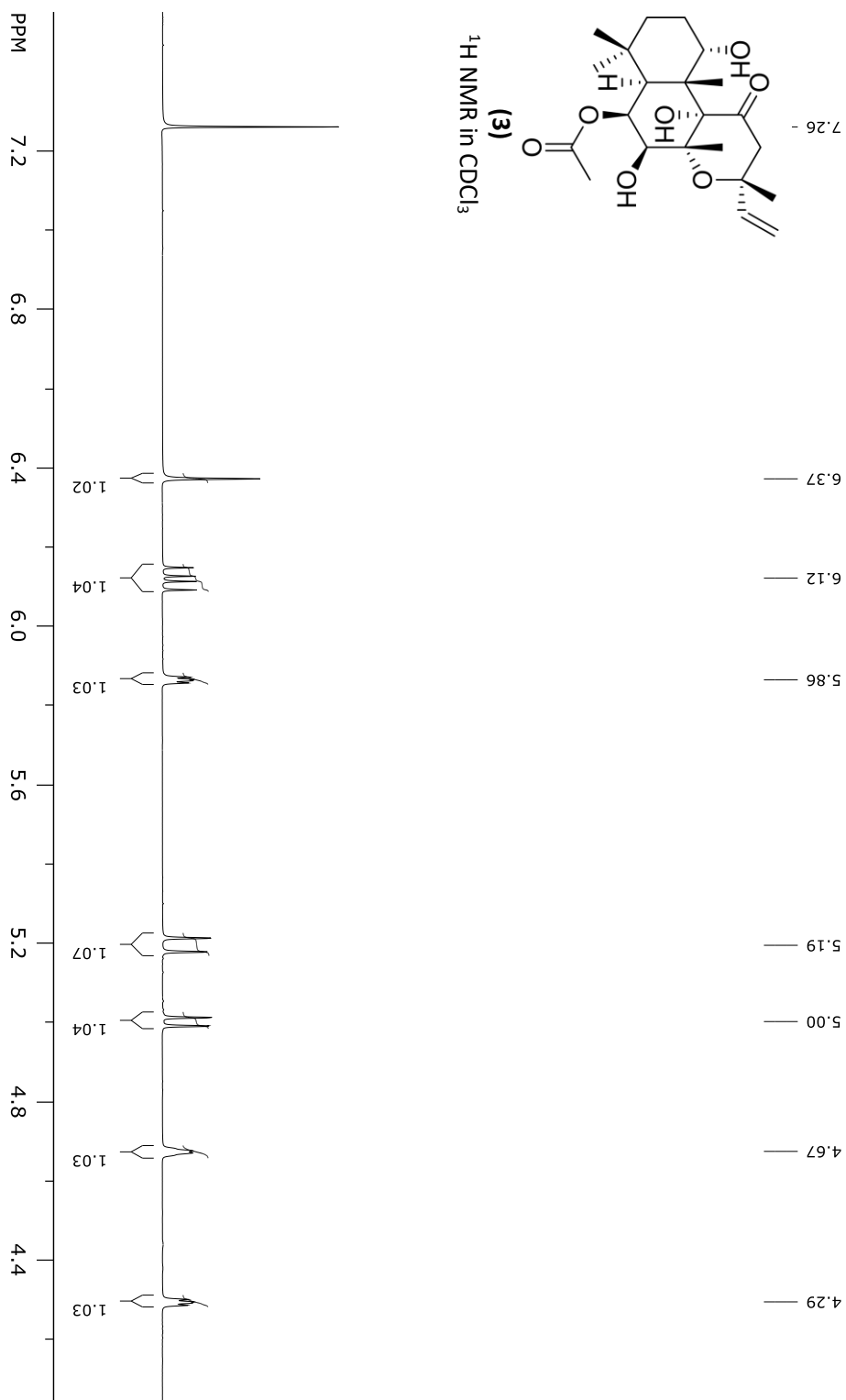
NMR Spectra

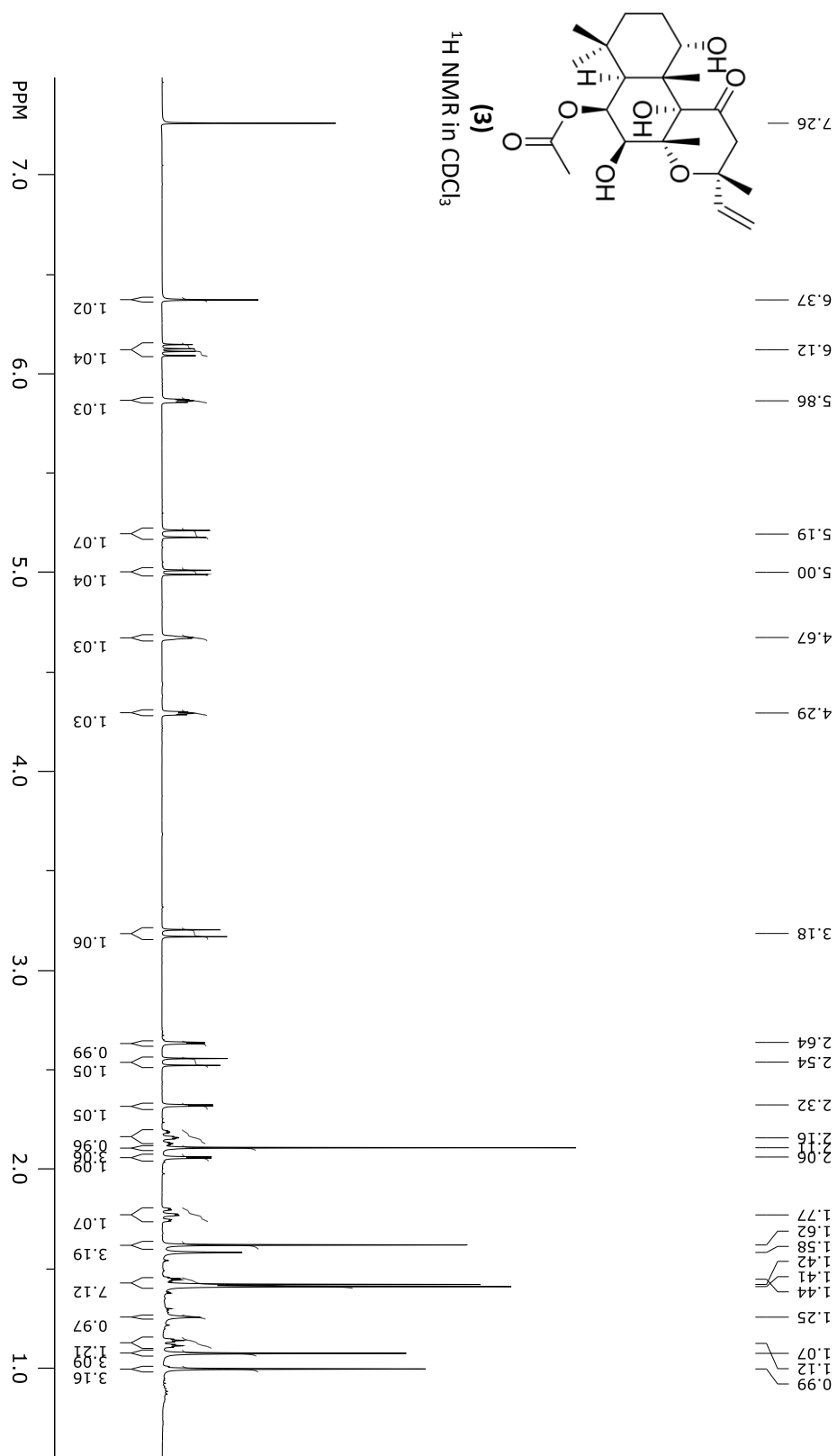


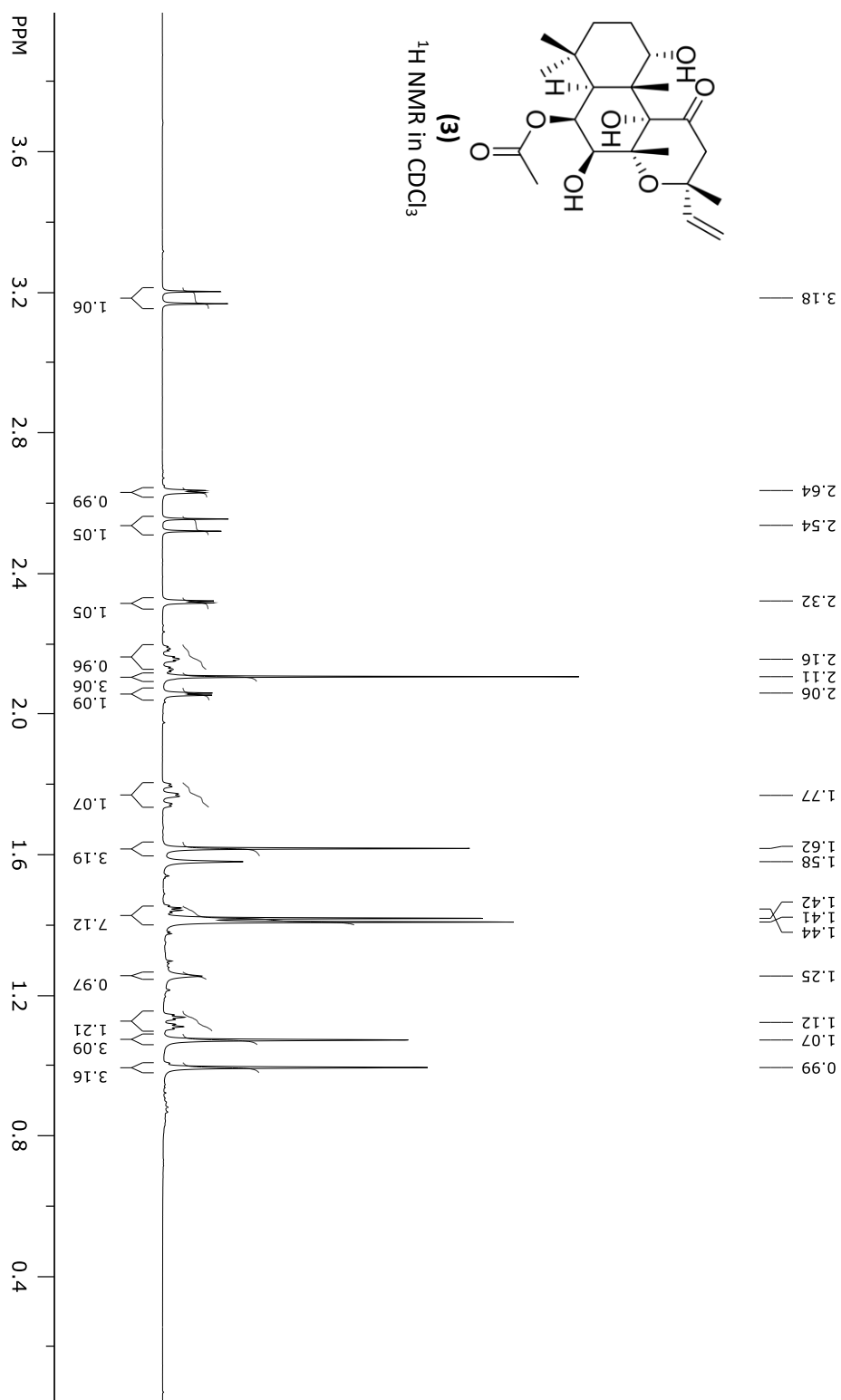


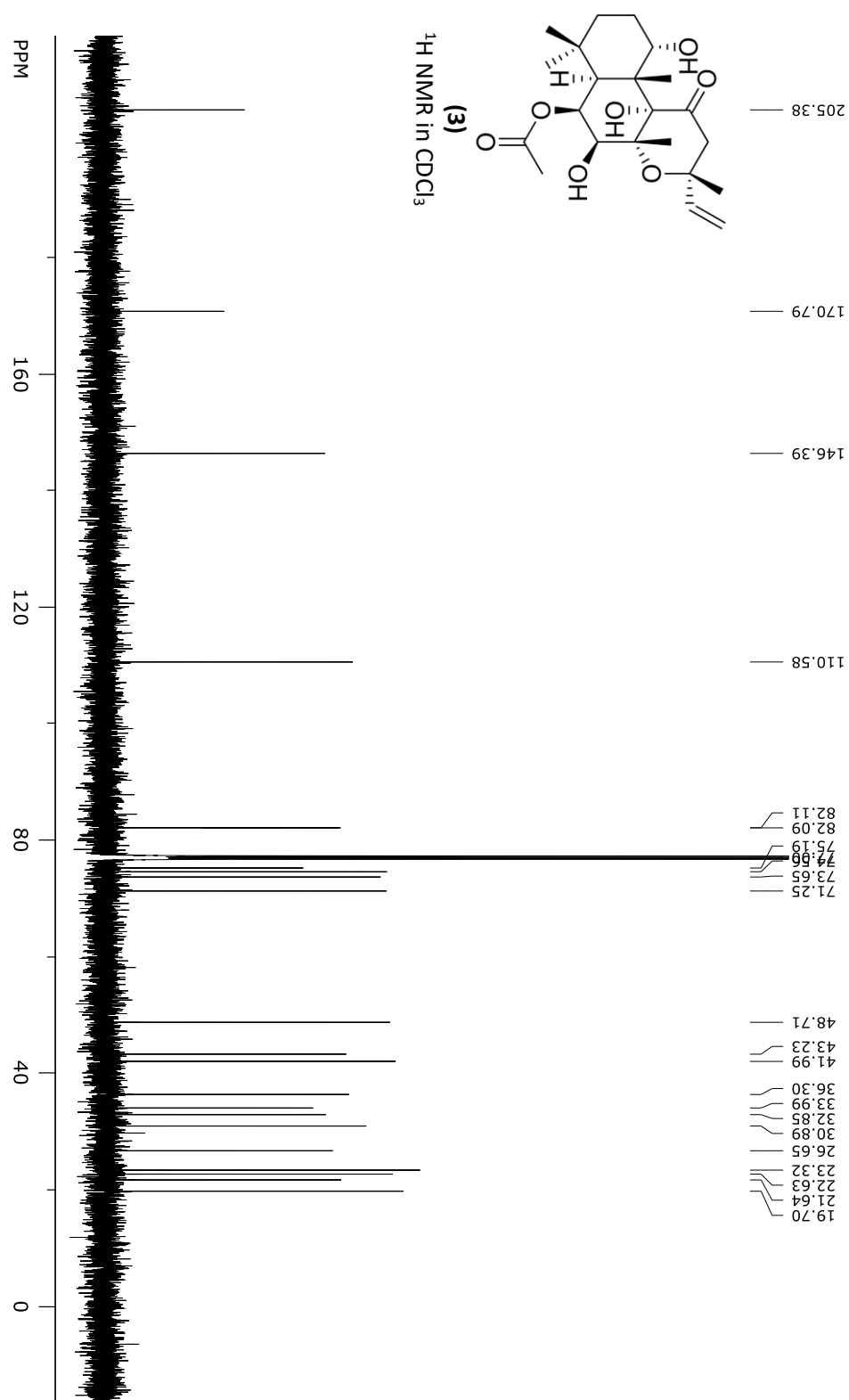


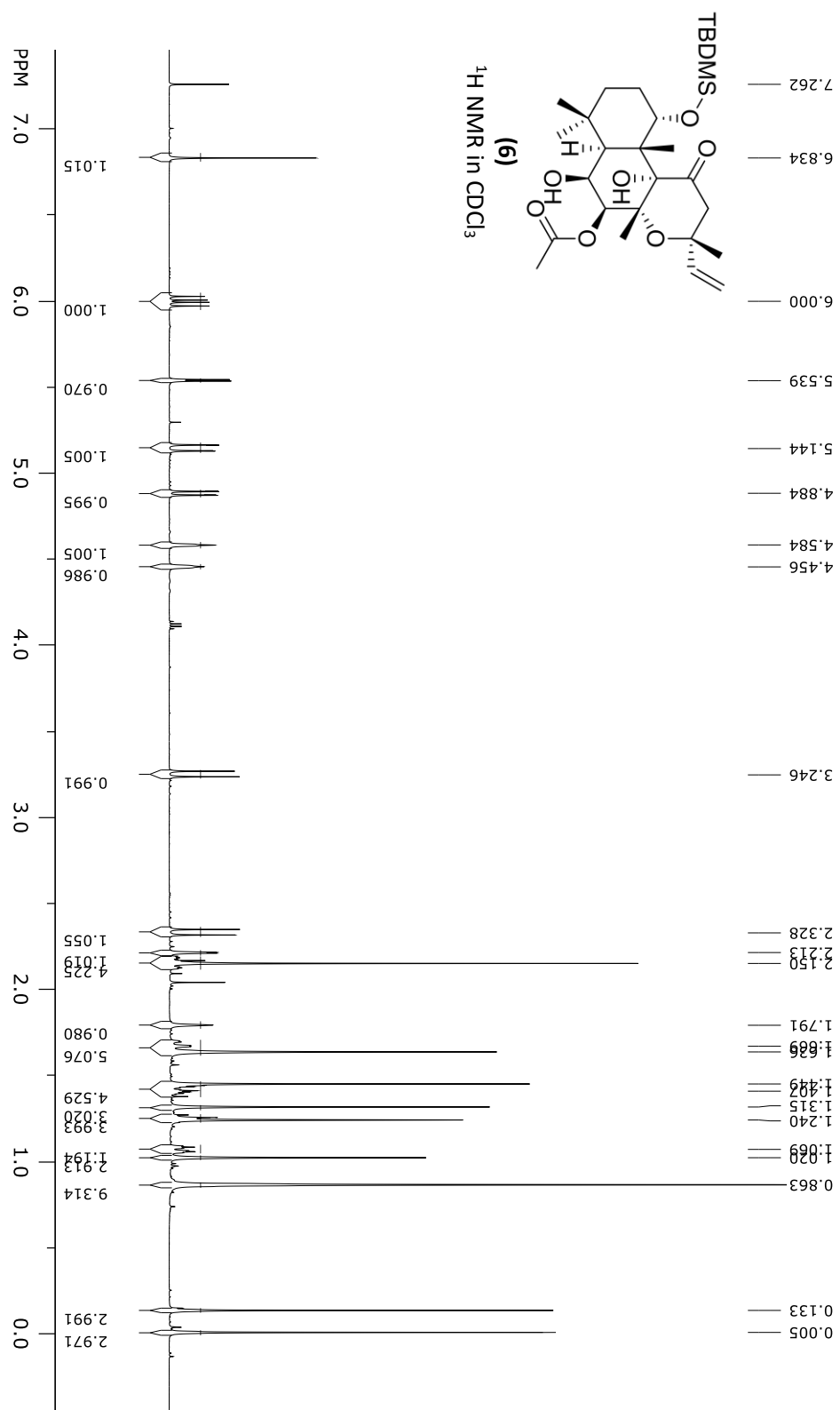


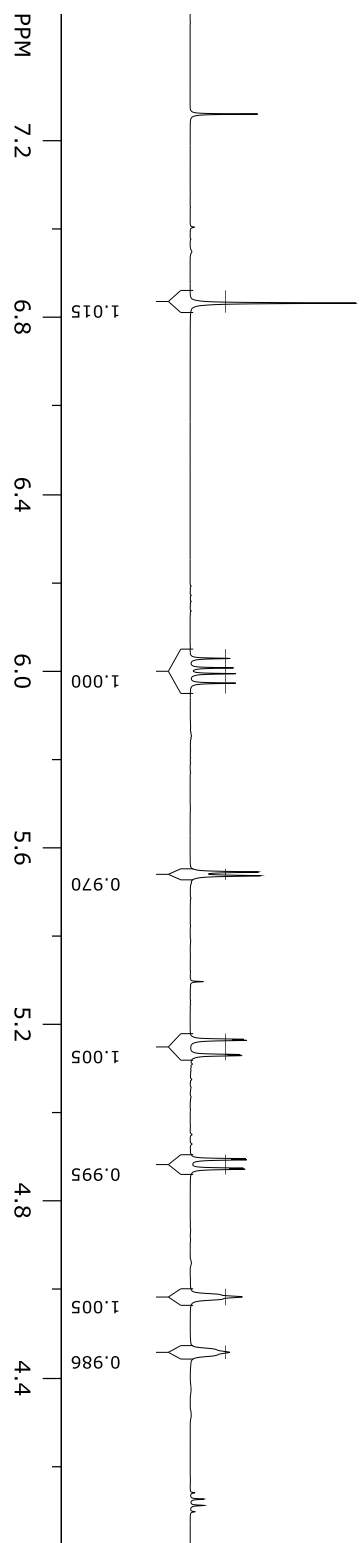
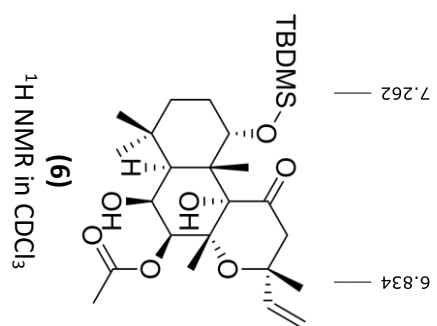


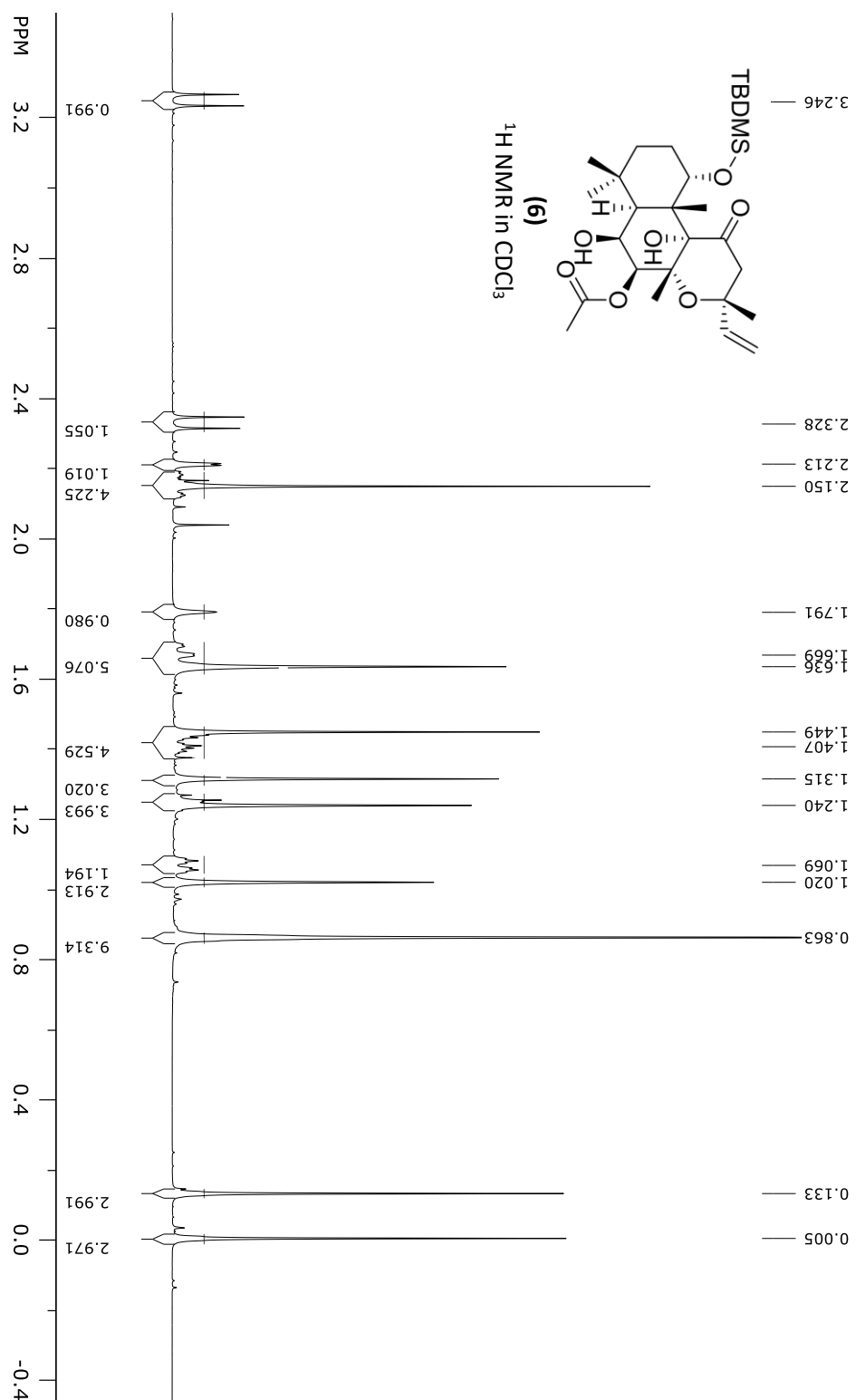


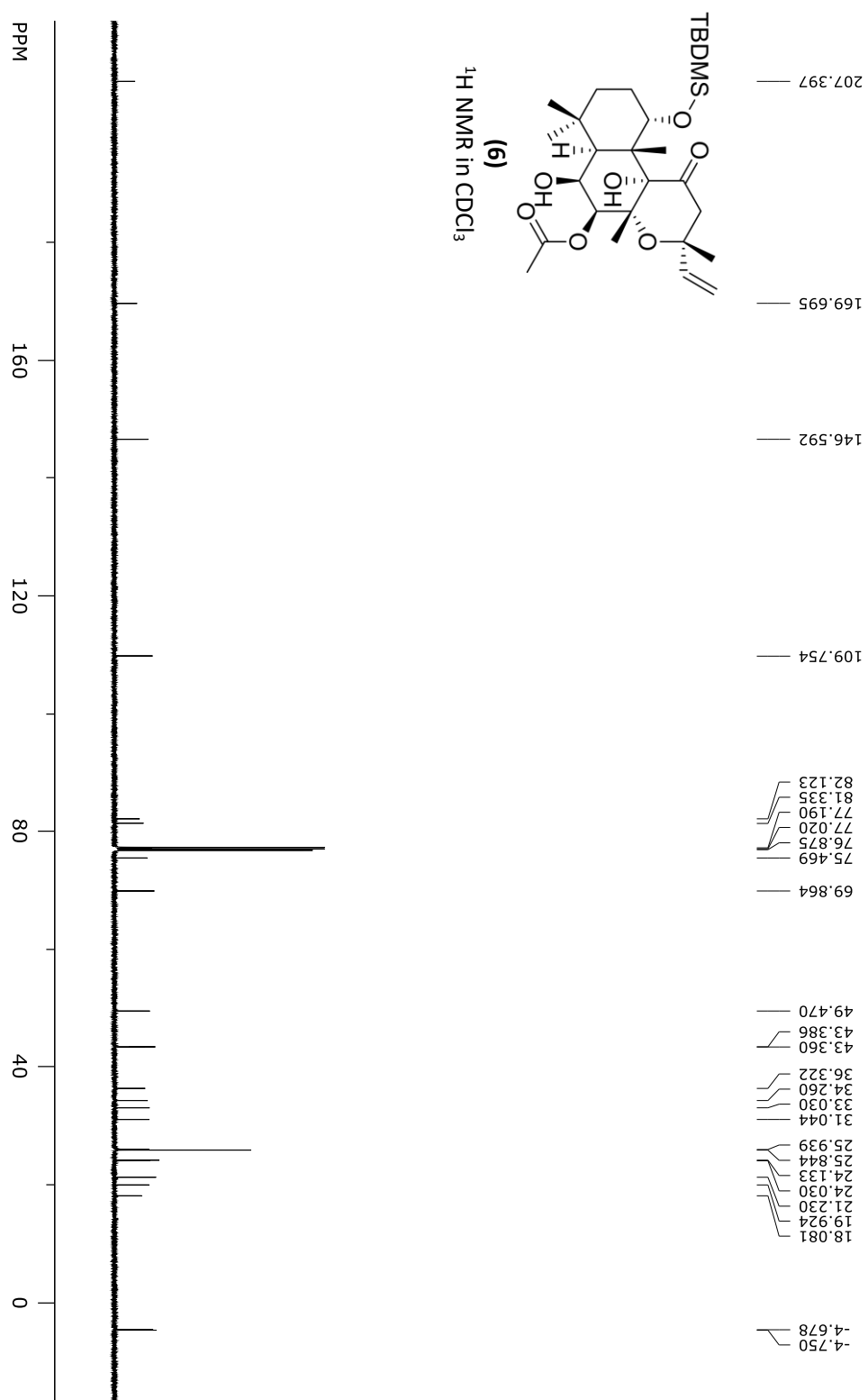


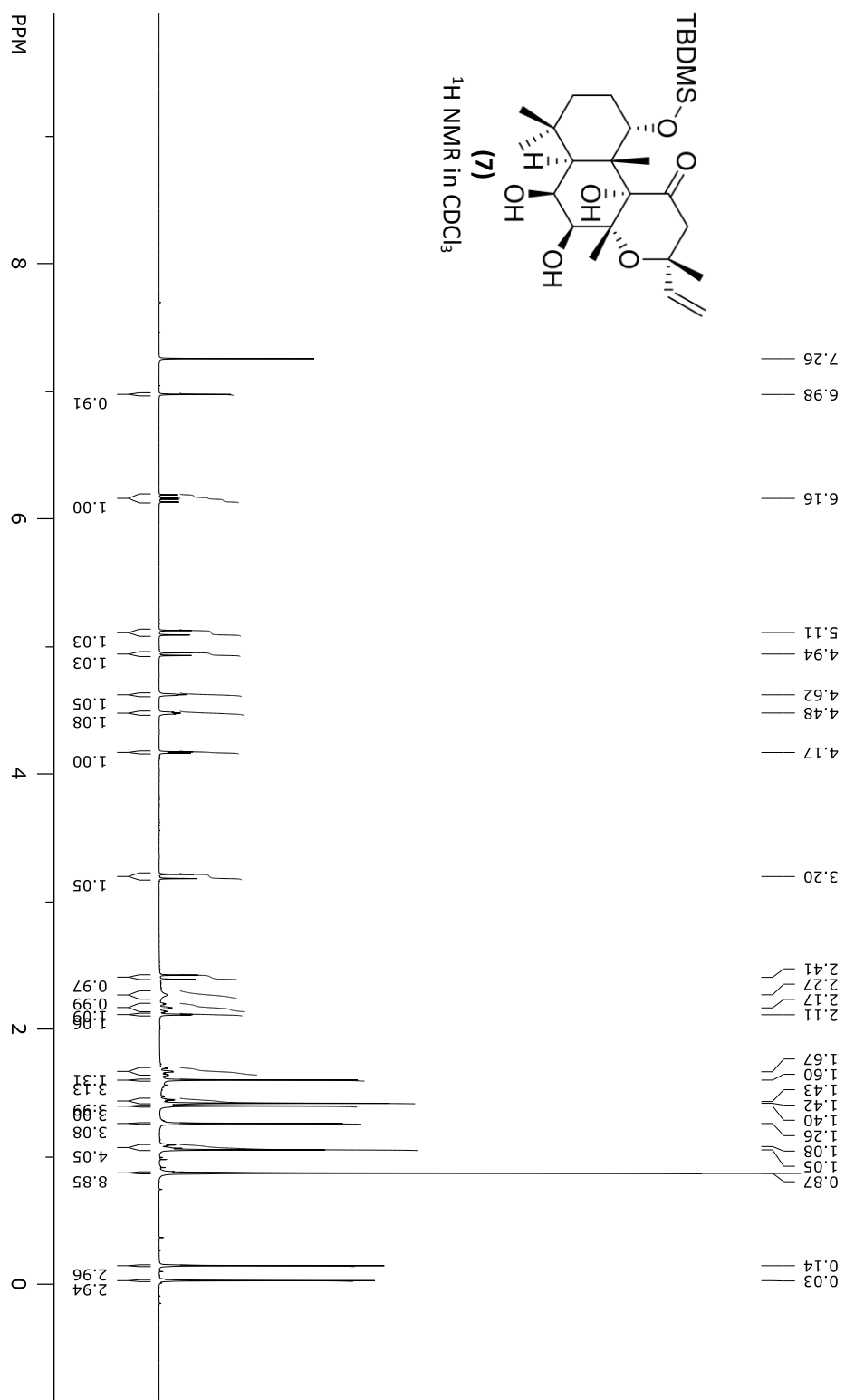


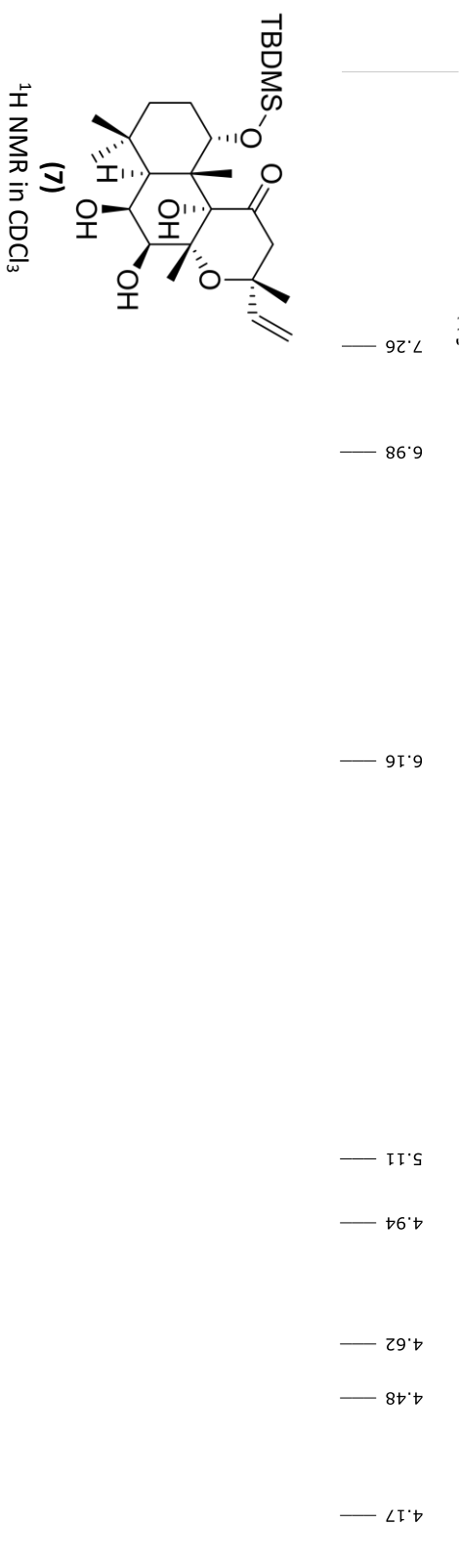


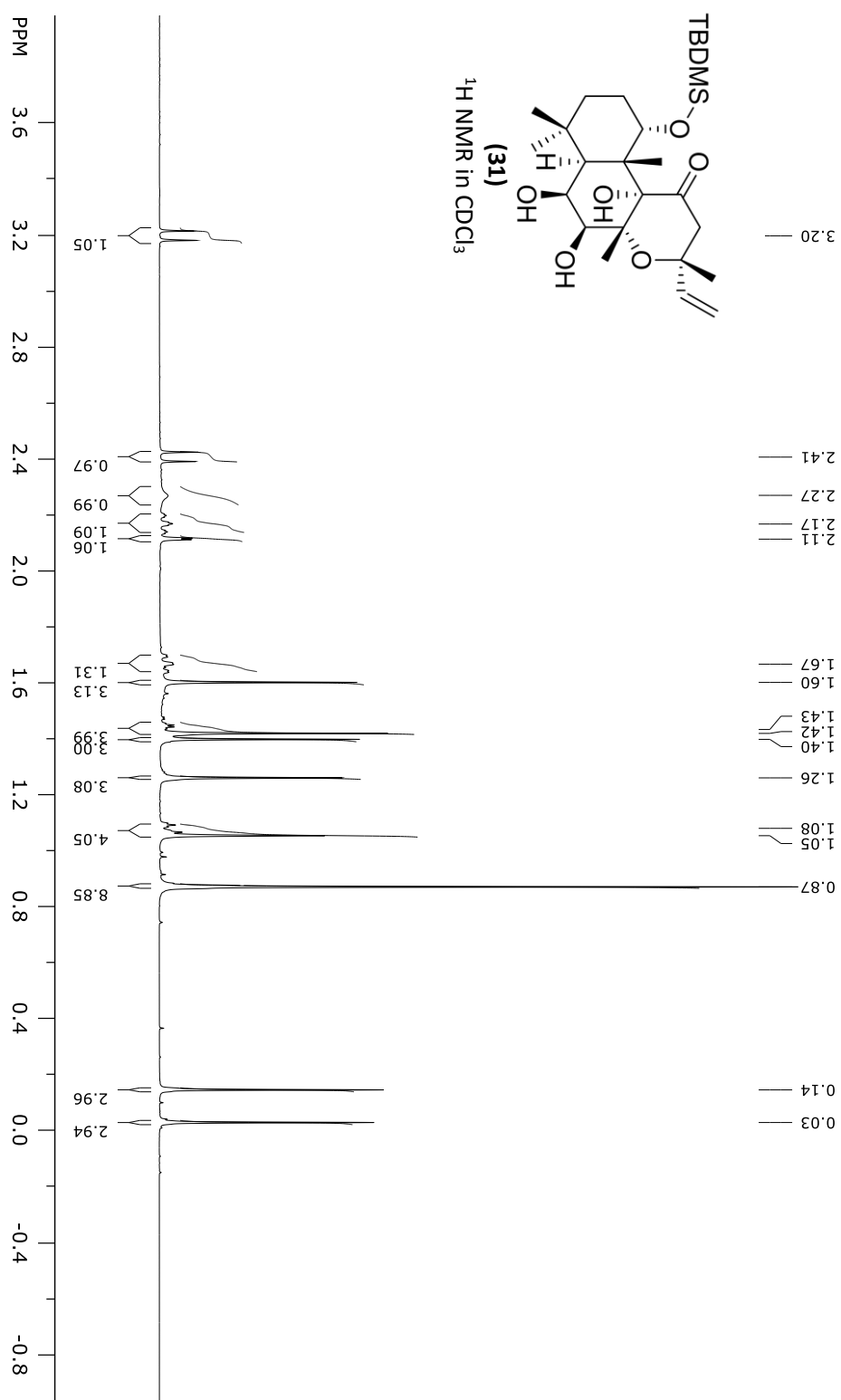


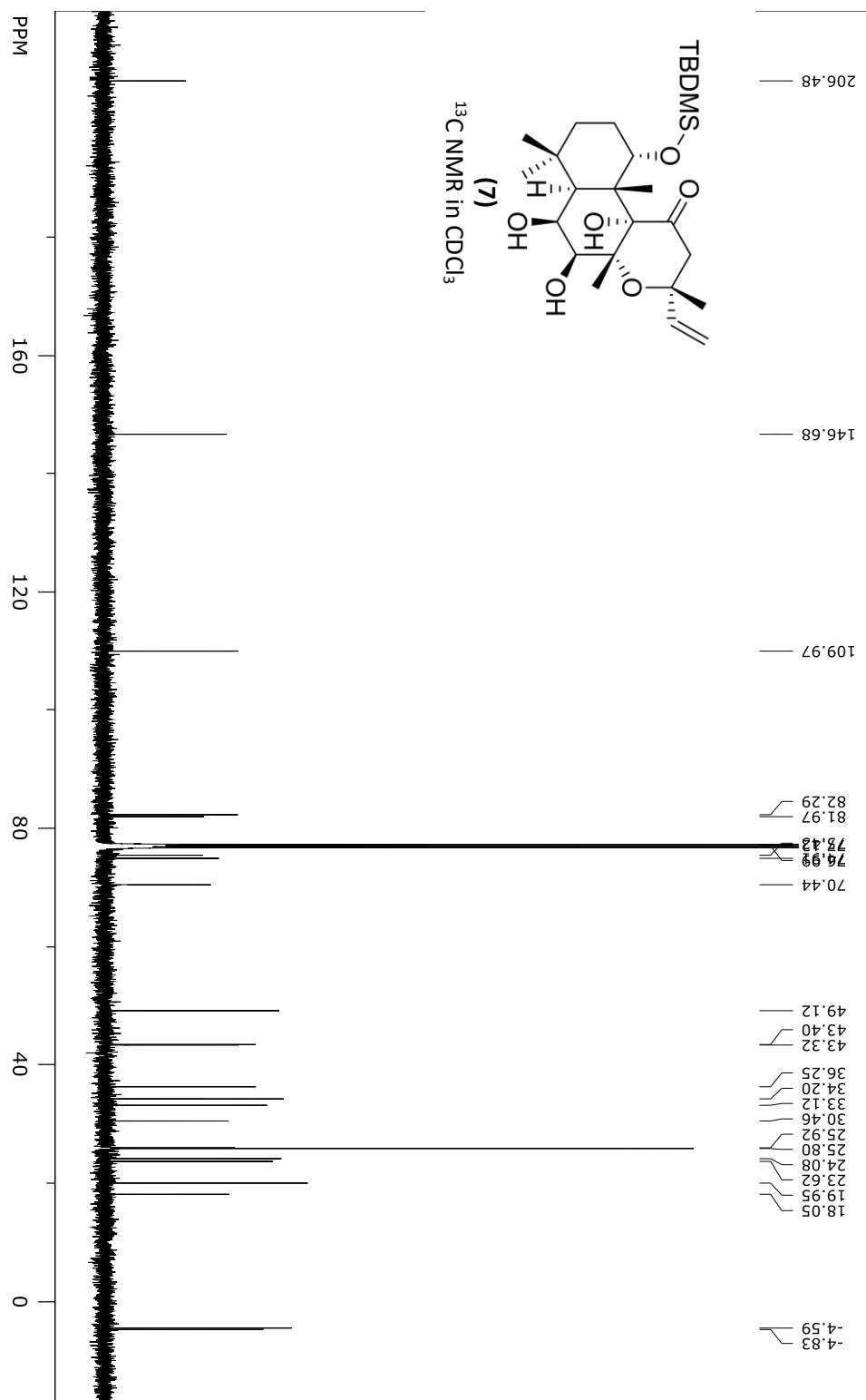


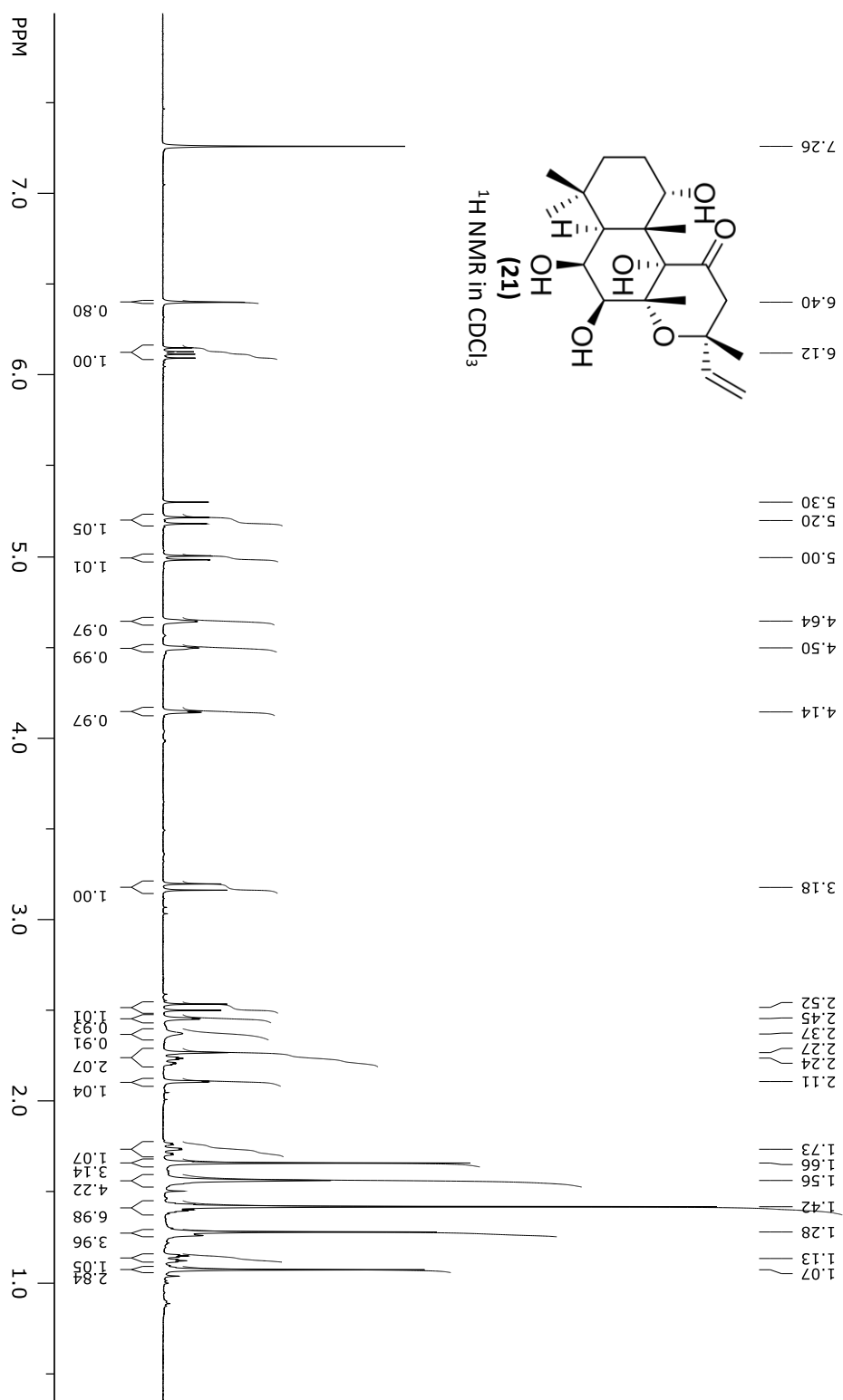


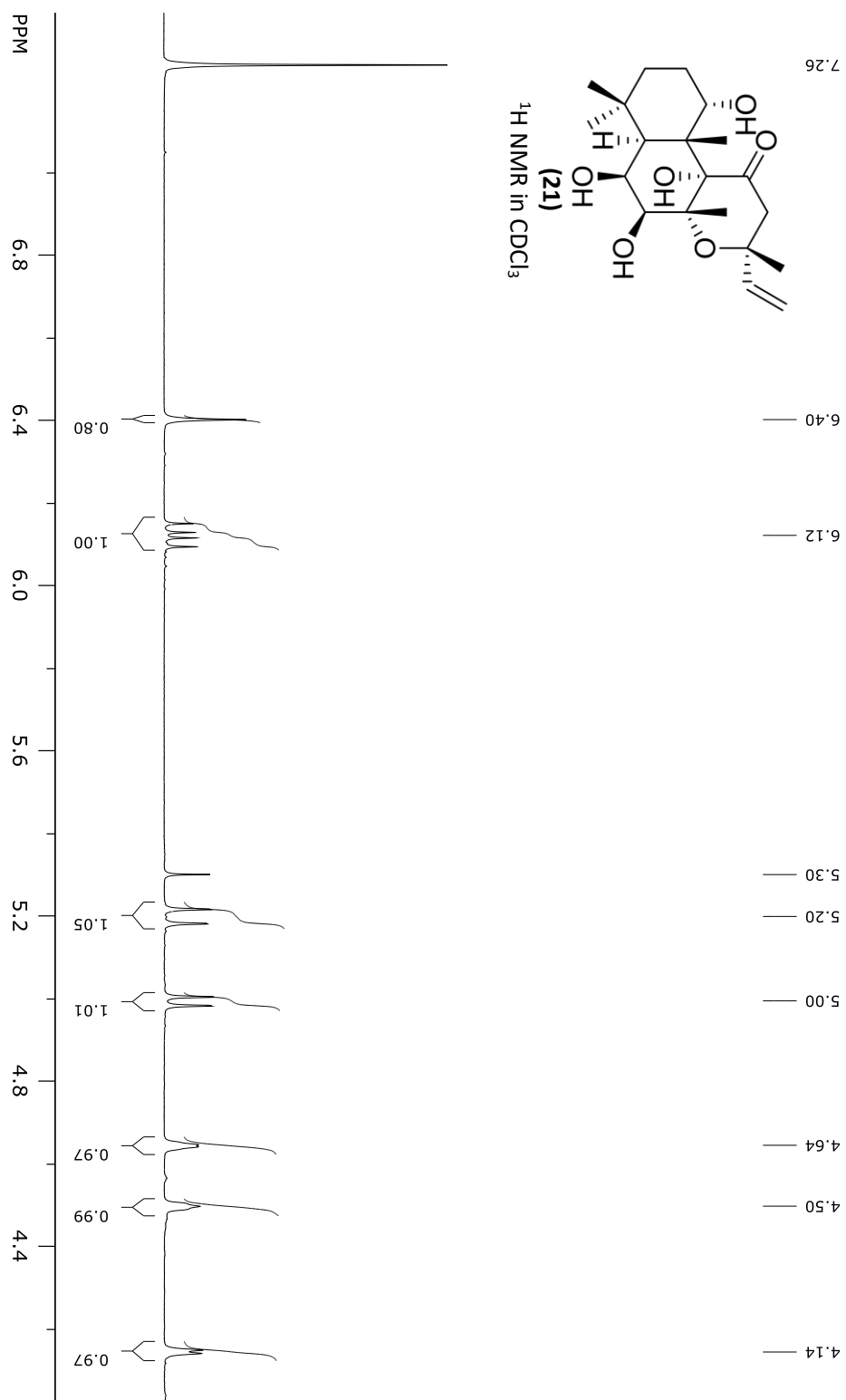


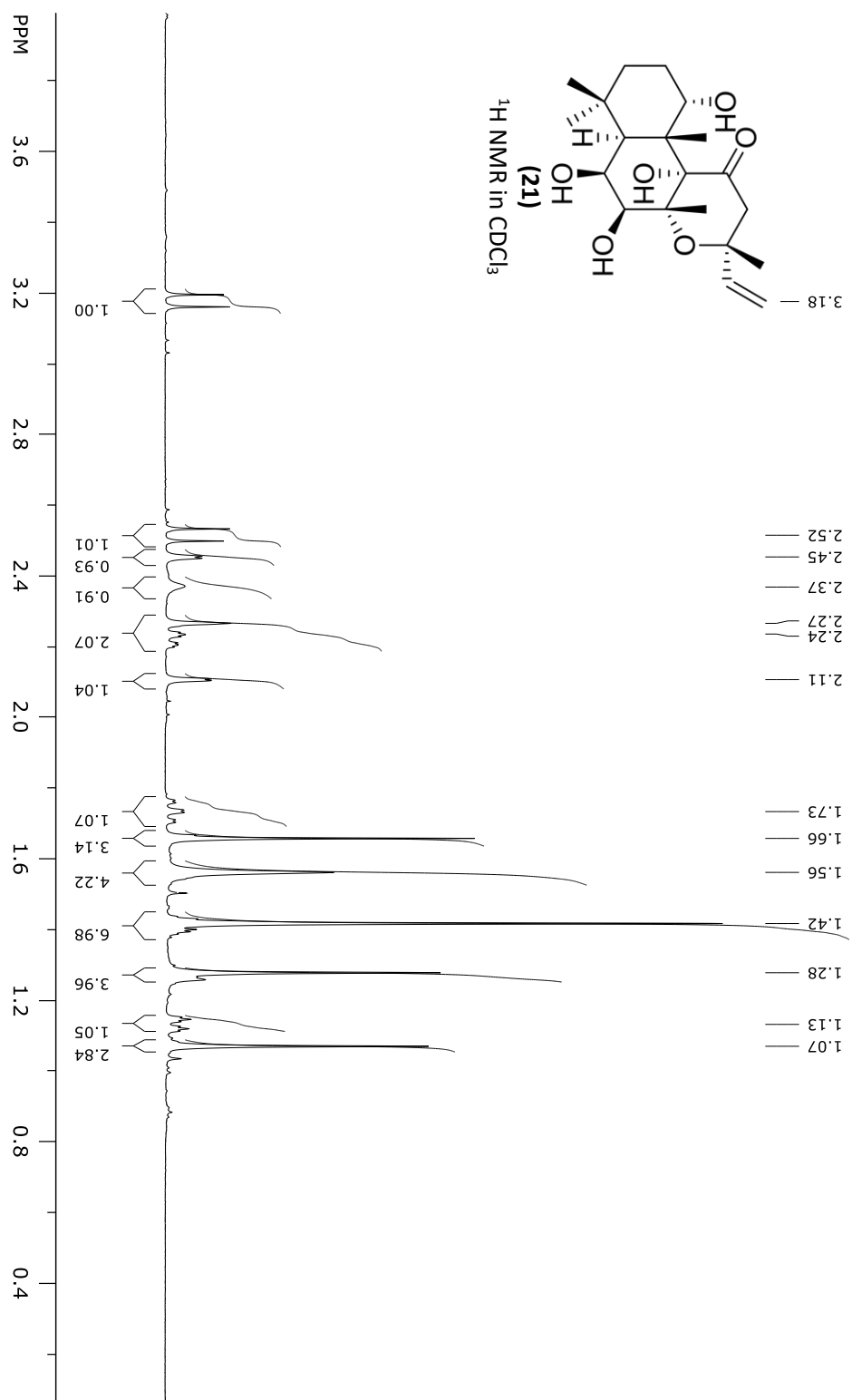


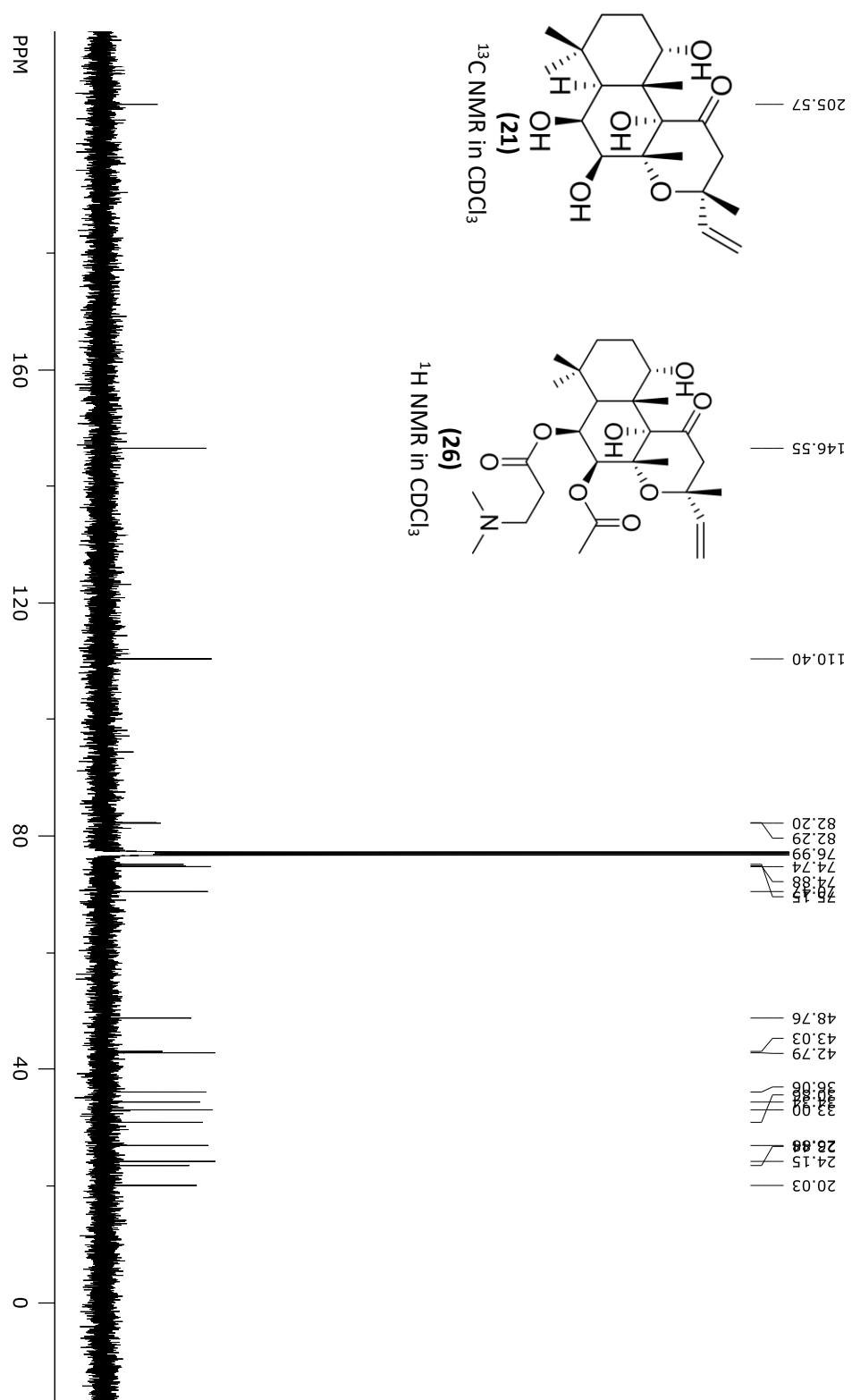


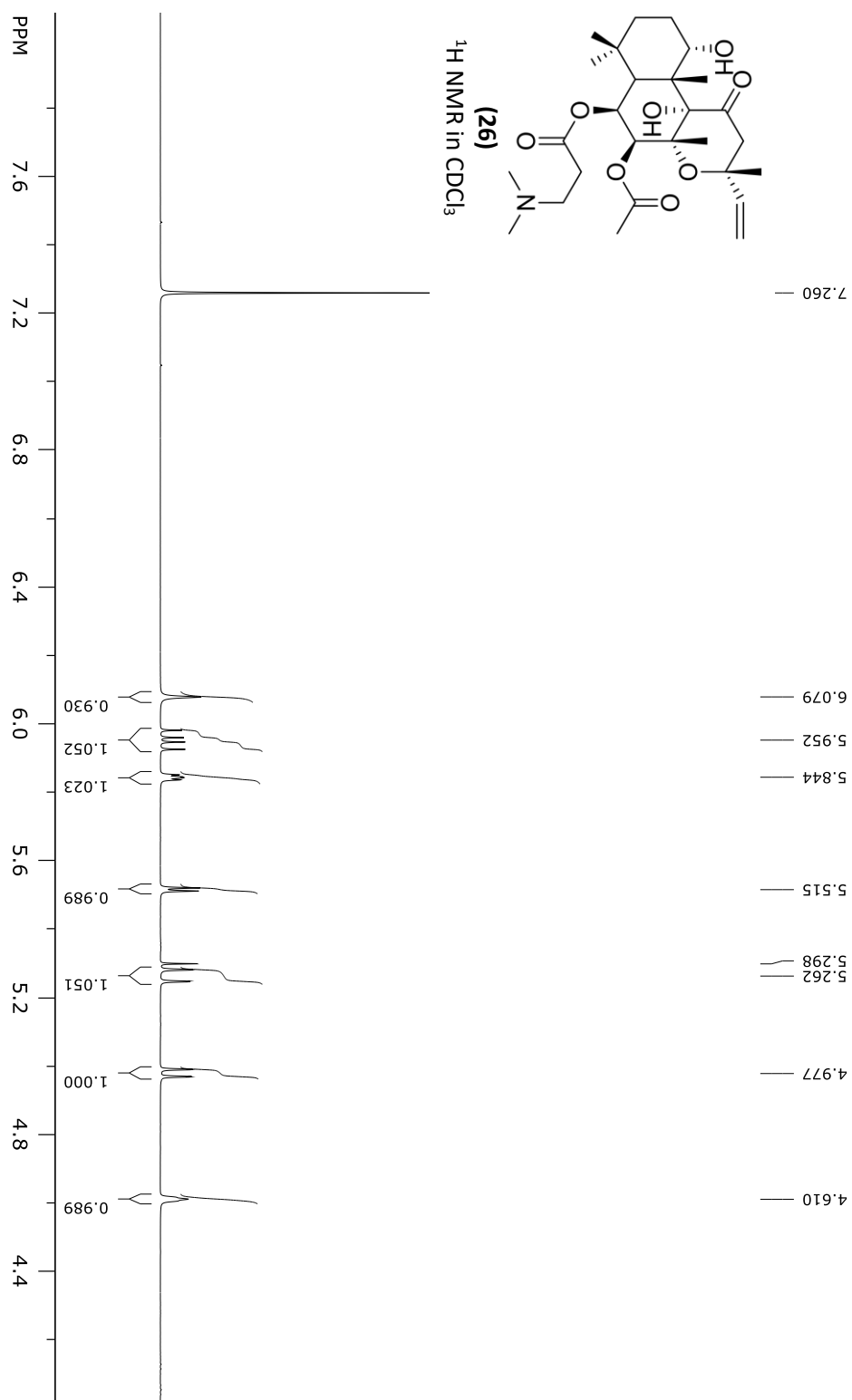


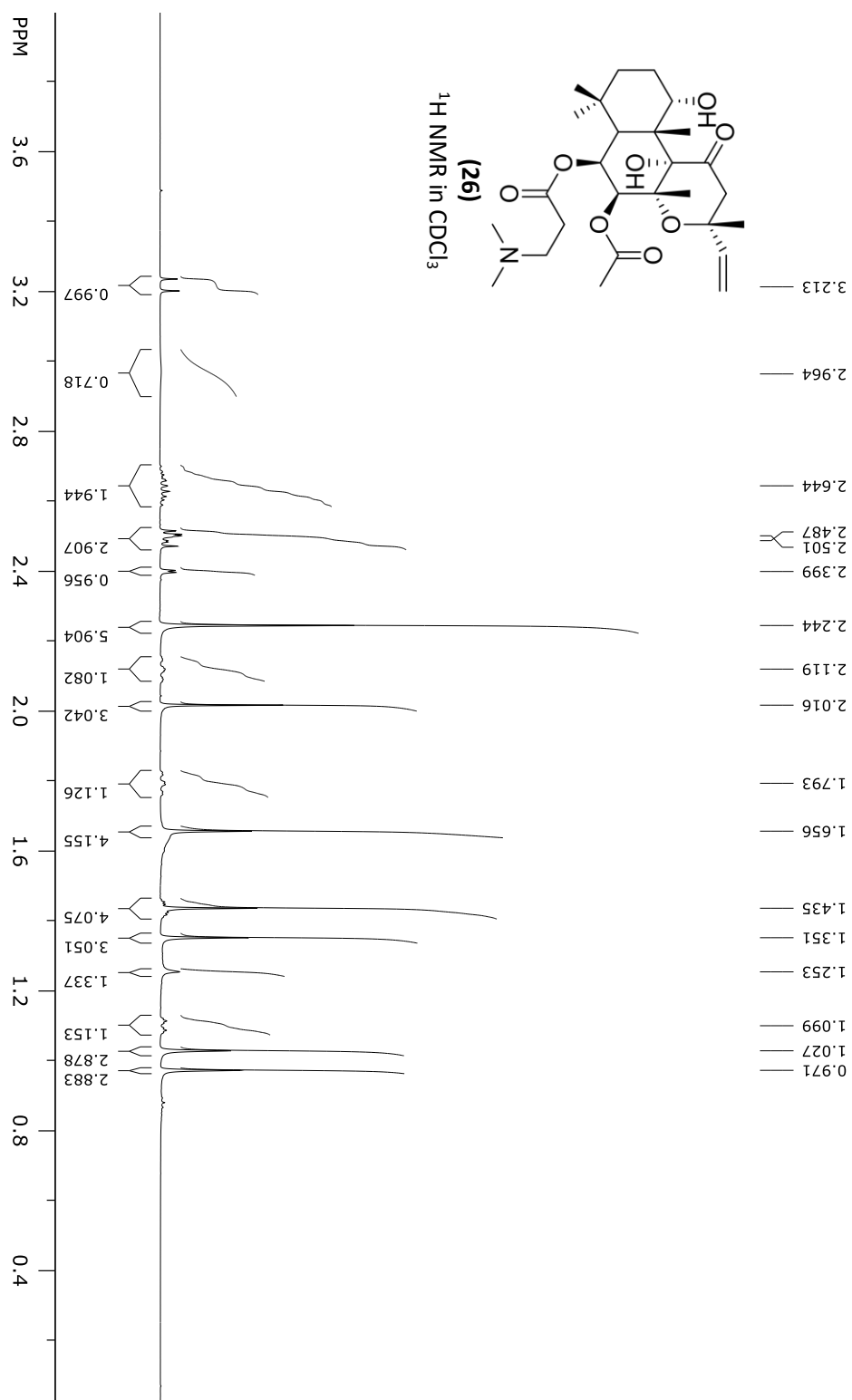


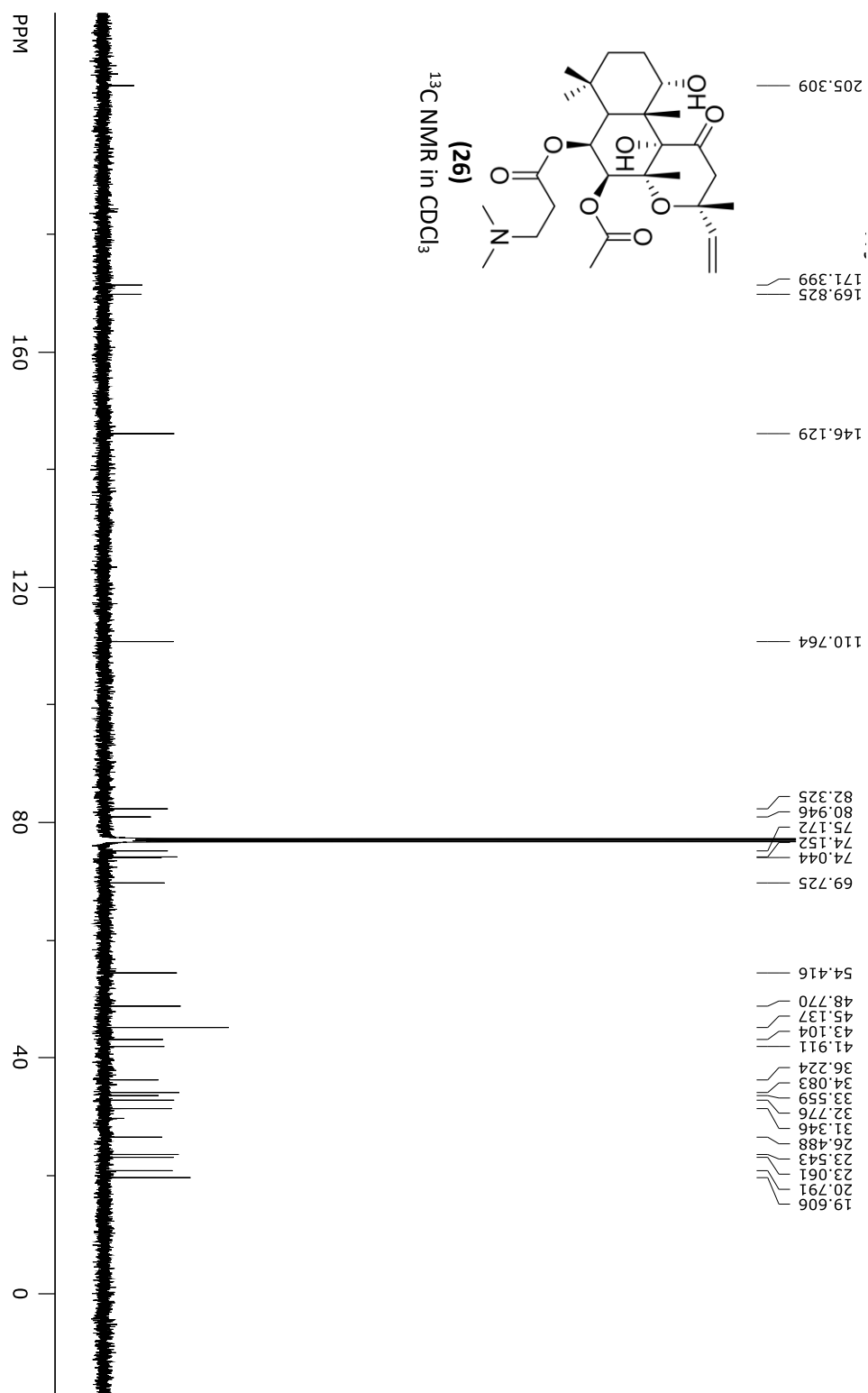


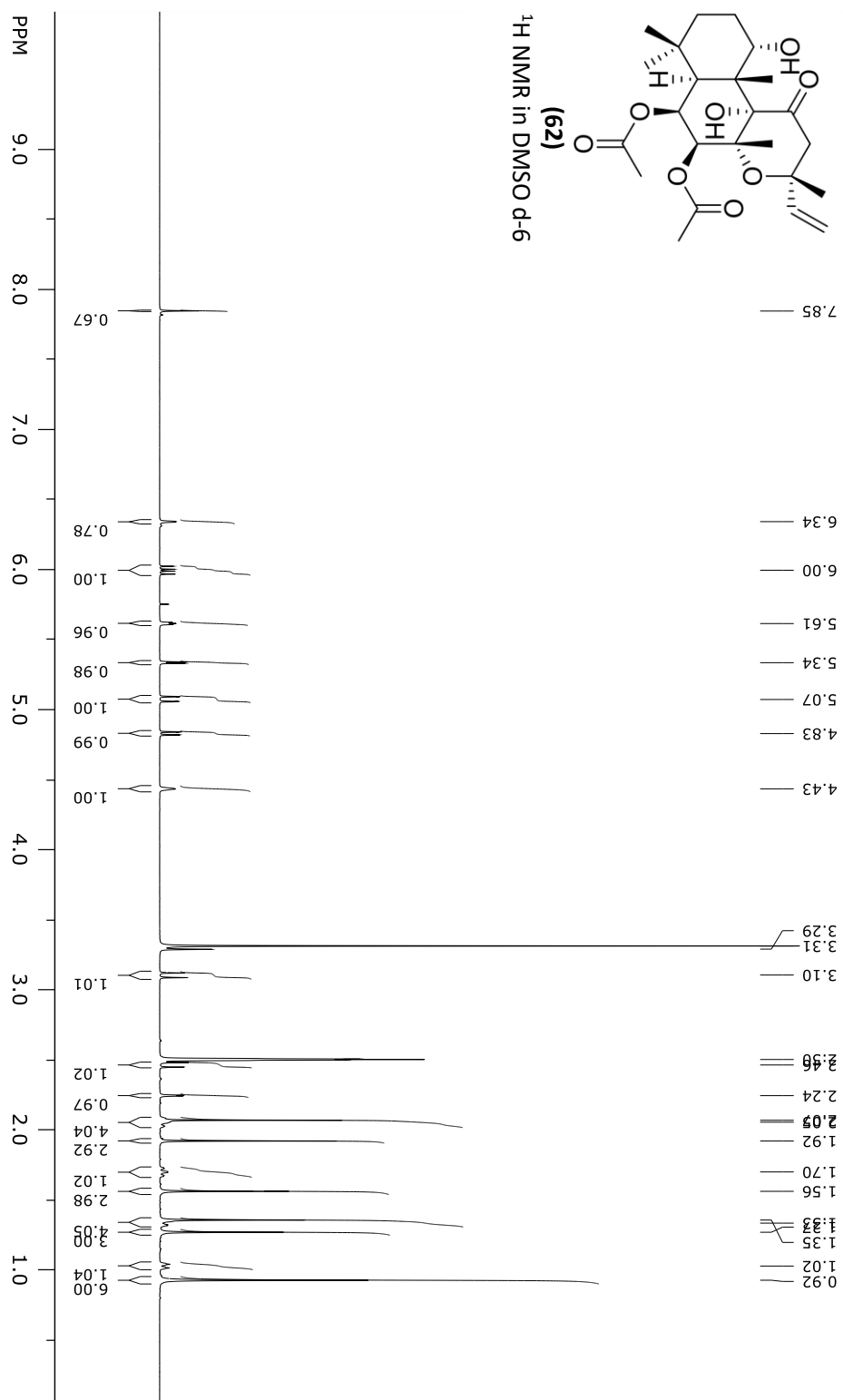


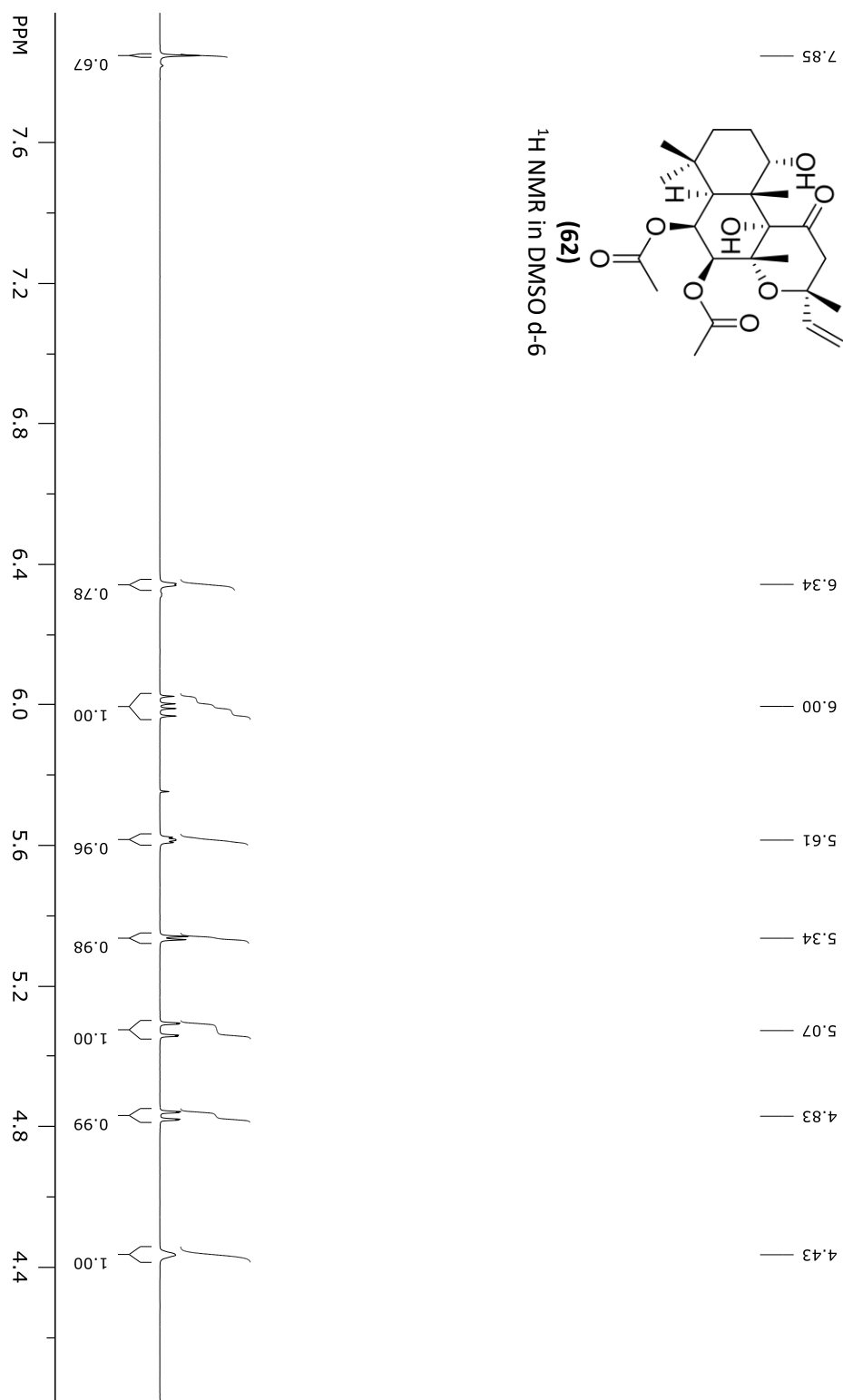


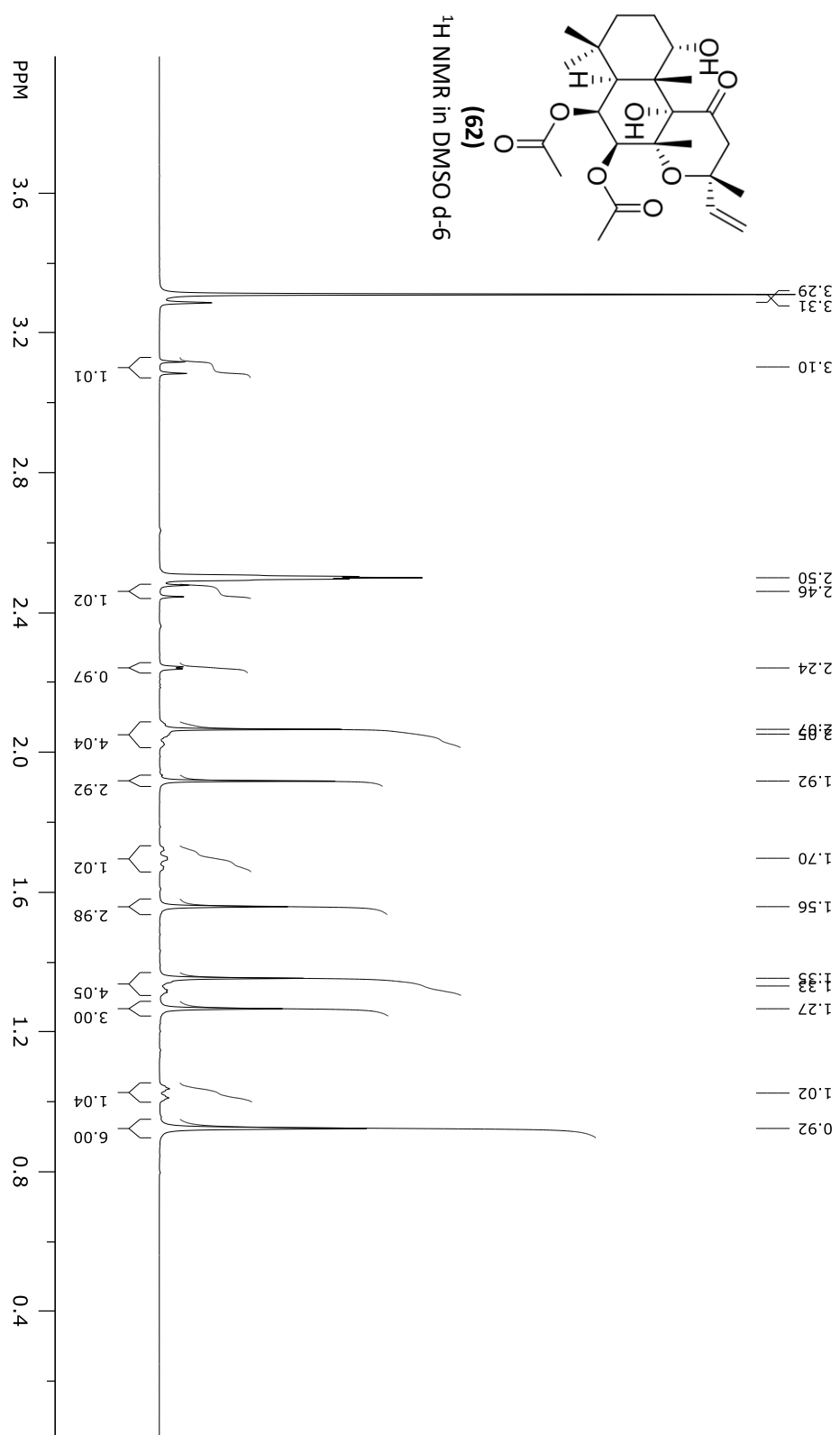


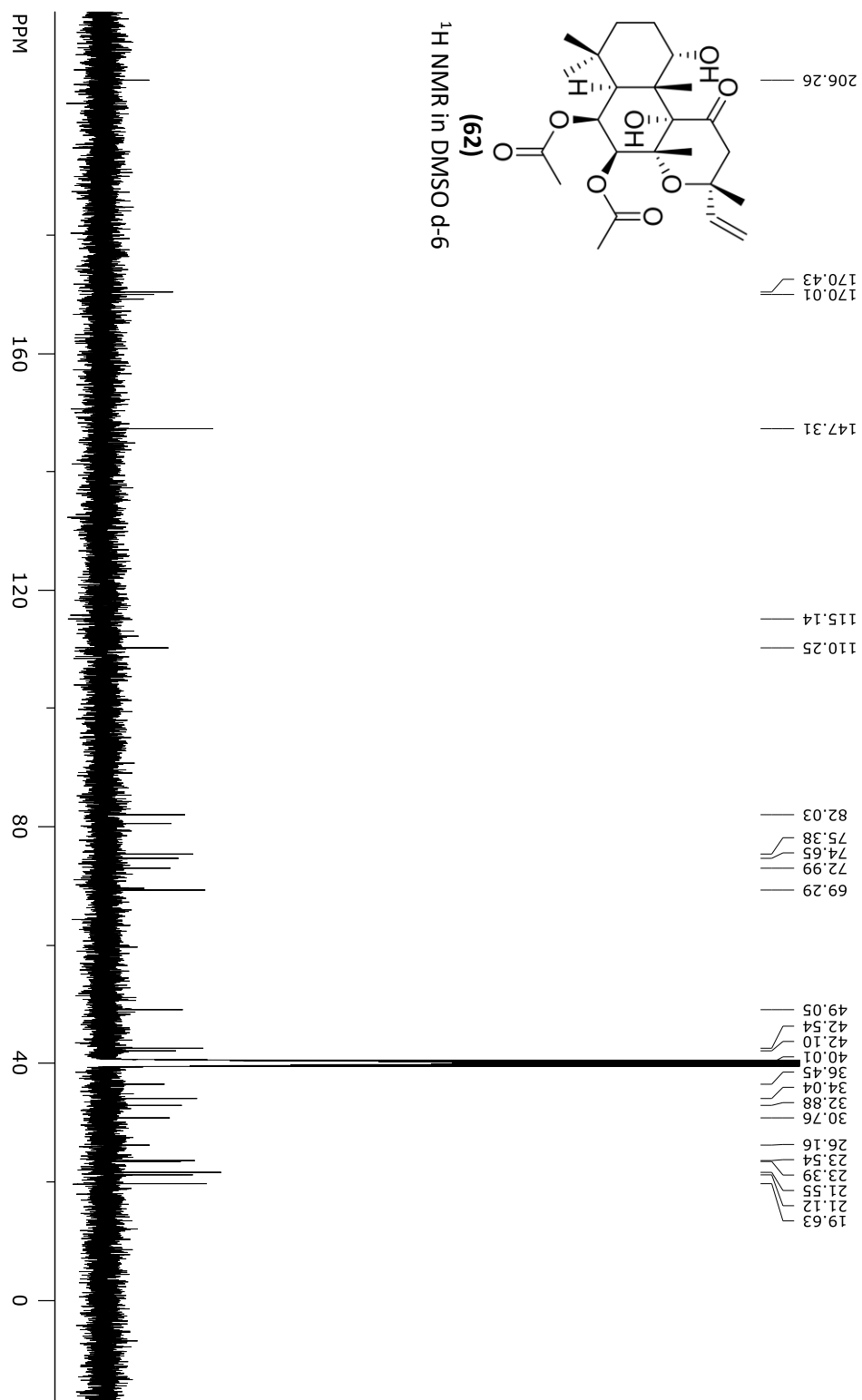


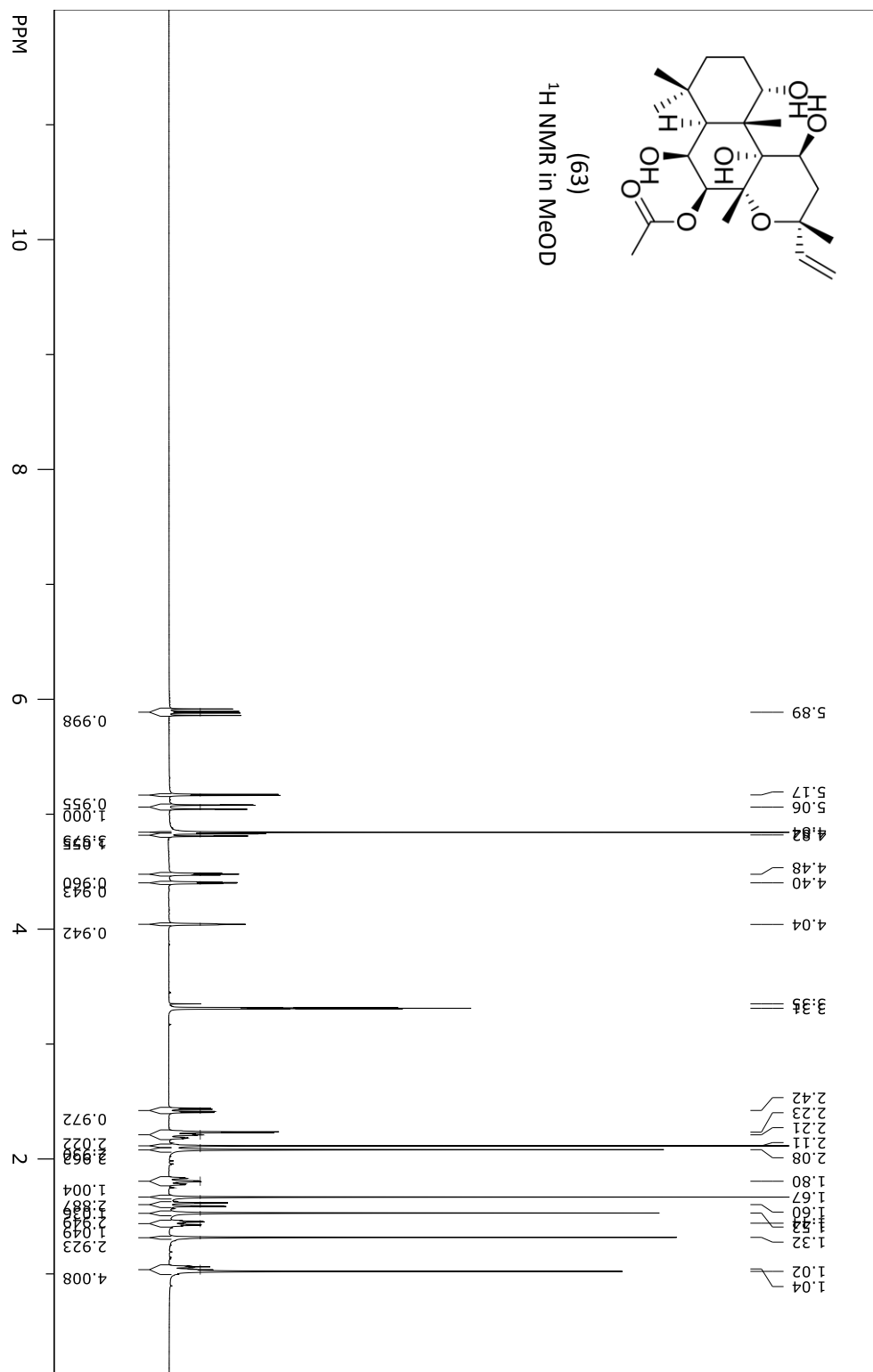


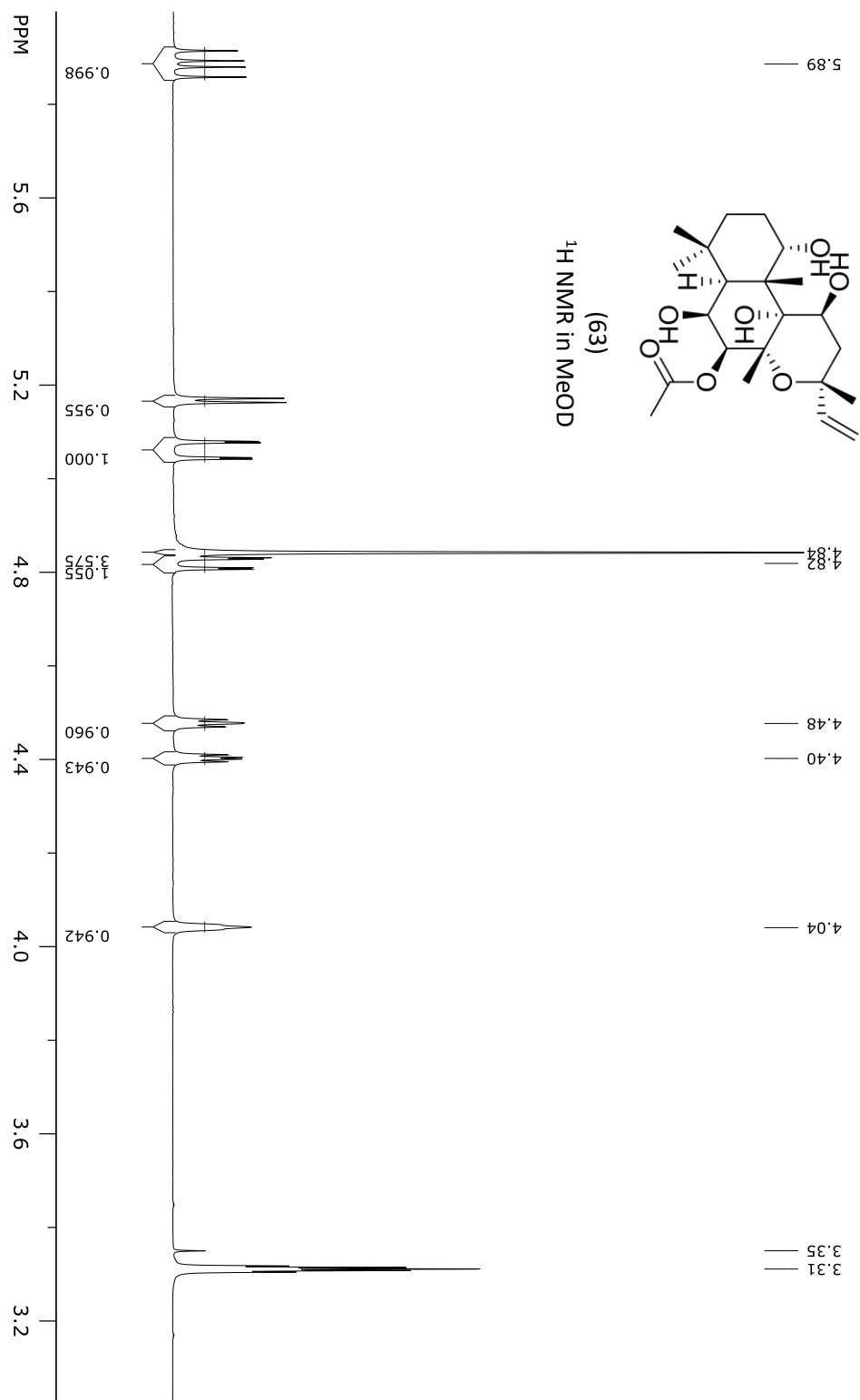


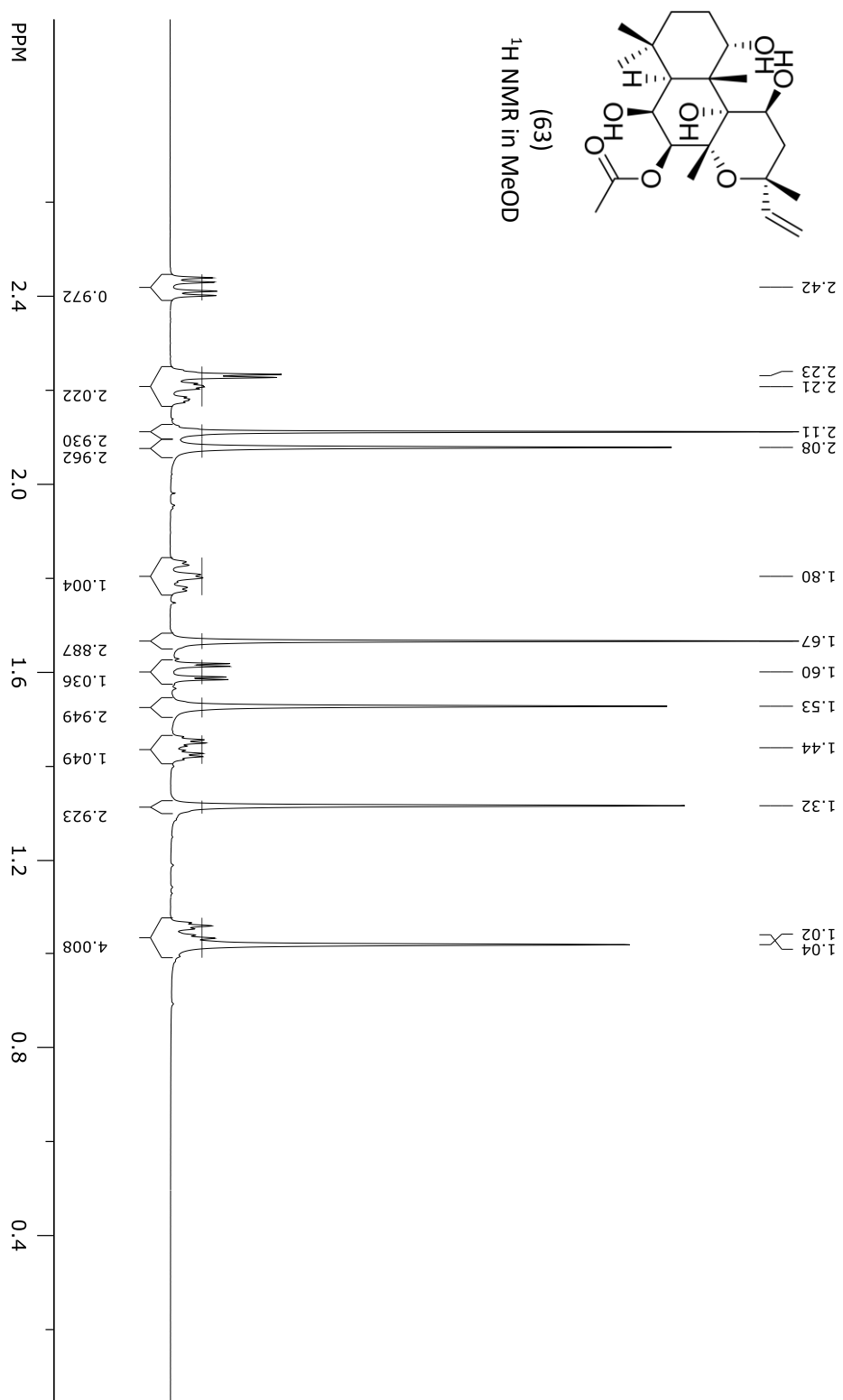


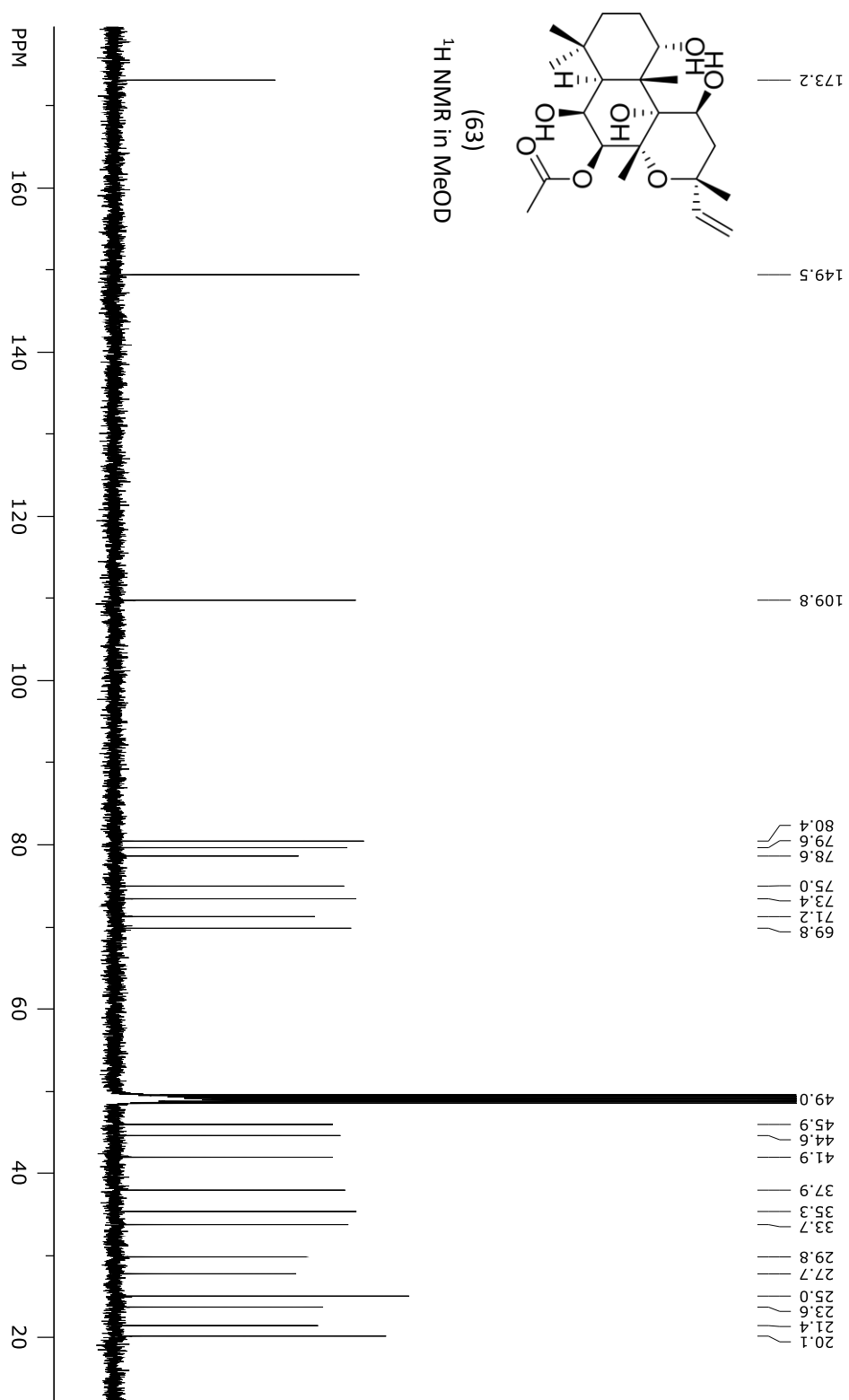


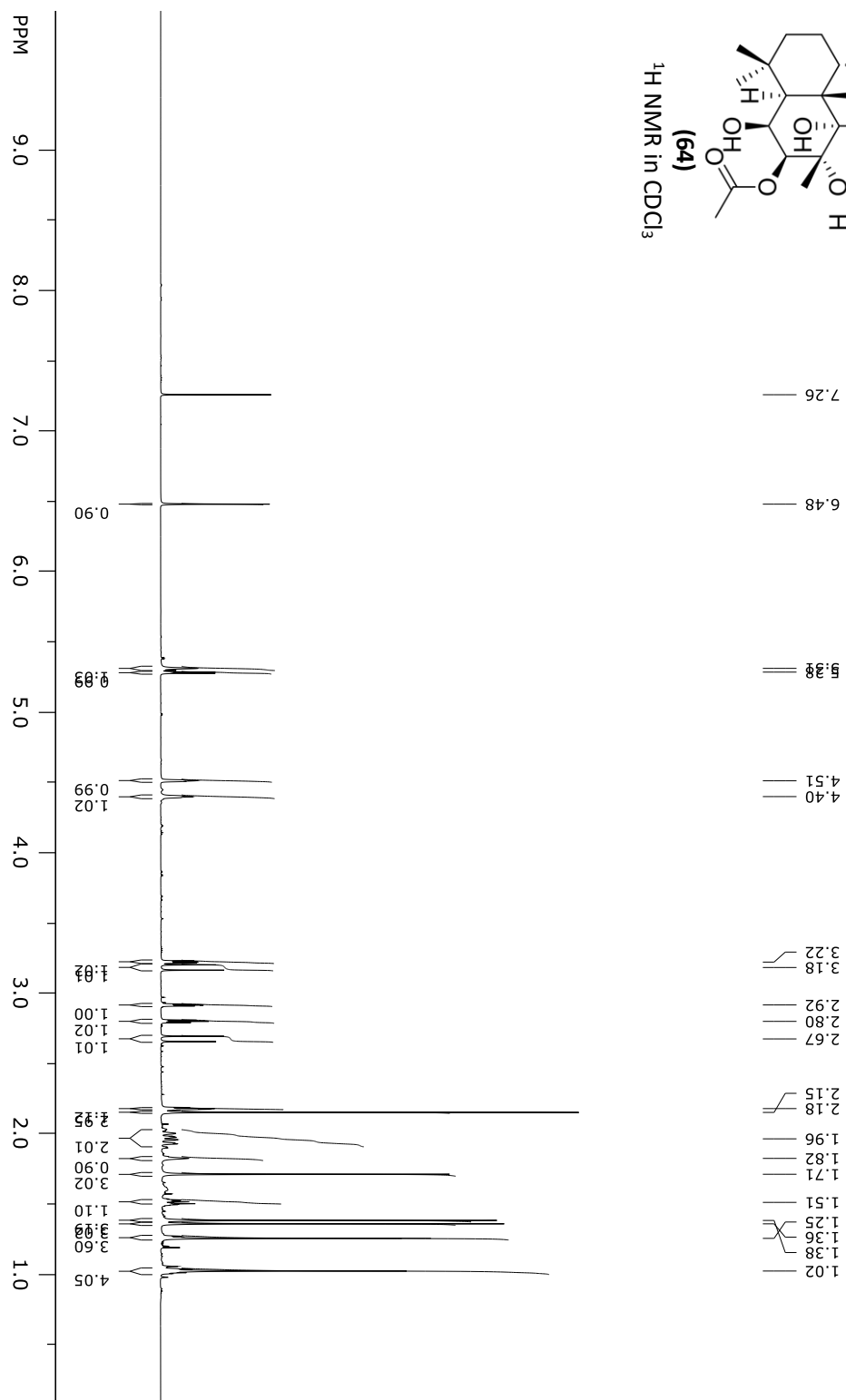
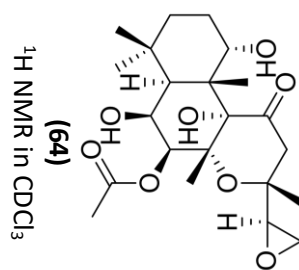


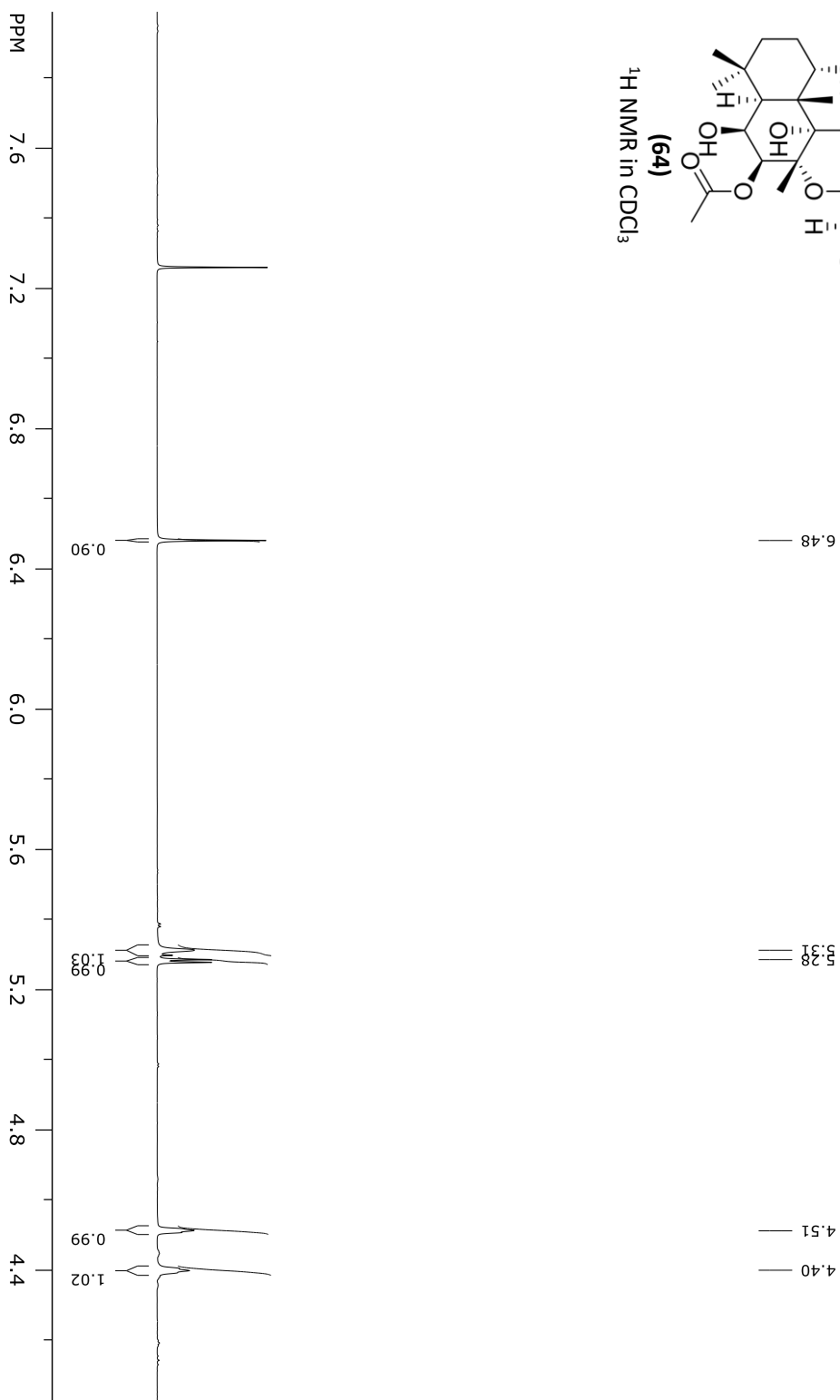
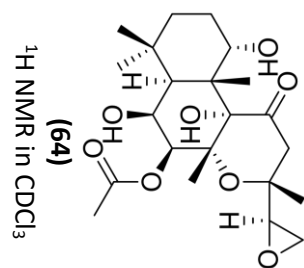


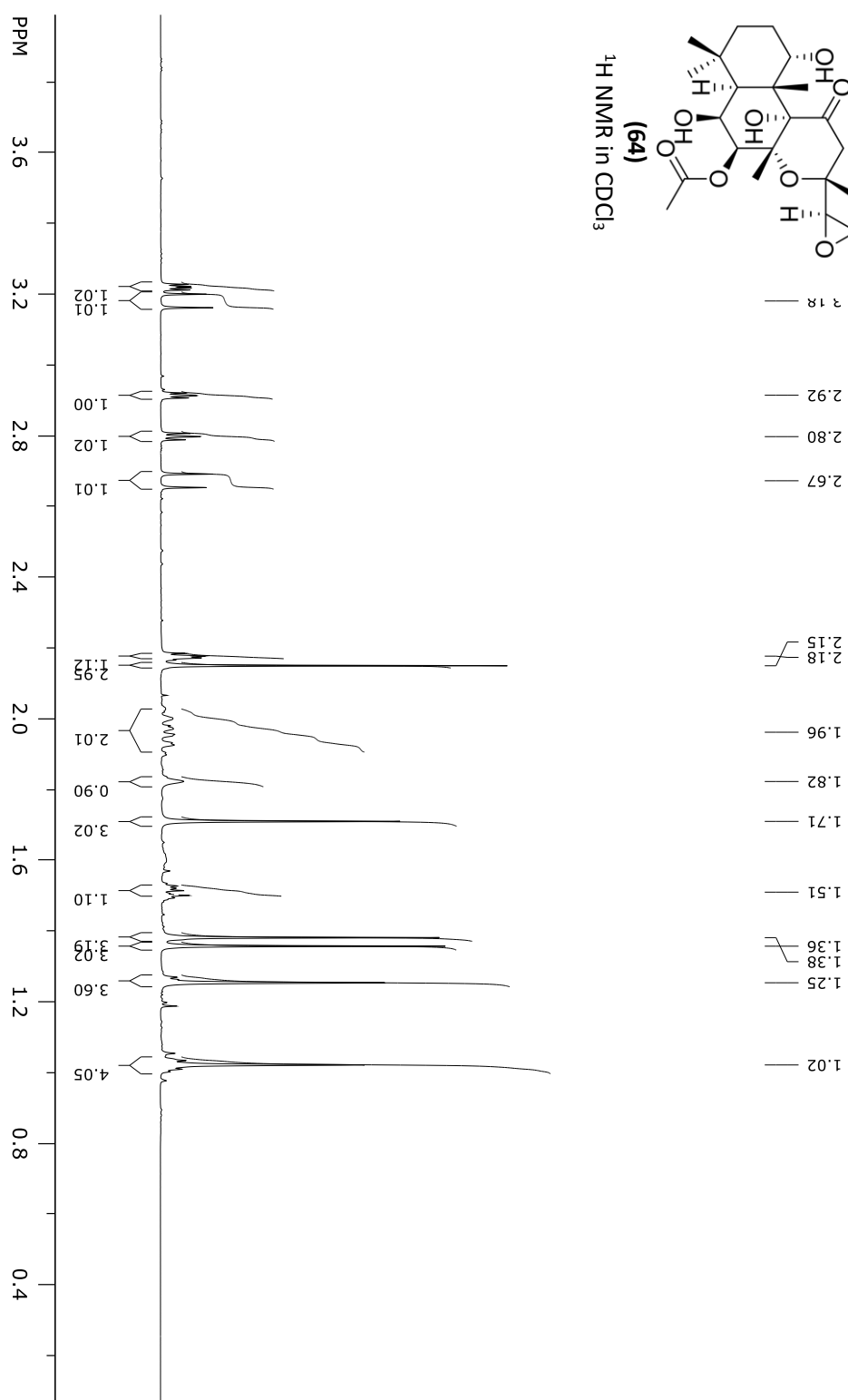
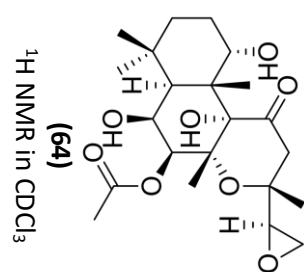


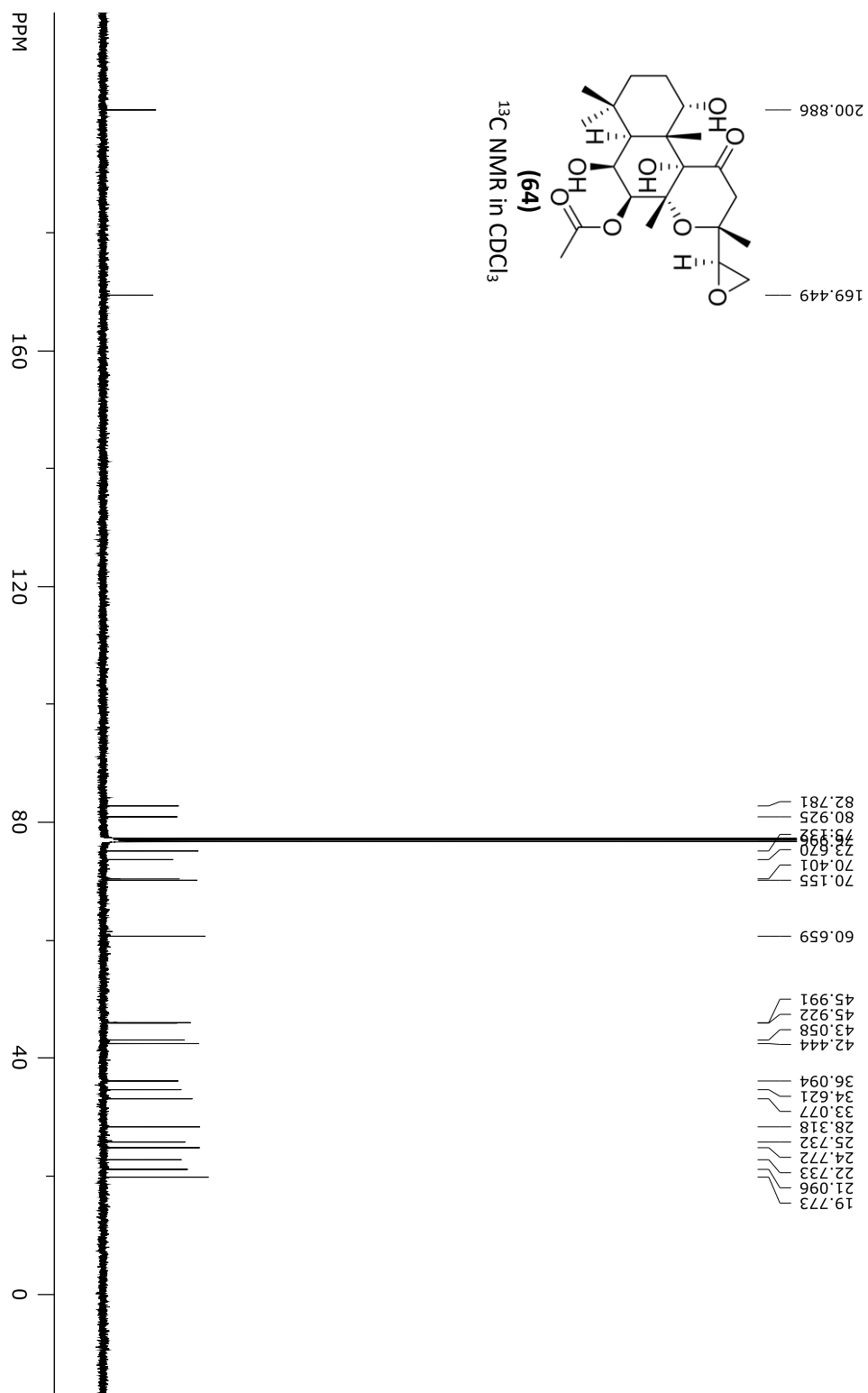


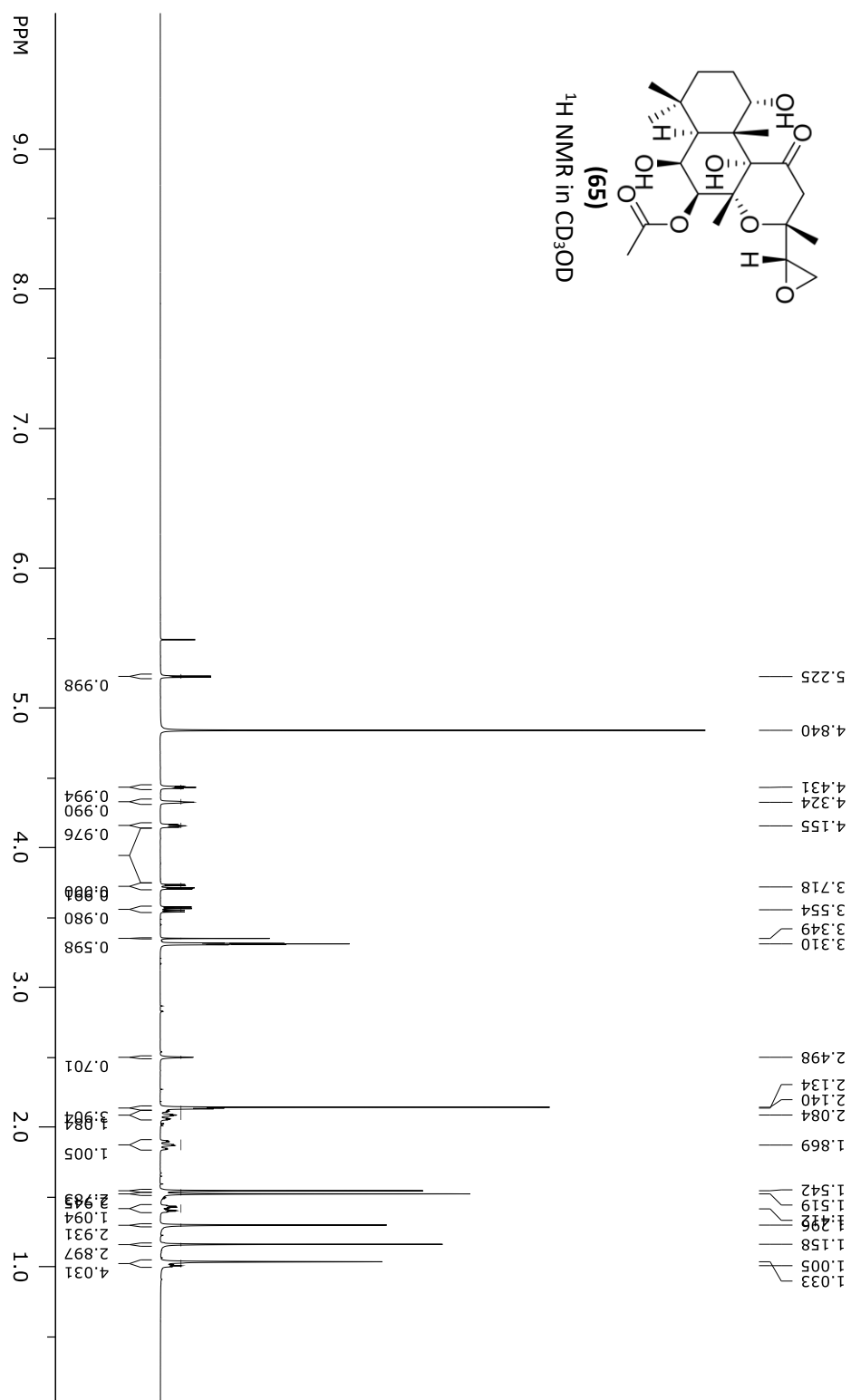


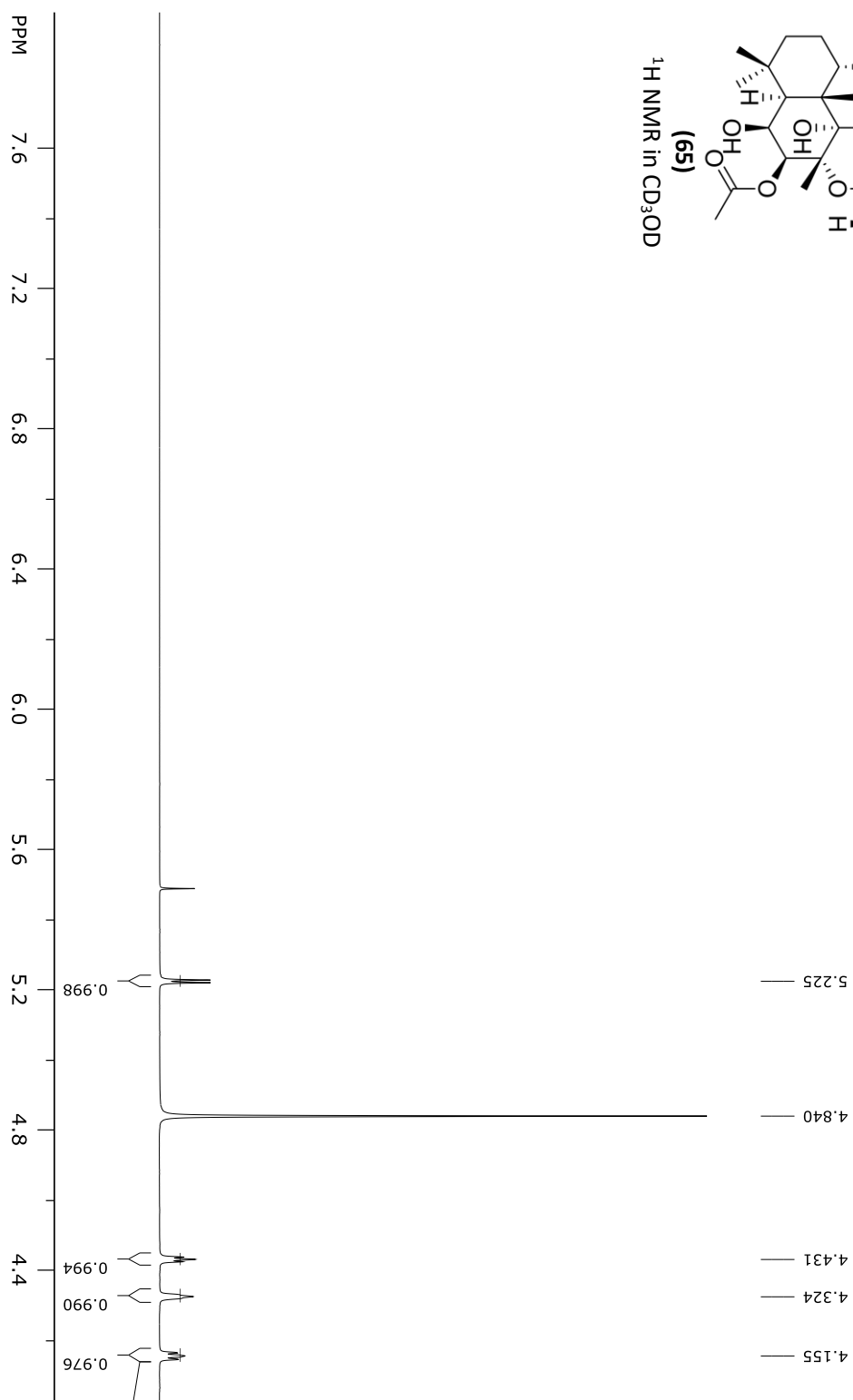
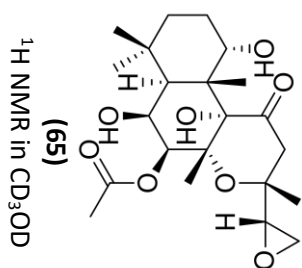


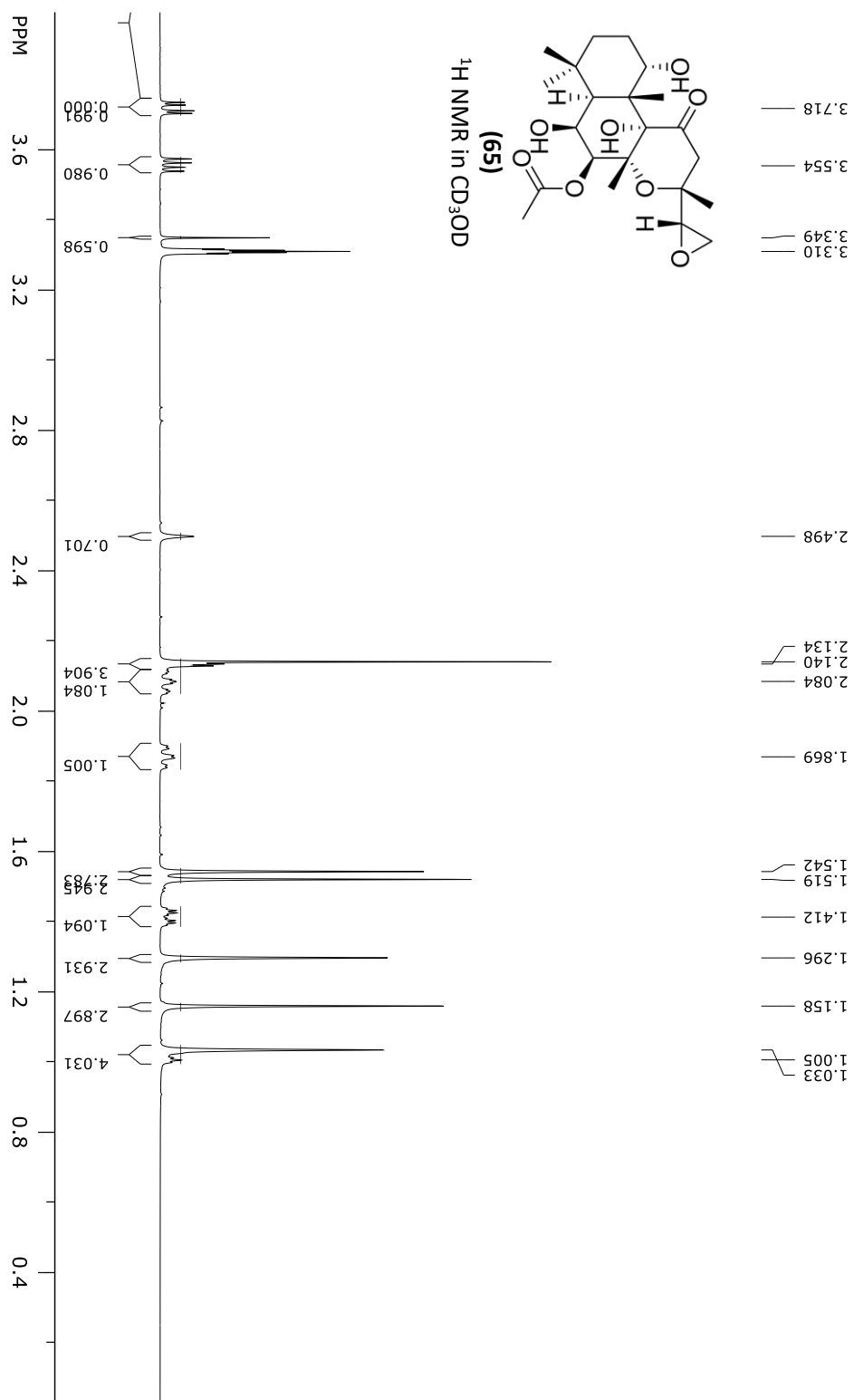


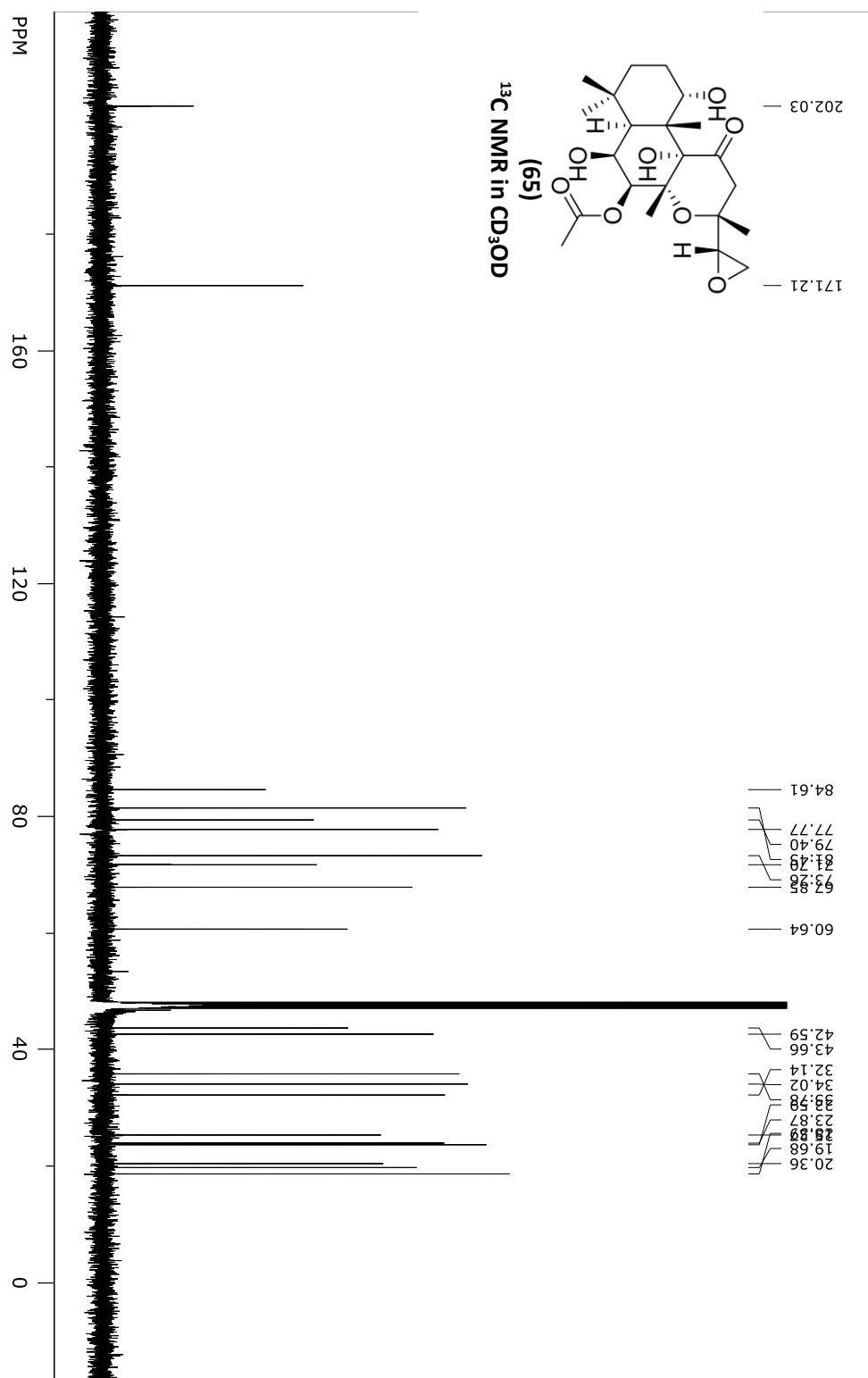


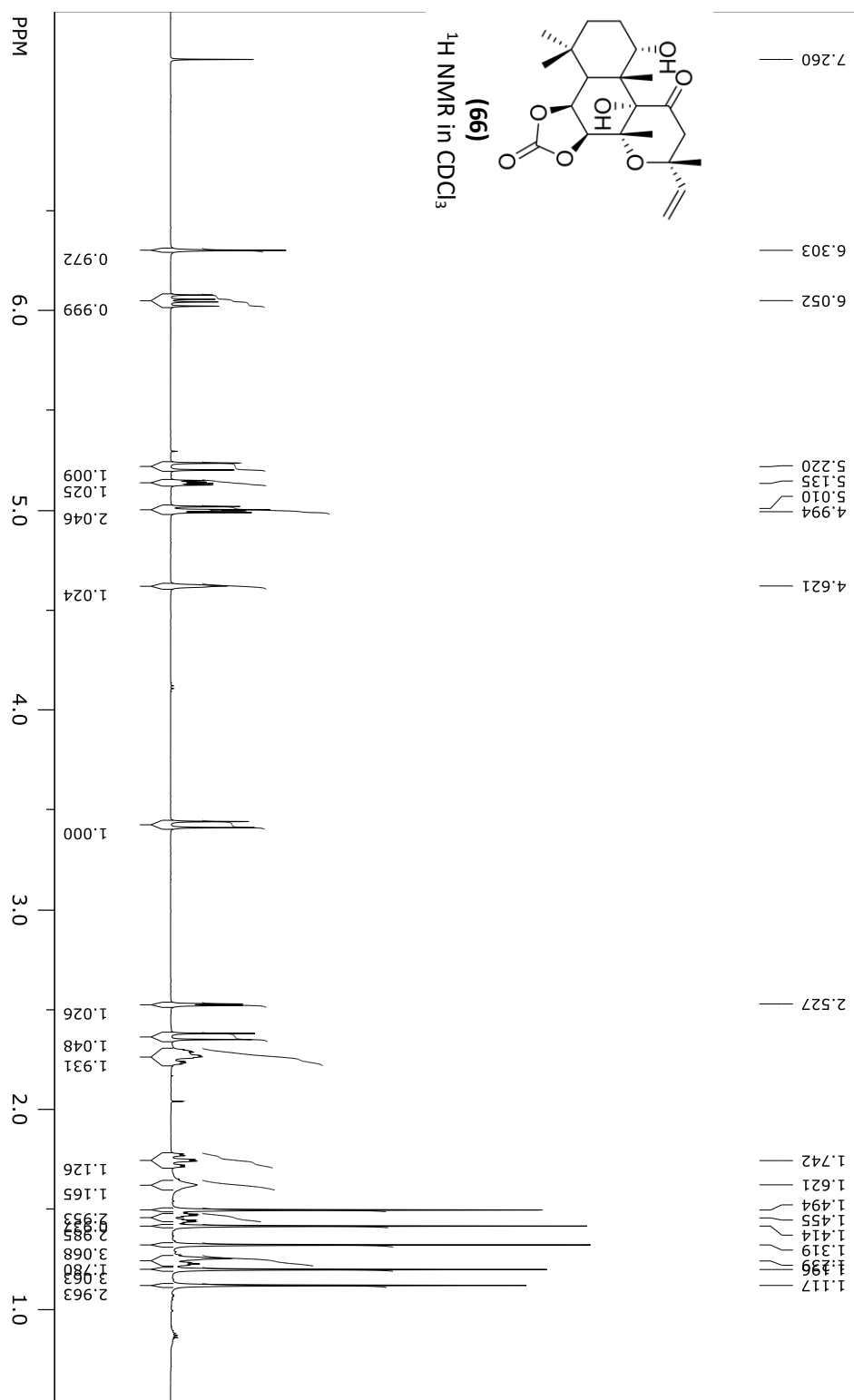


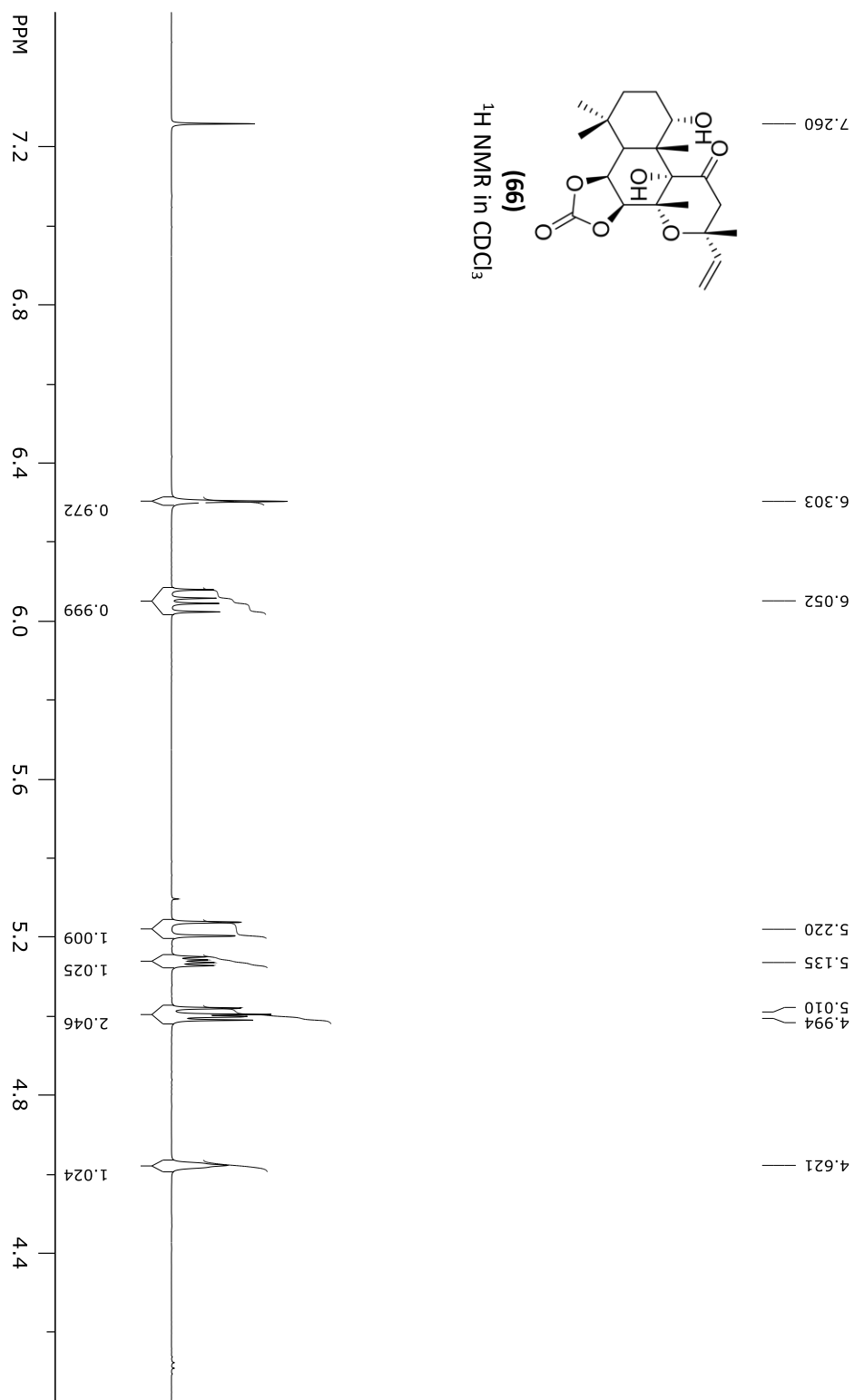


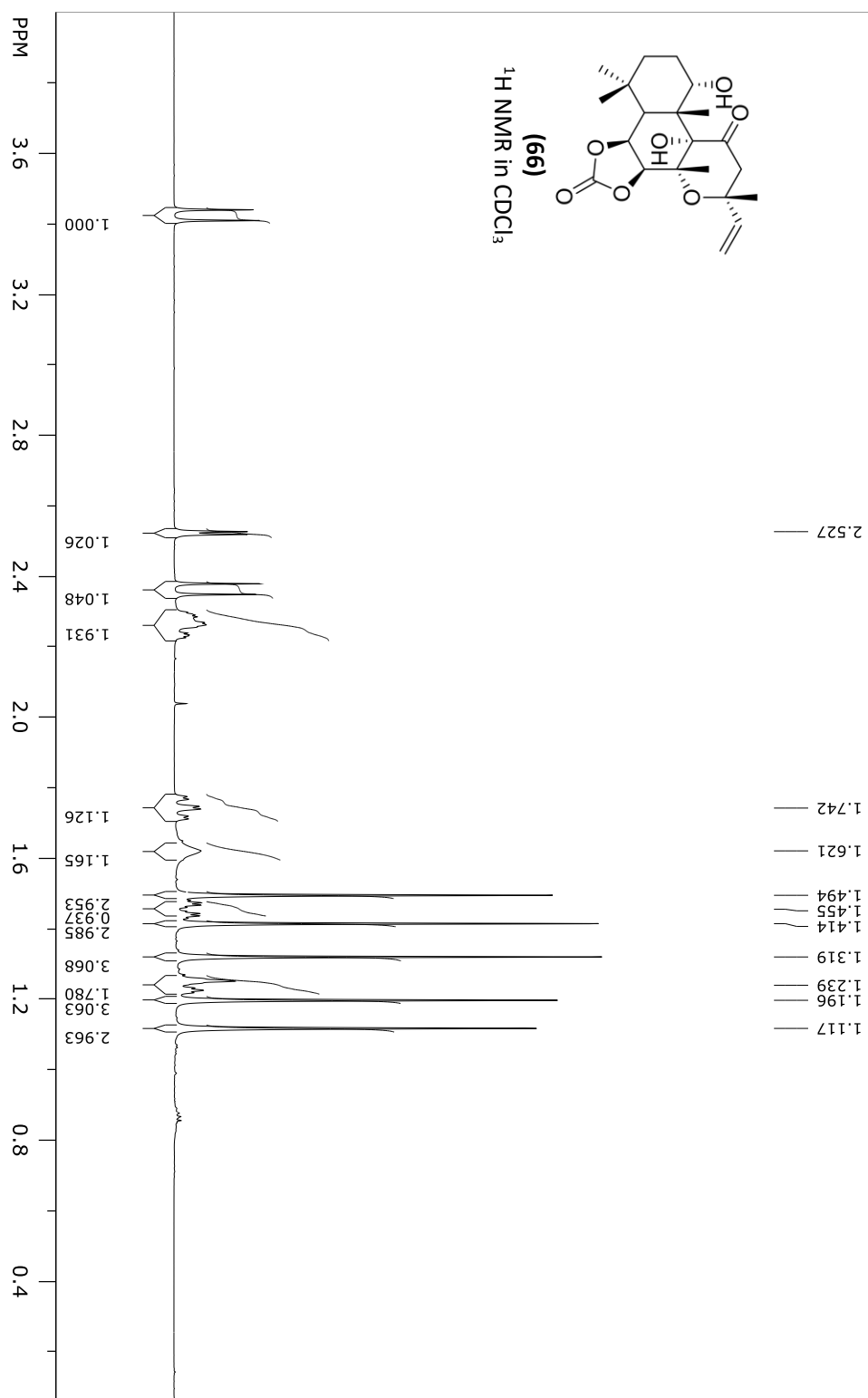


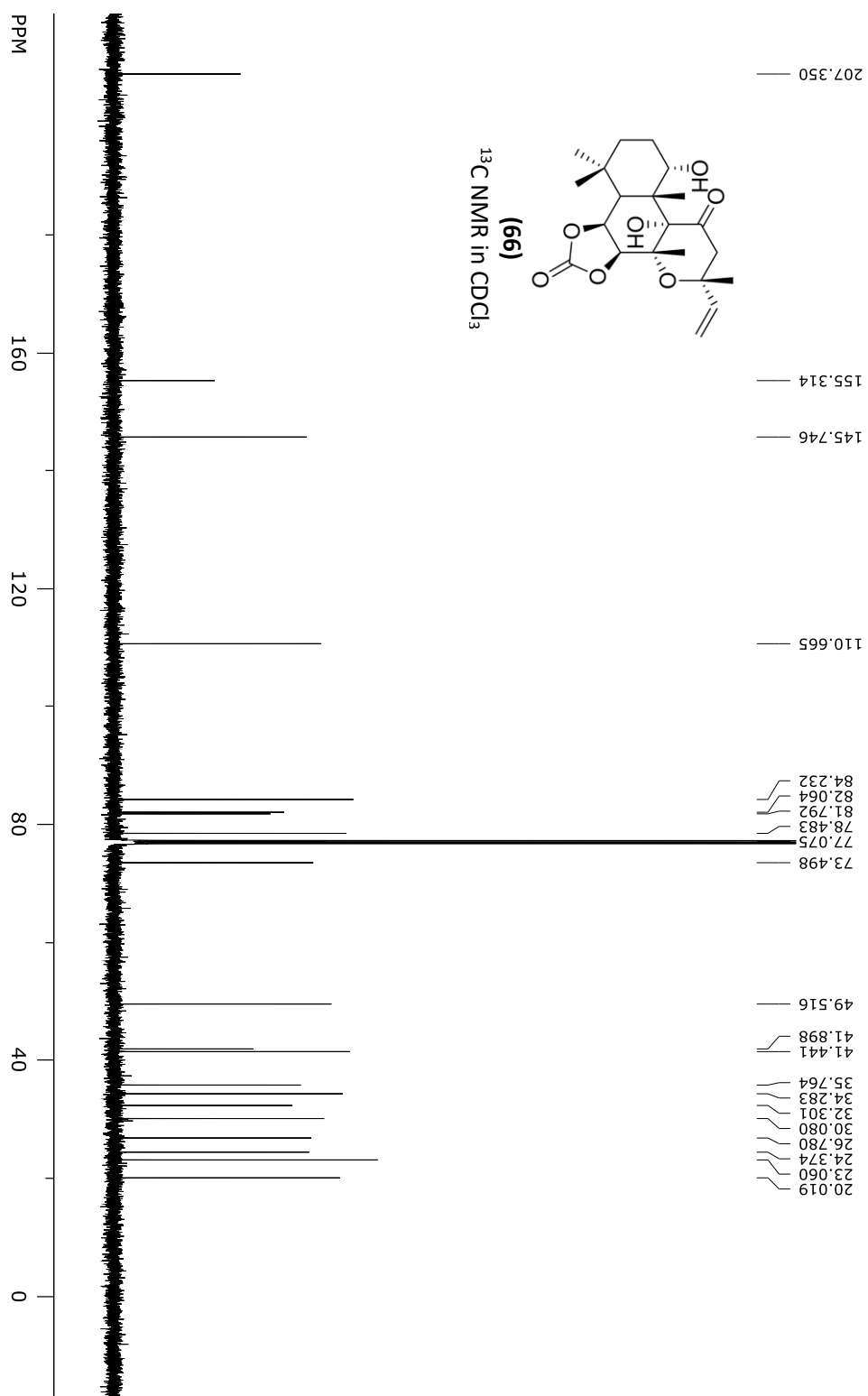


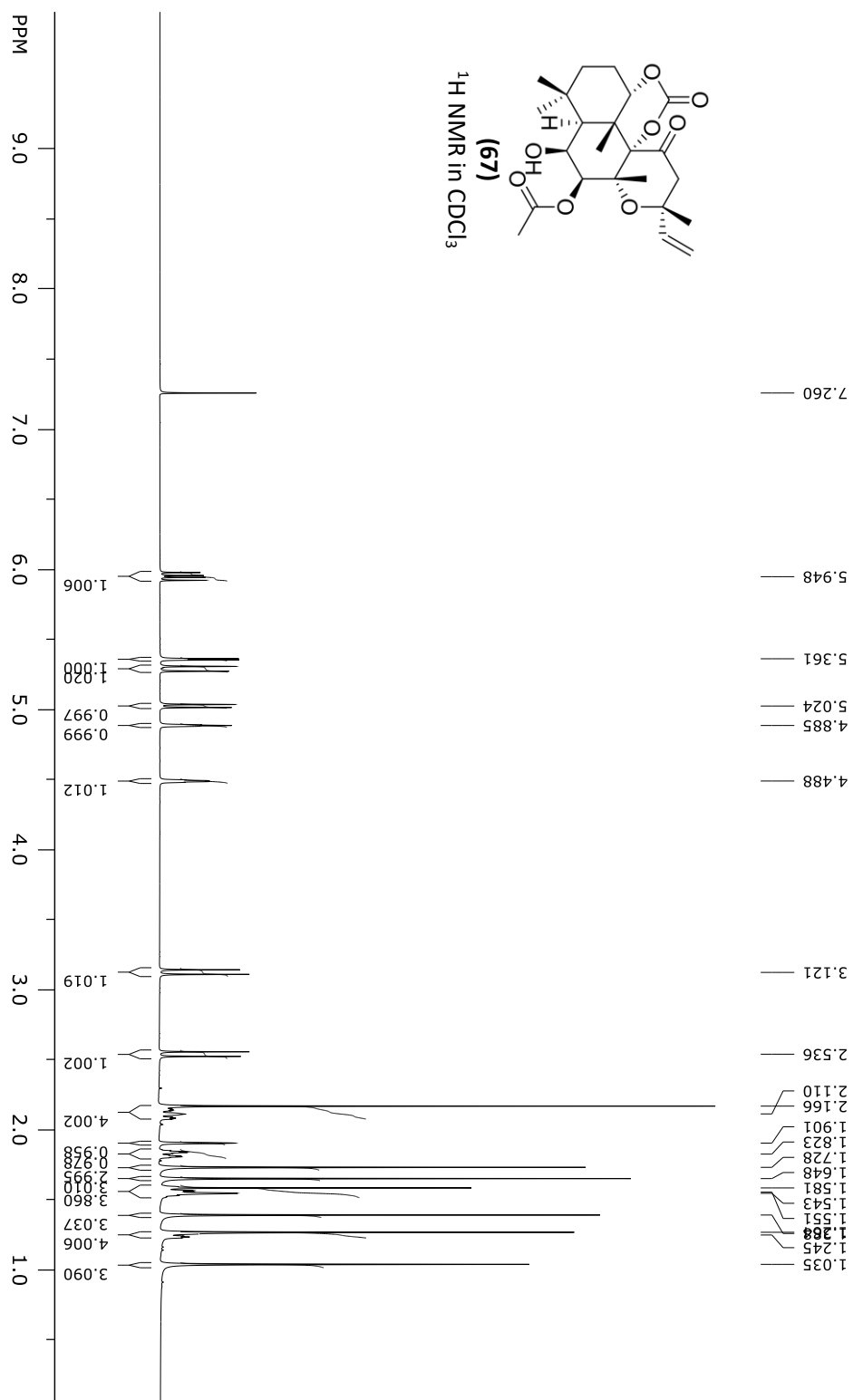


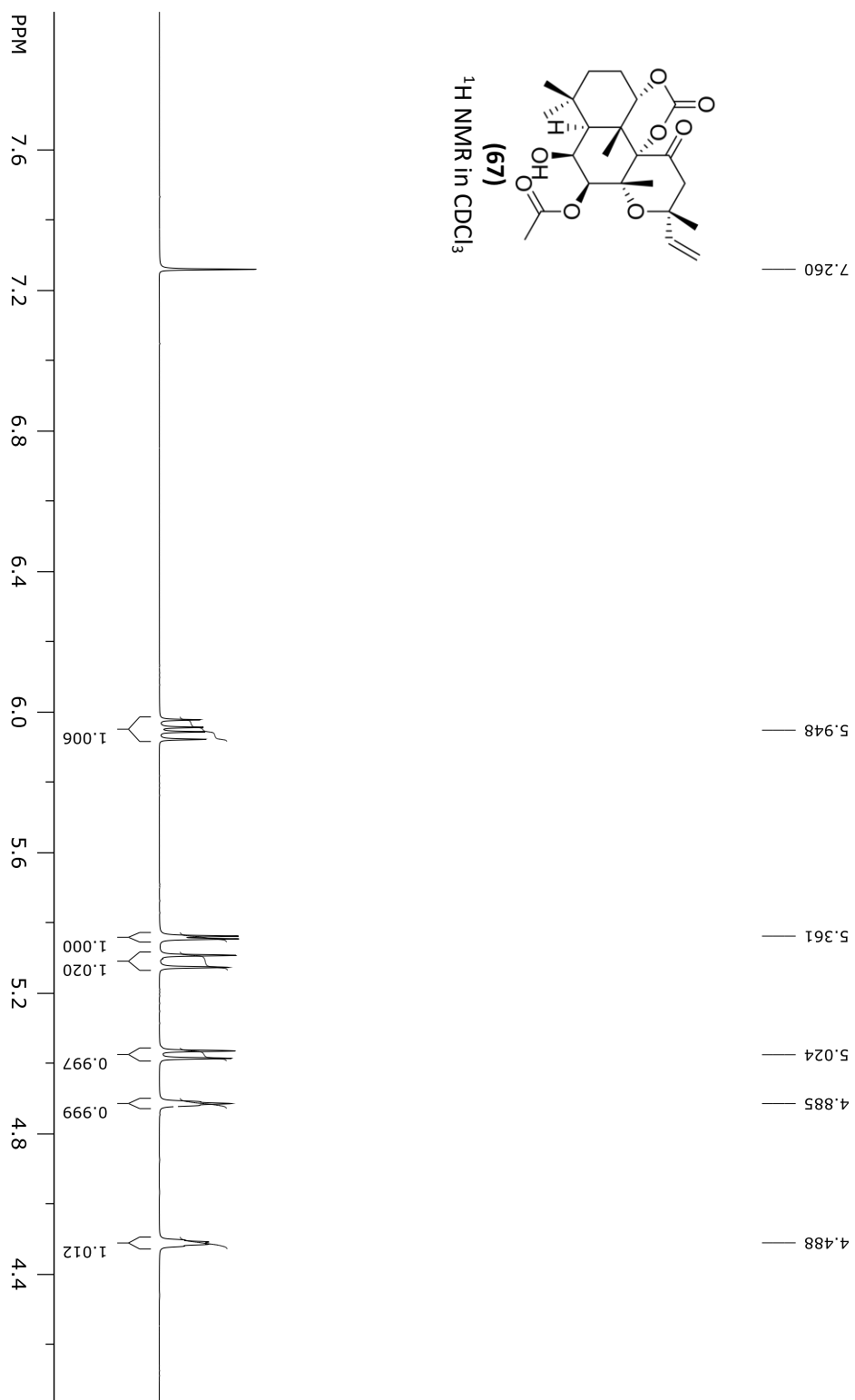
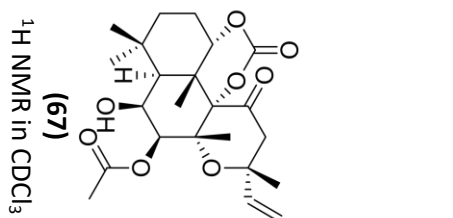


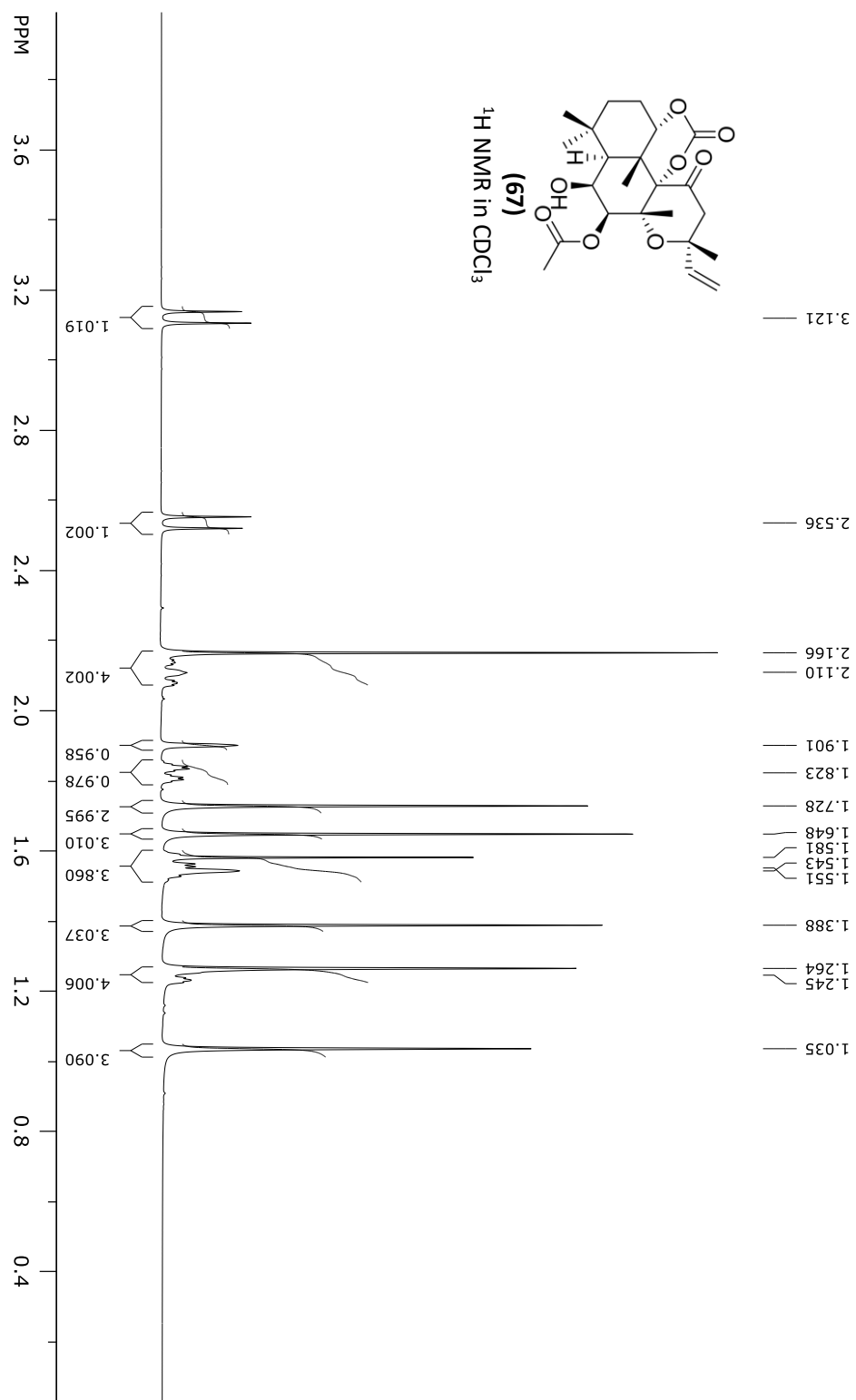


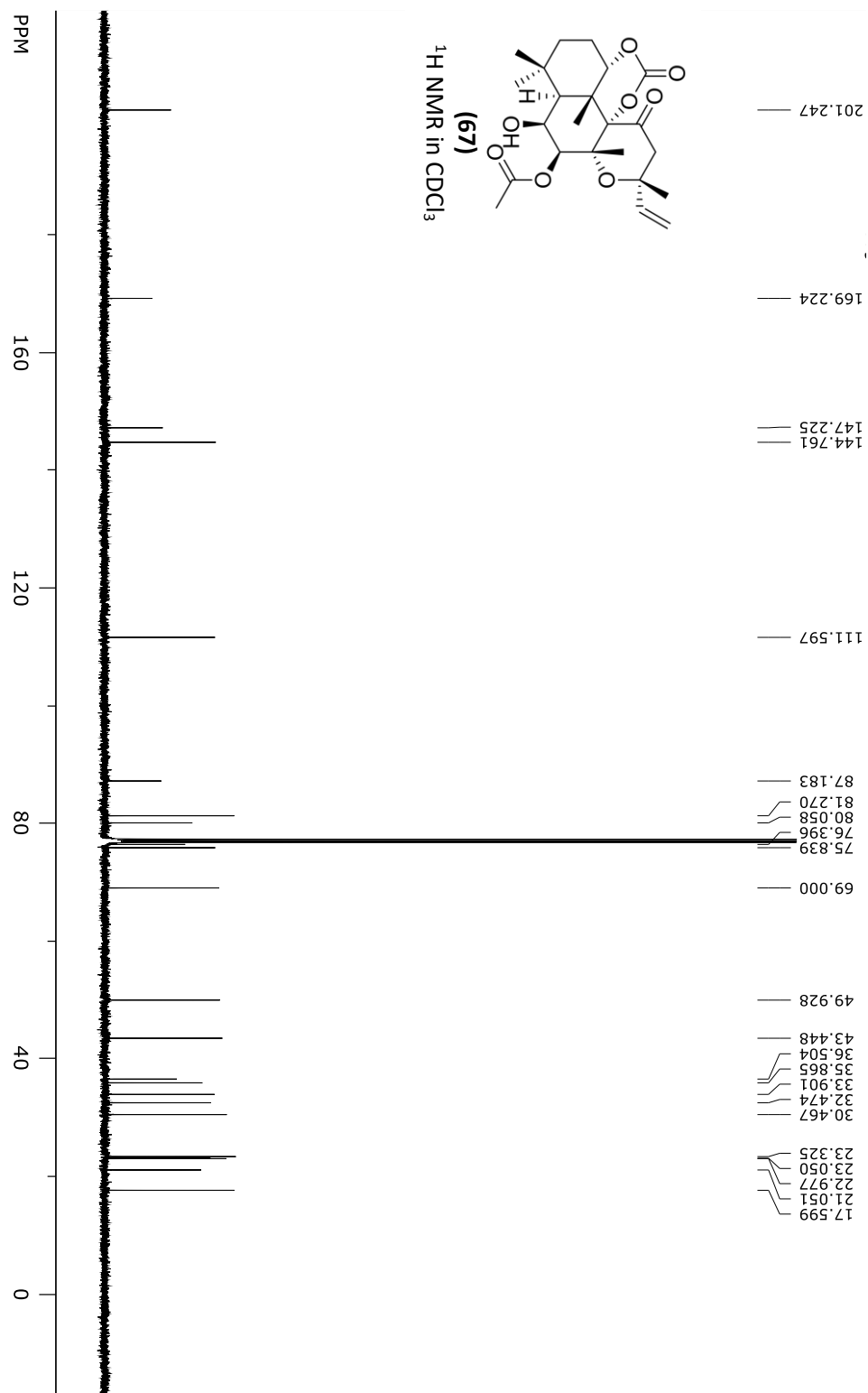


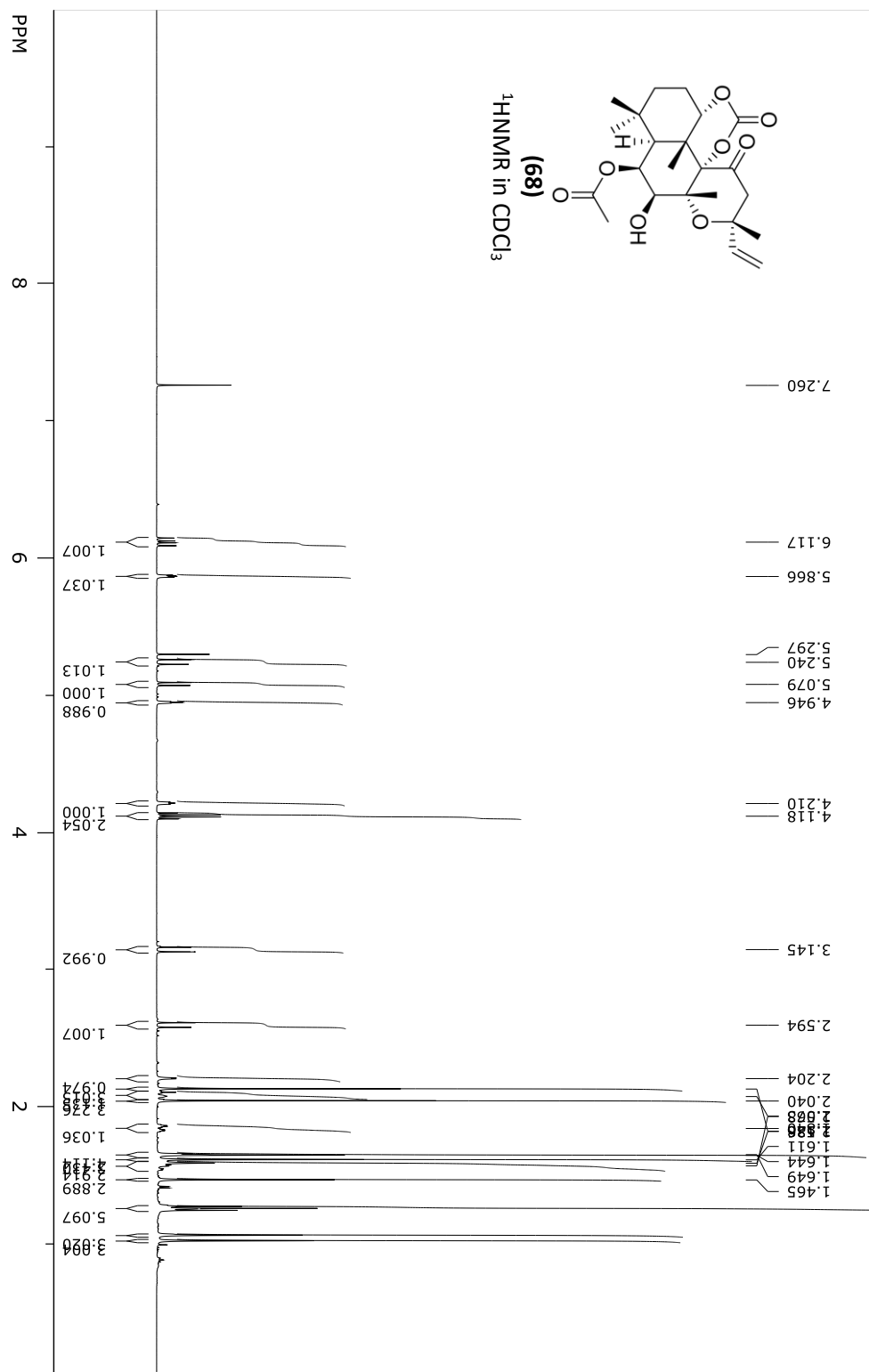


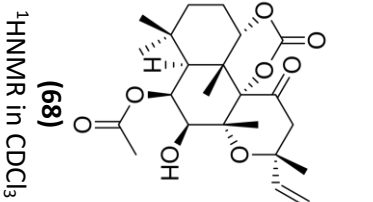


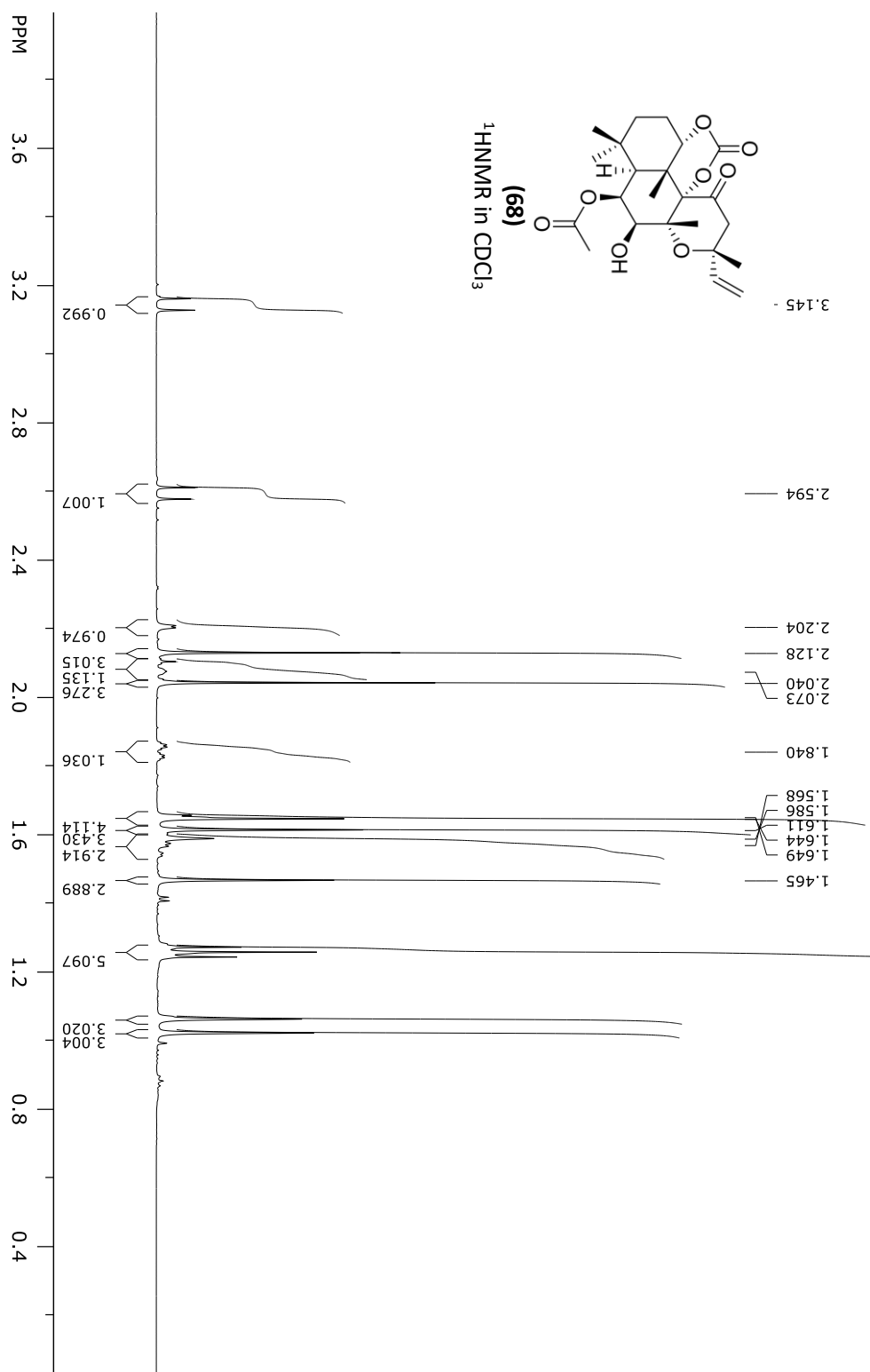


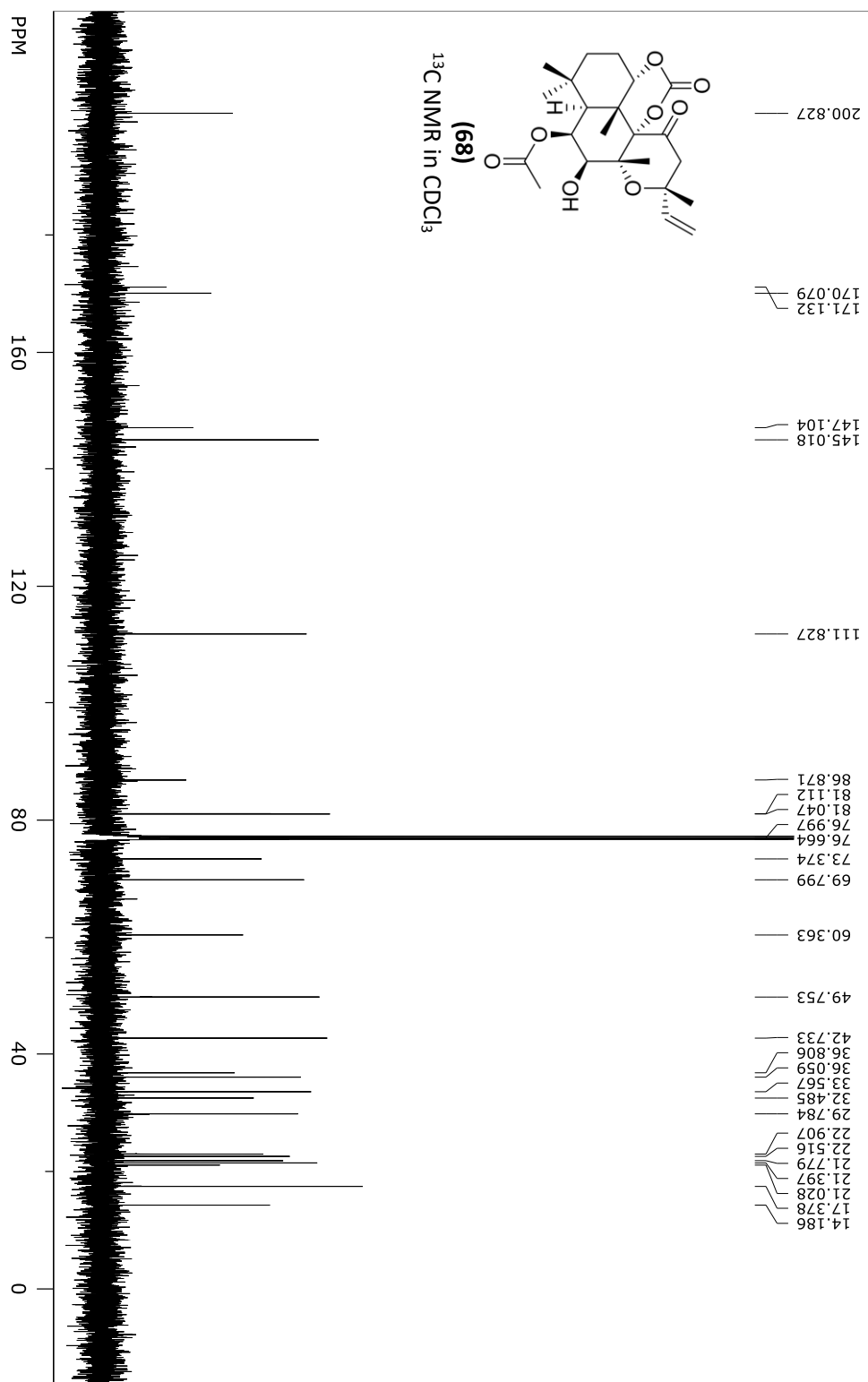


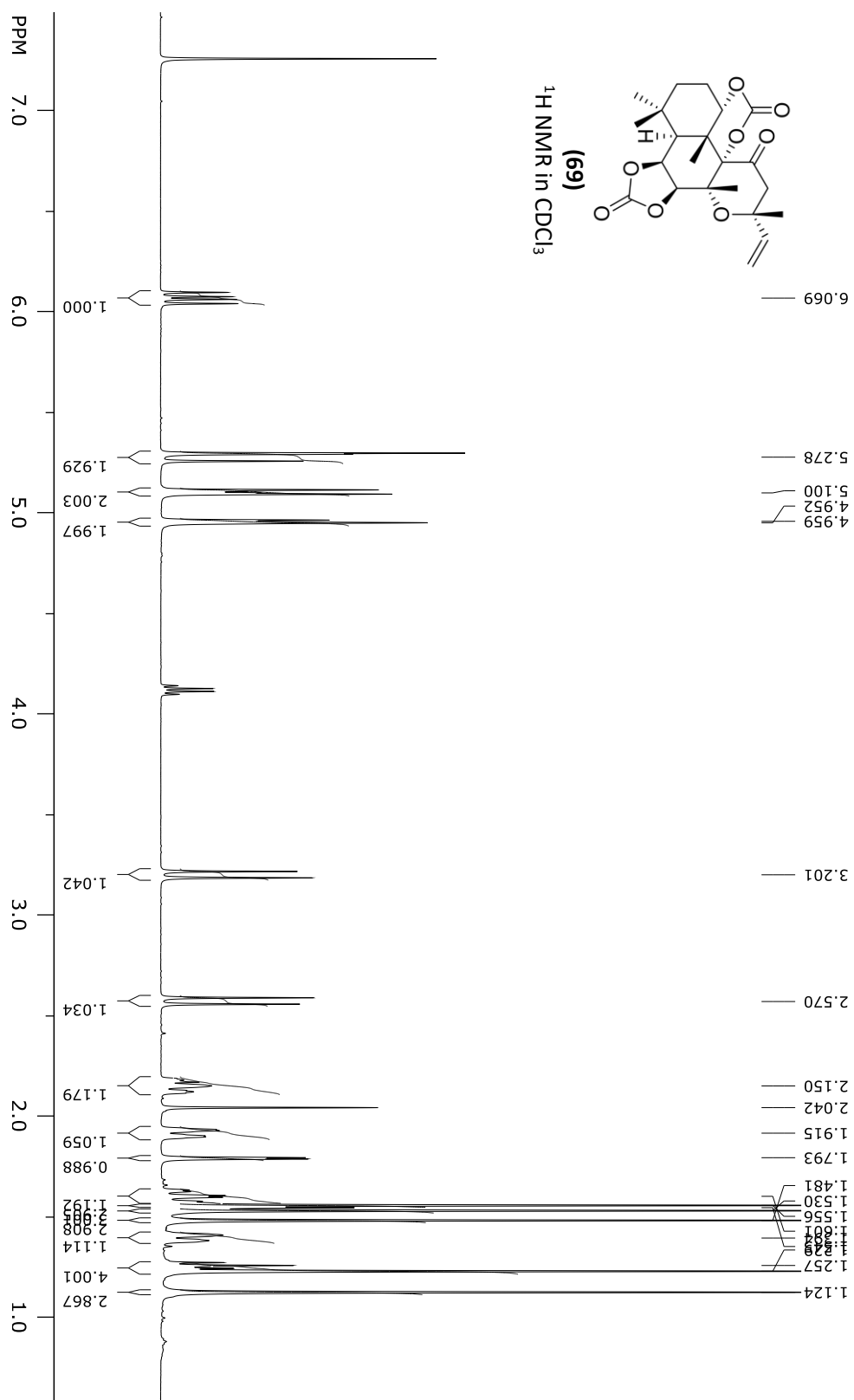


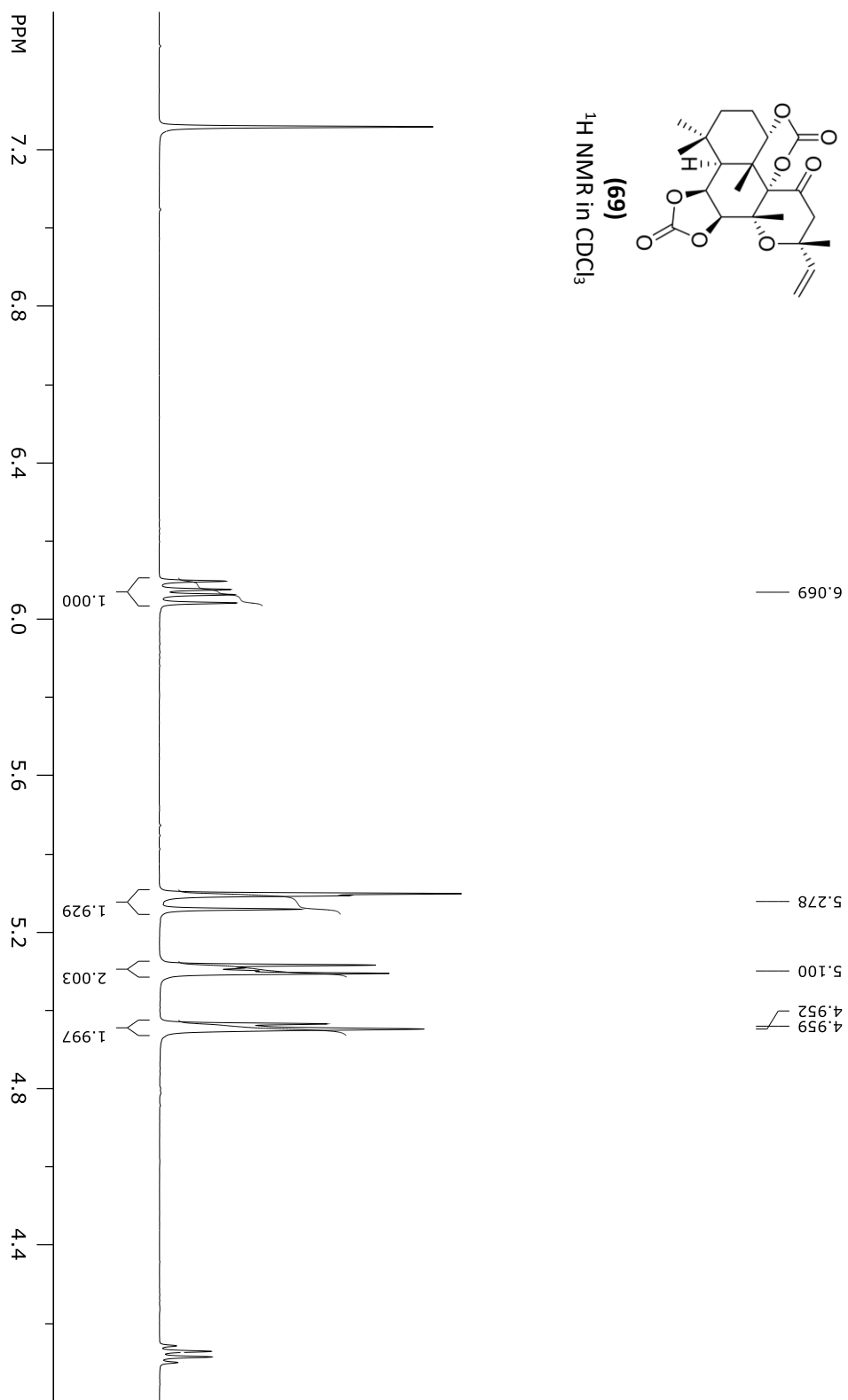
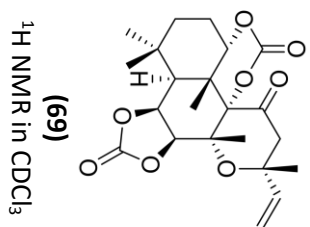


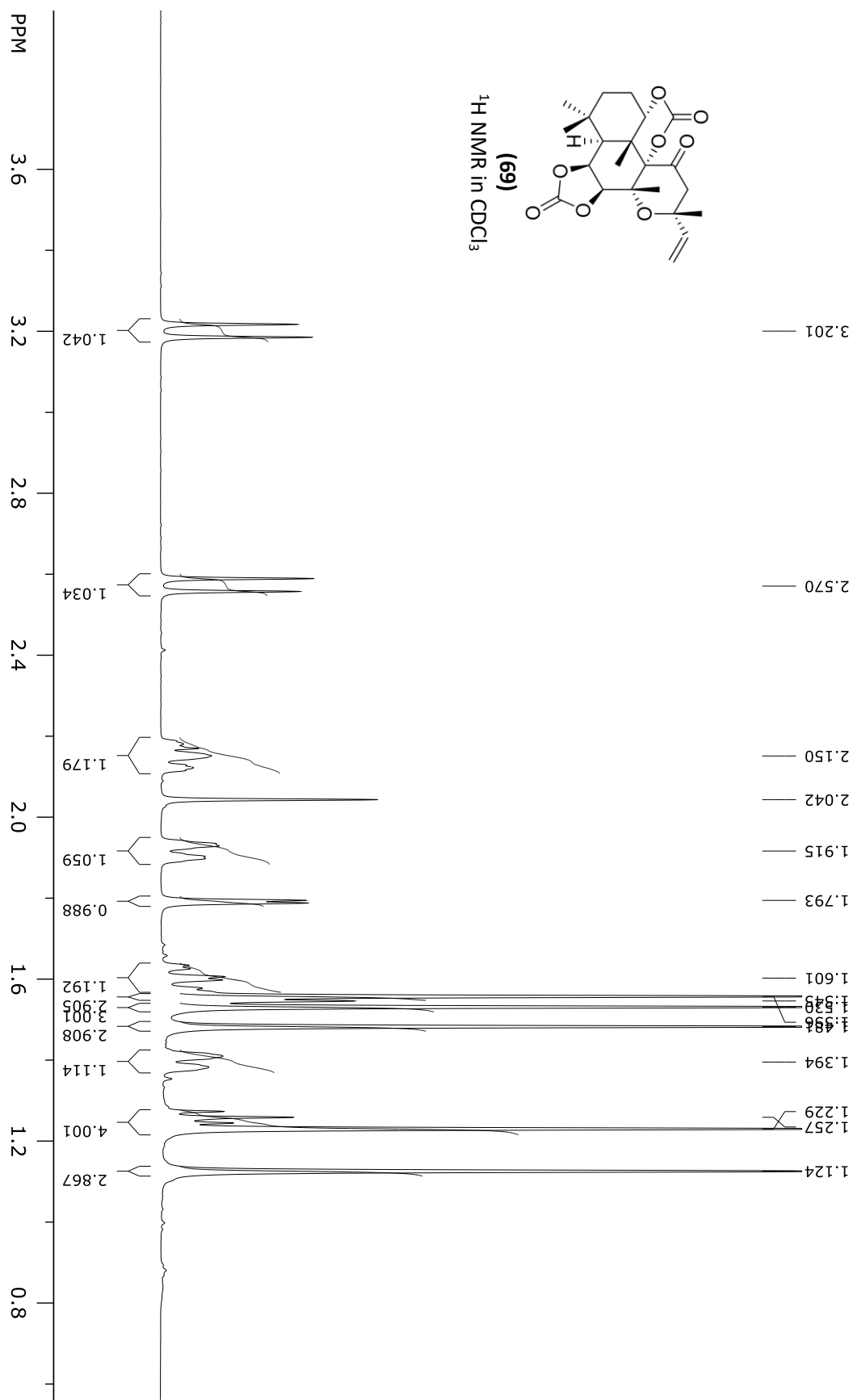




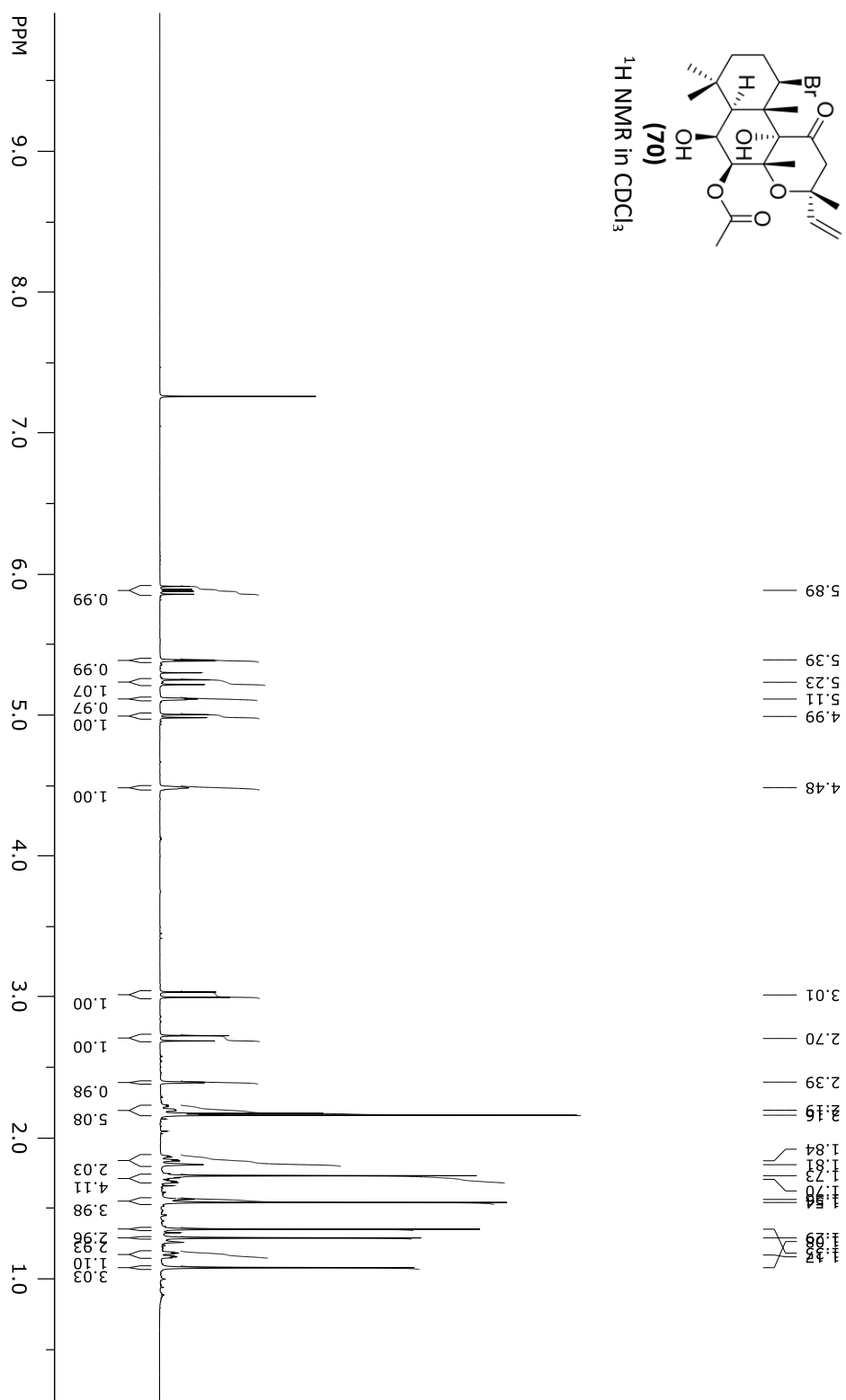
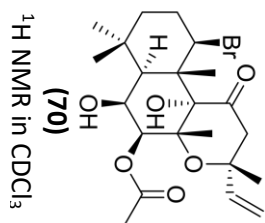


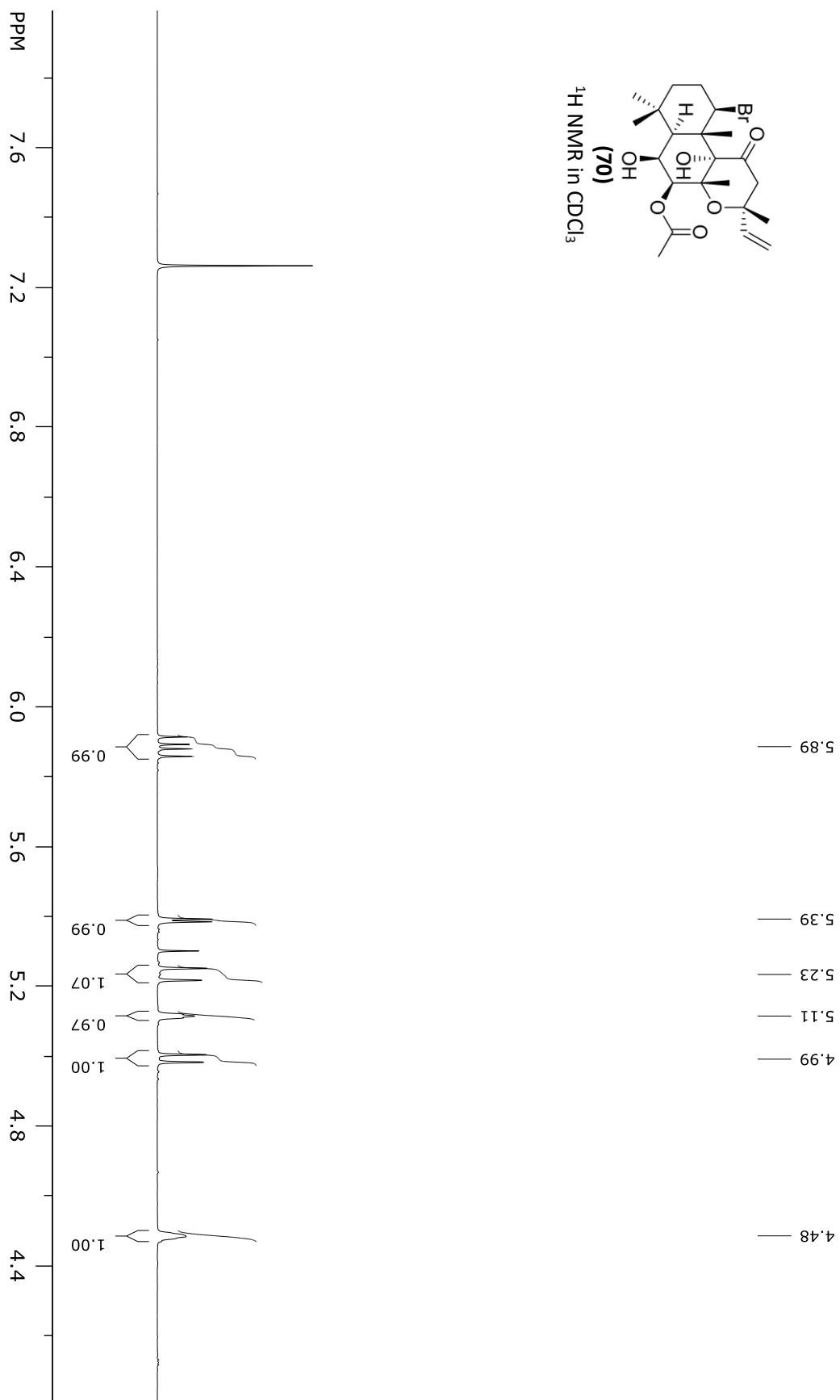
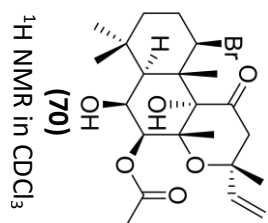


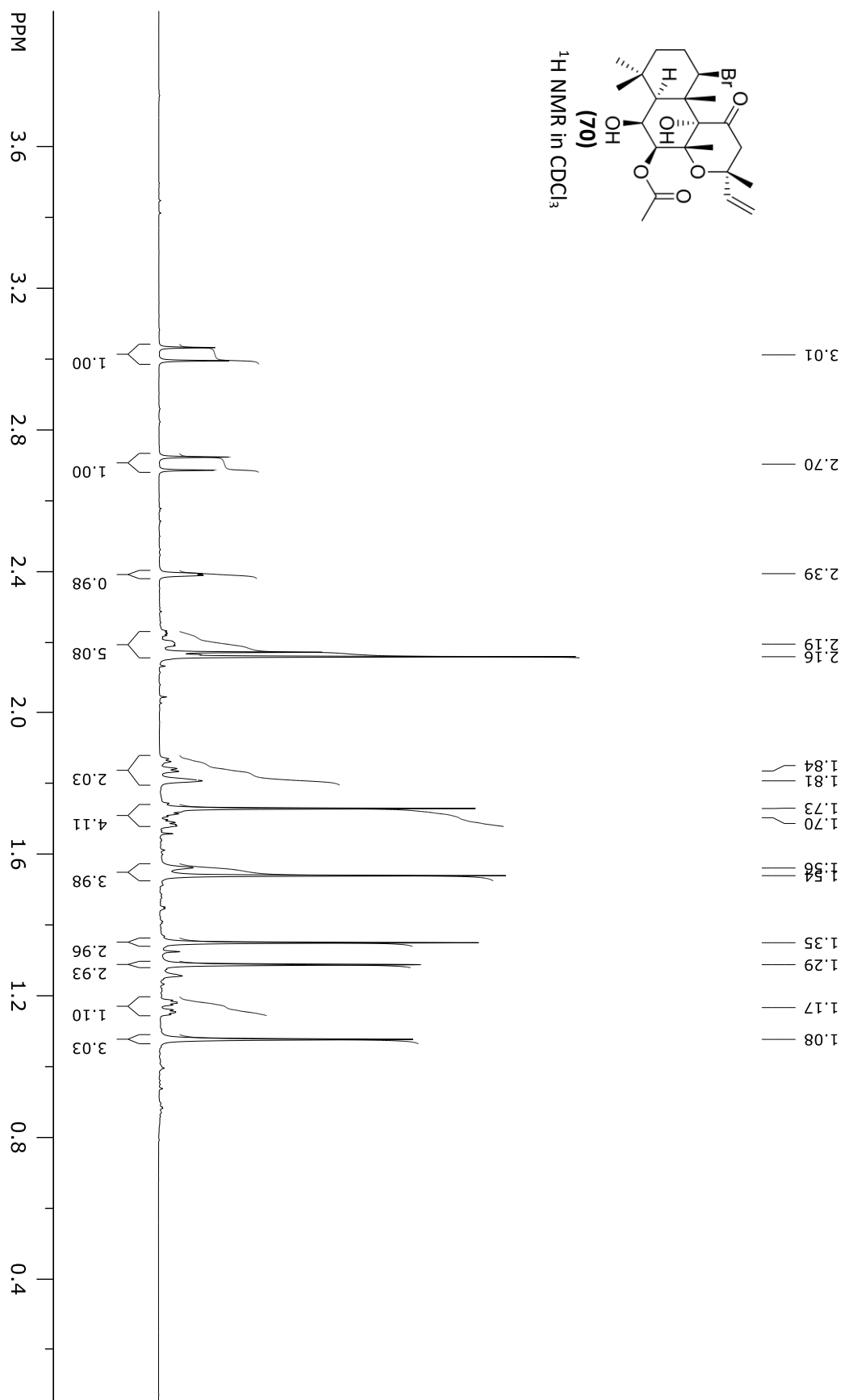


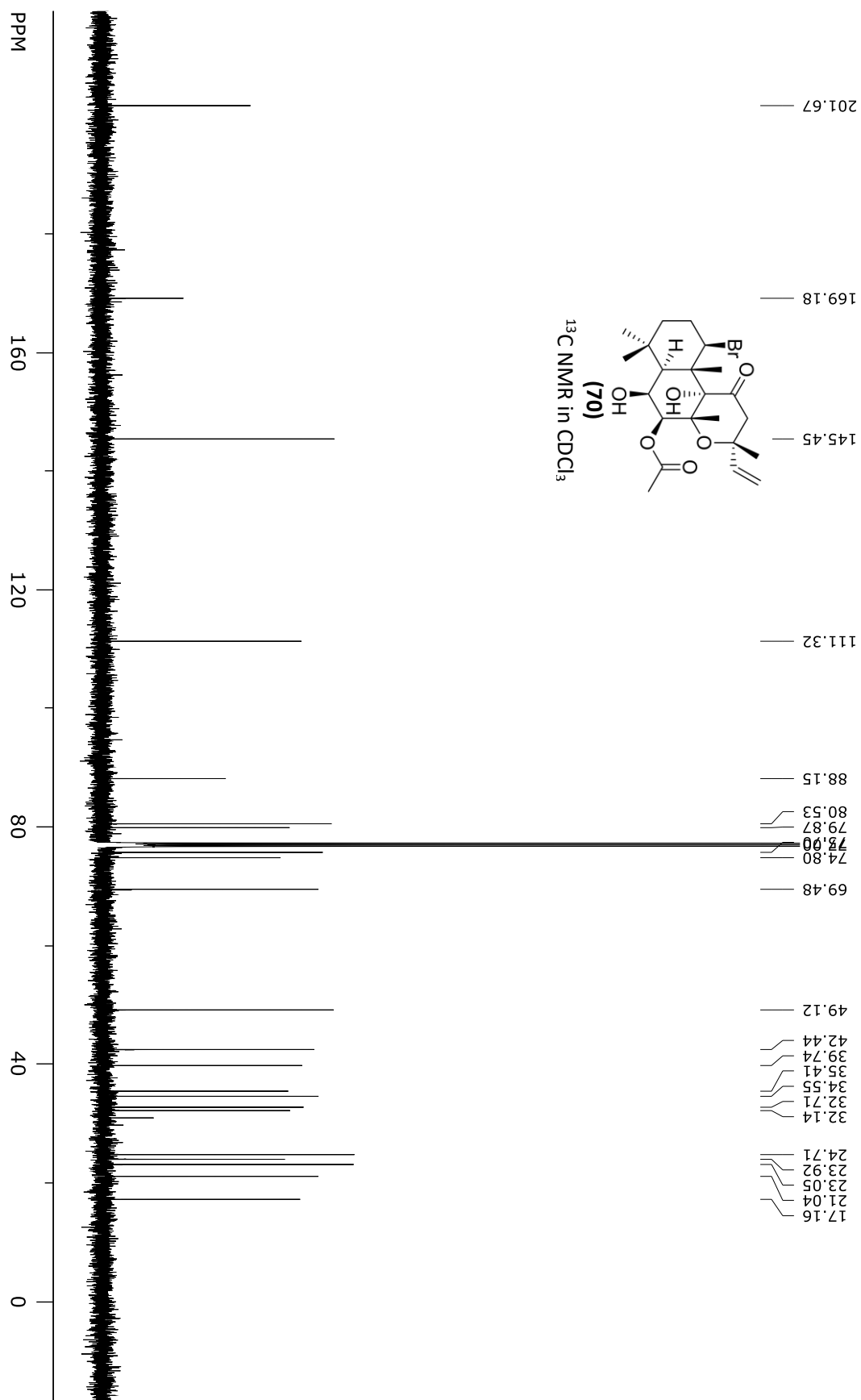


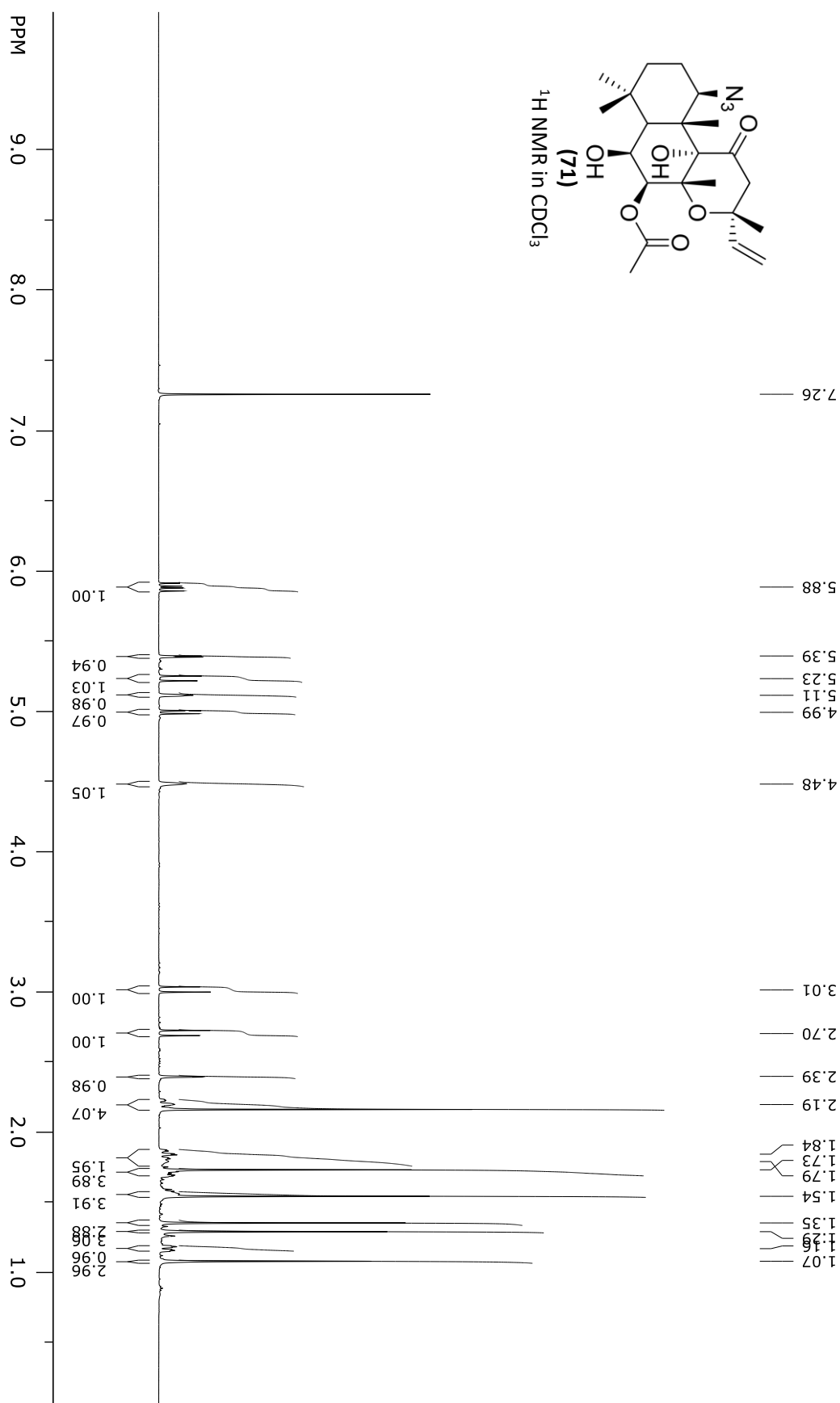


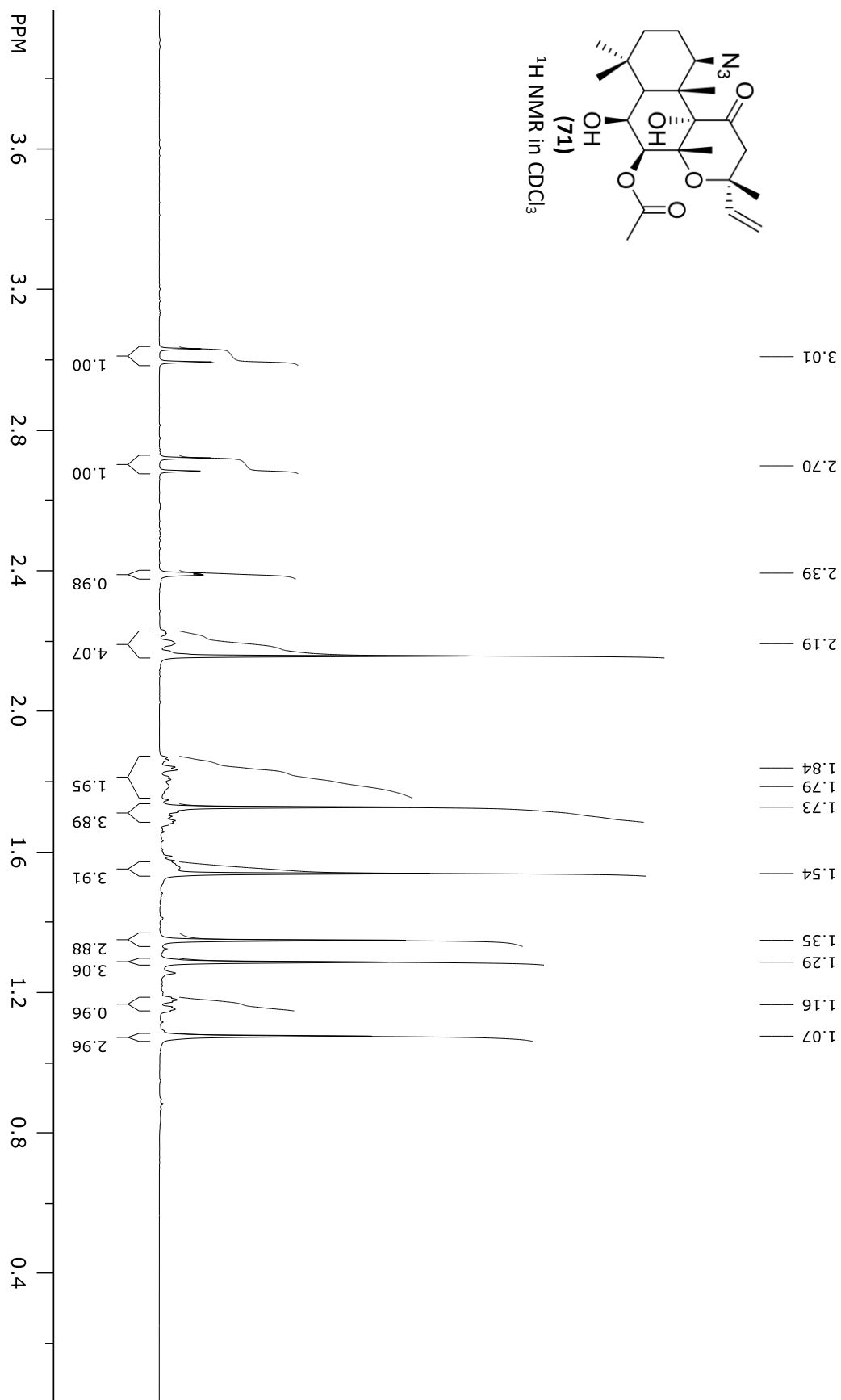


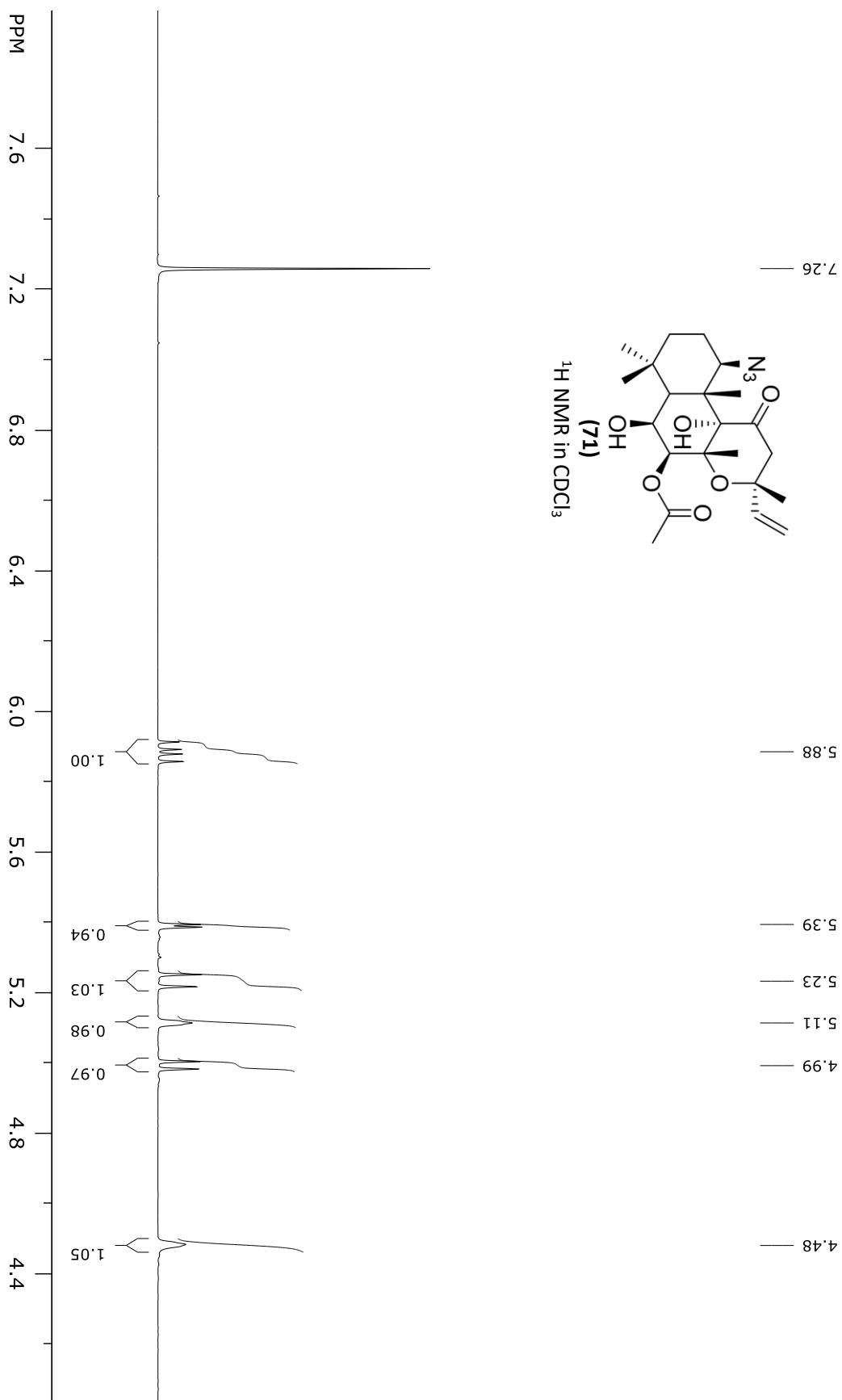


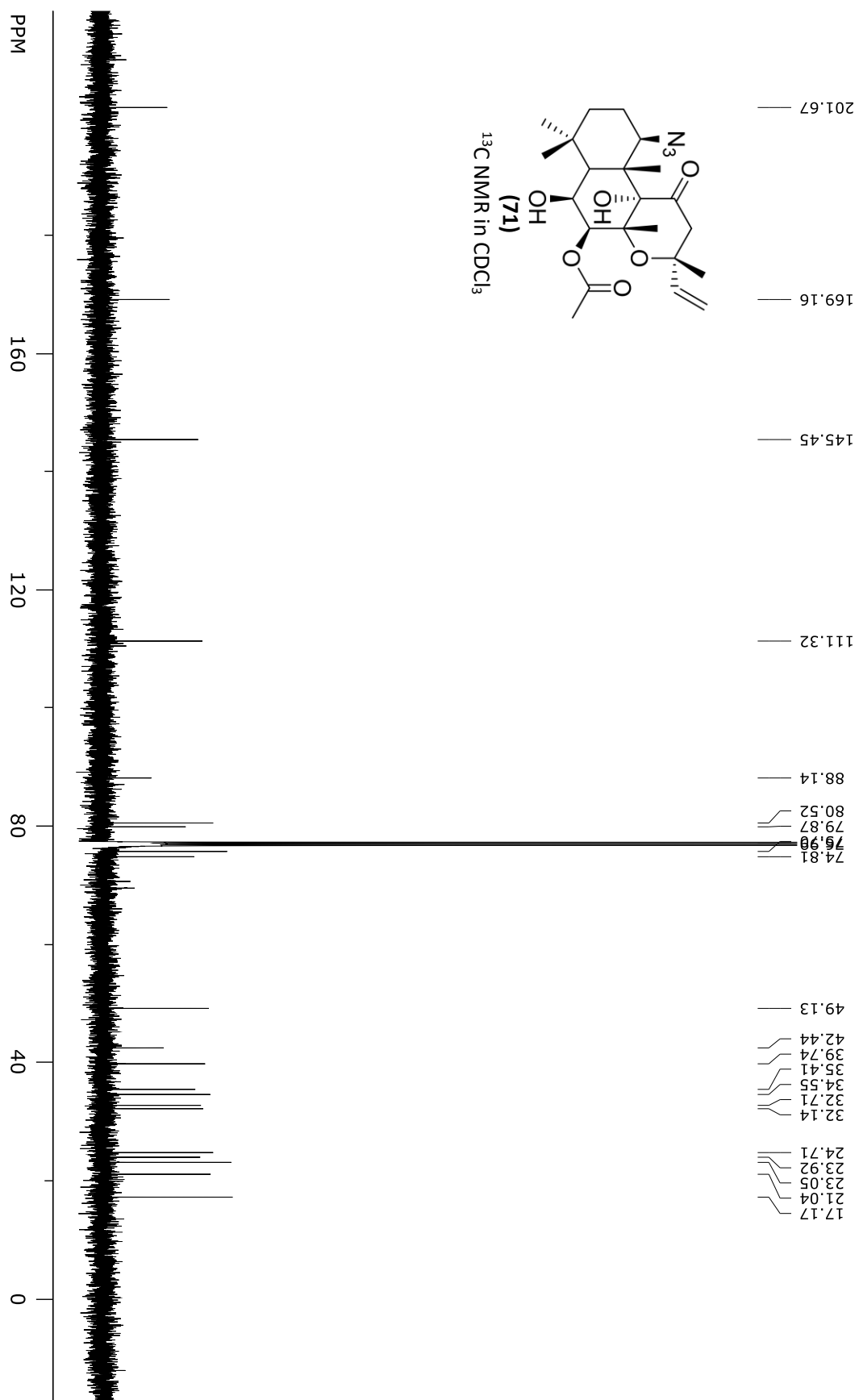


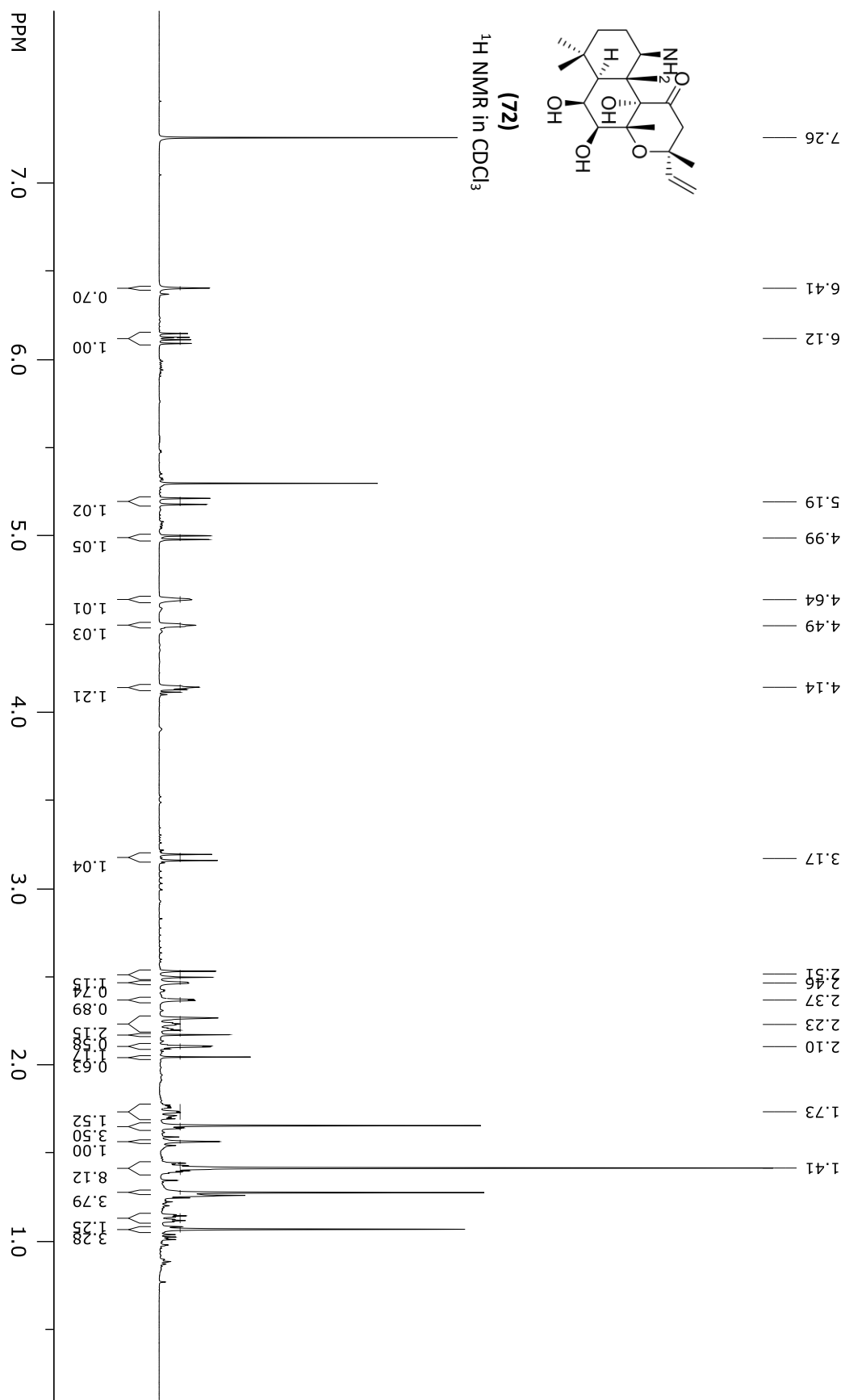


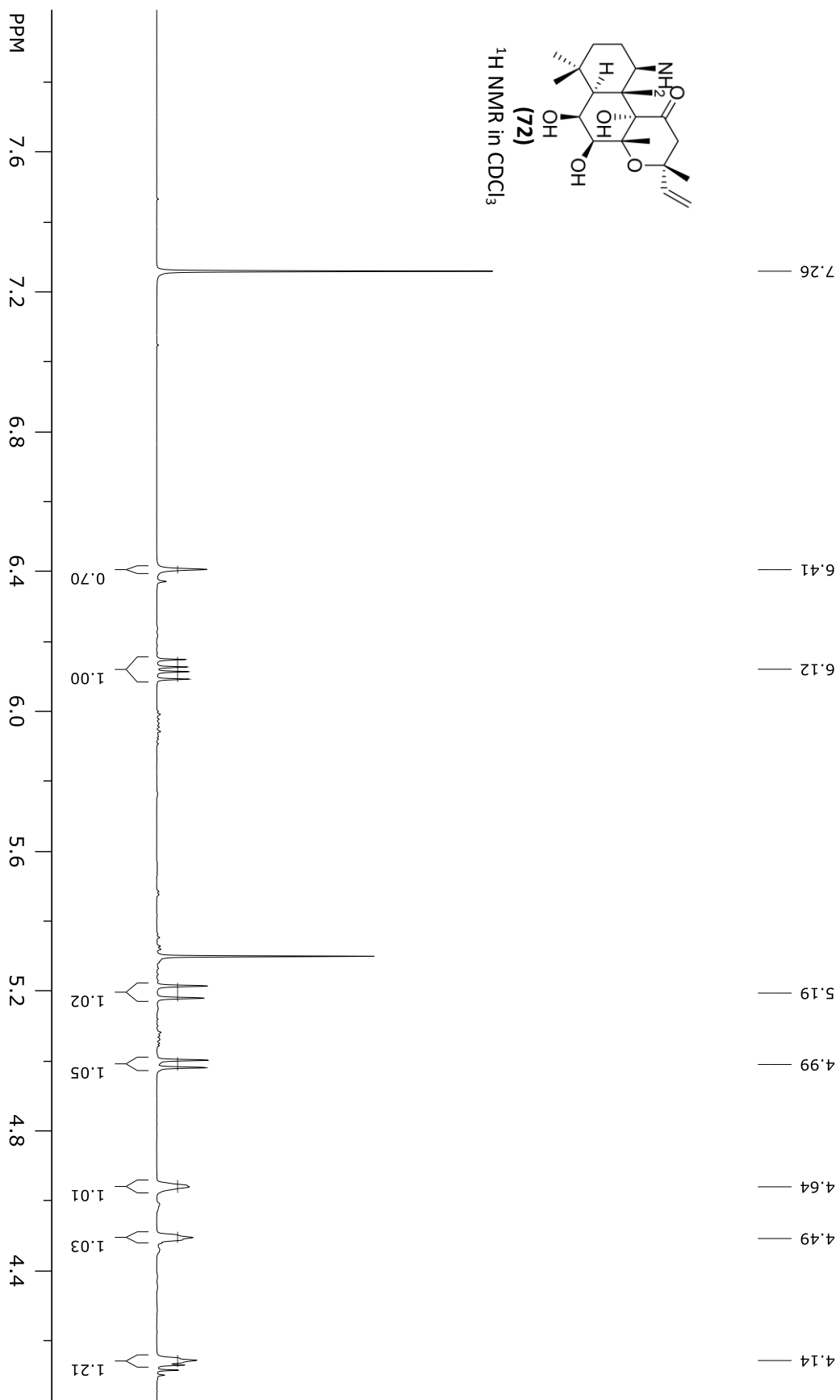


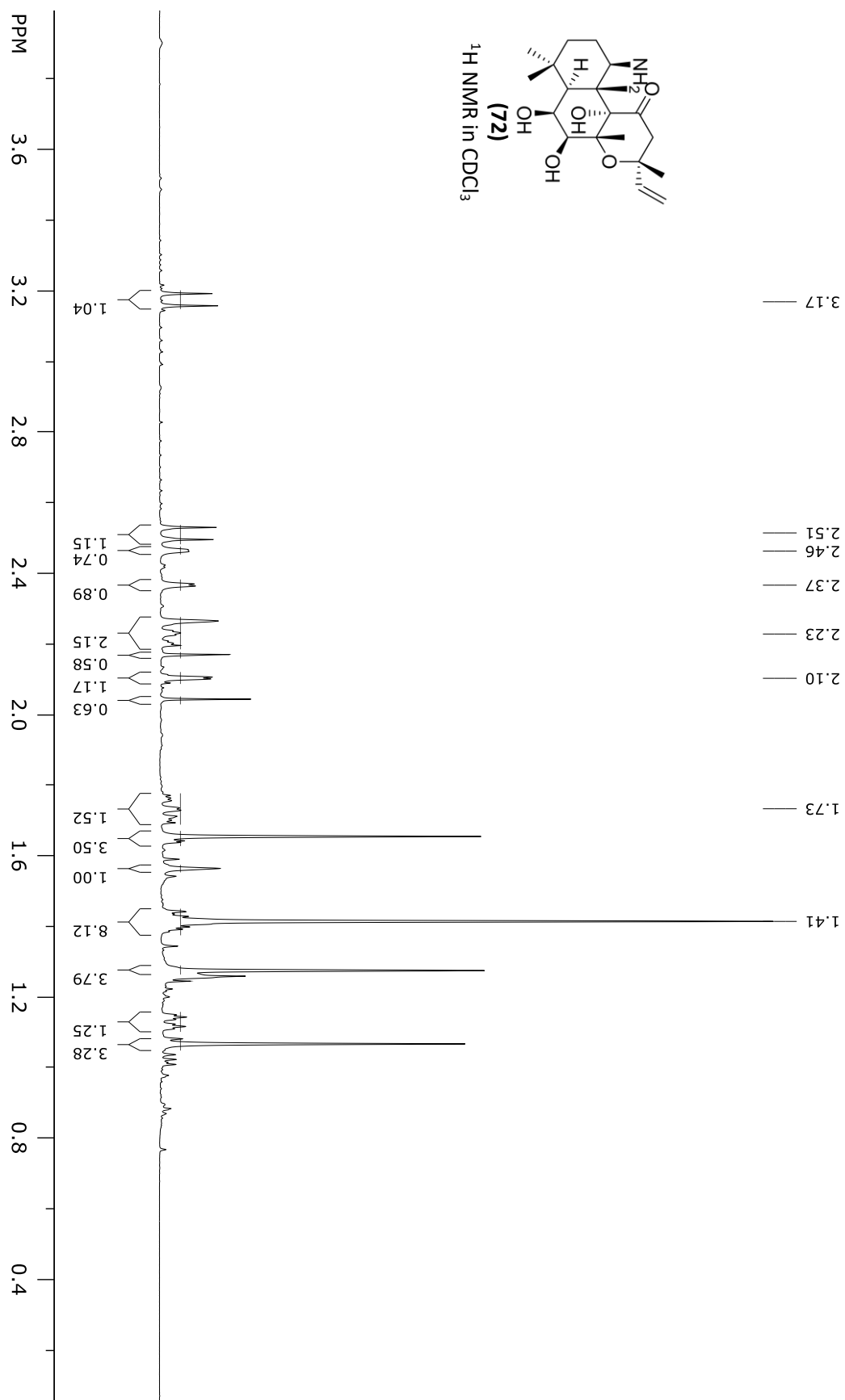


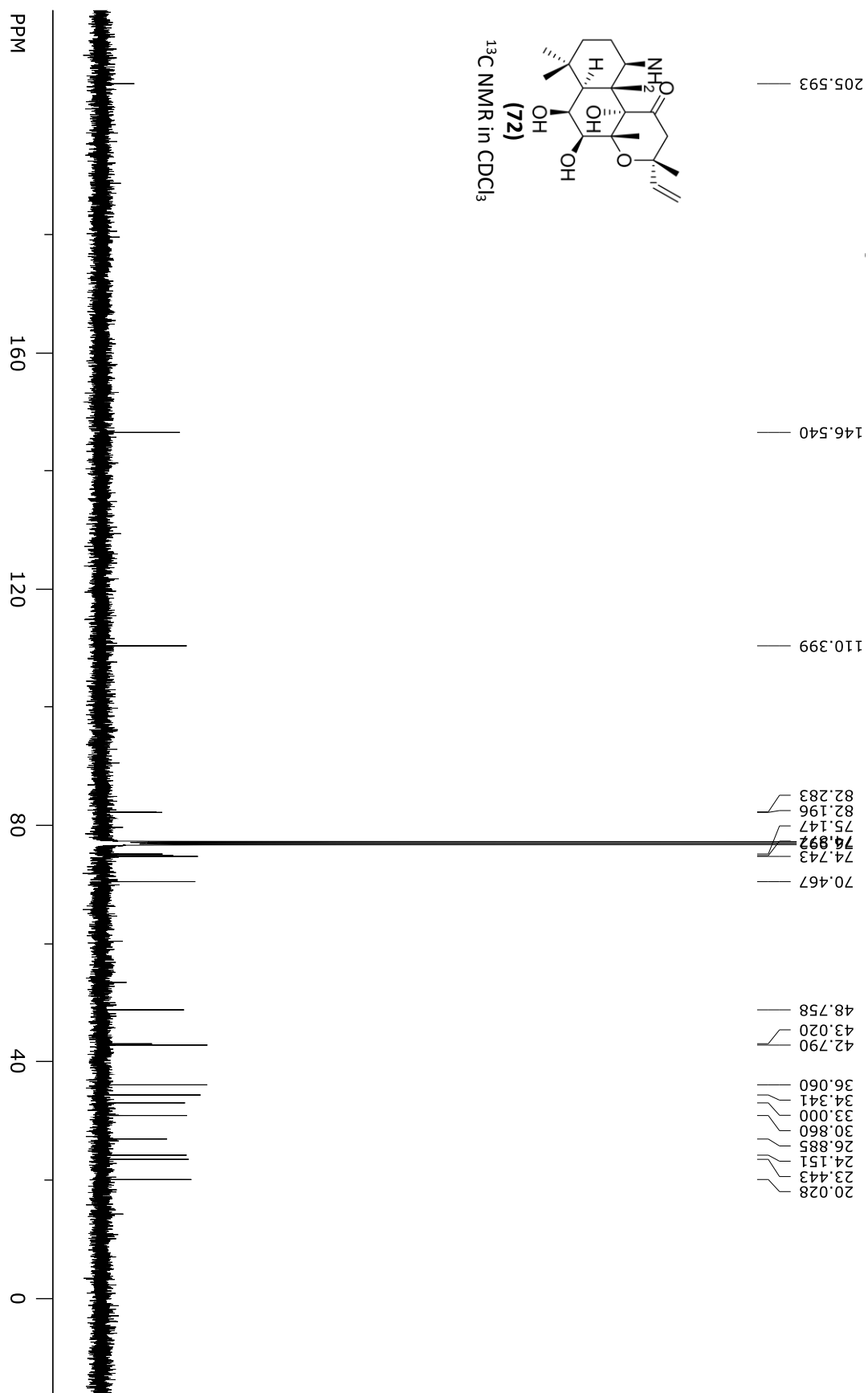


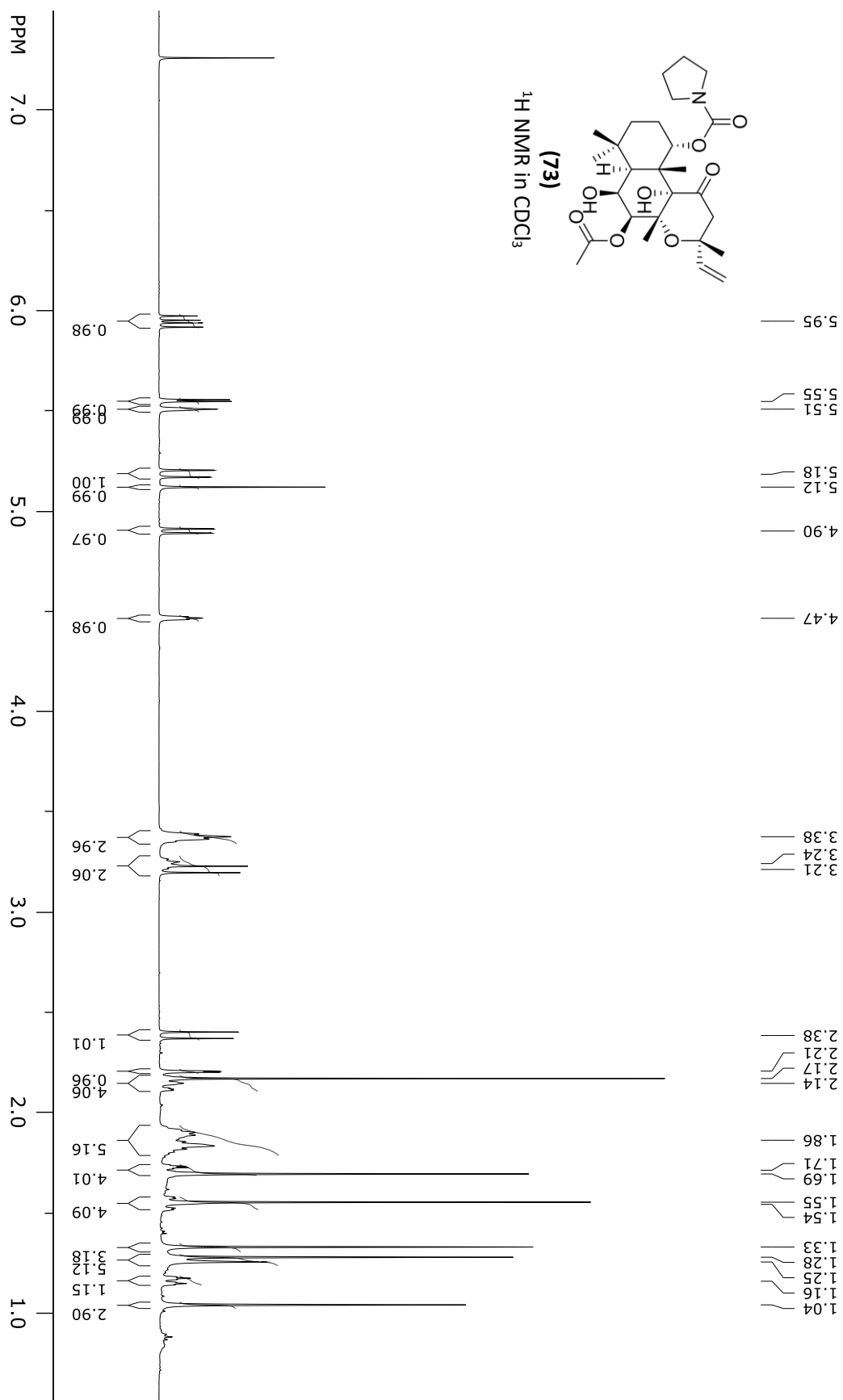


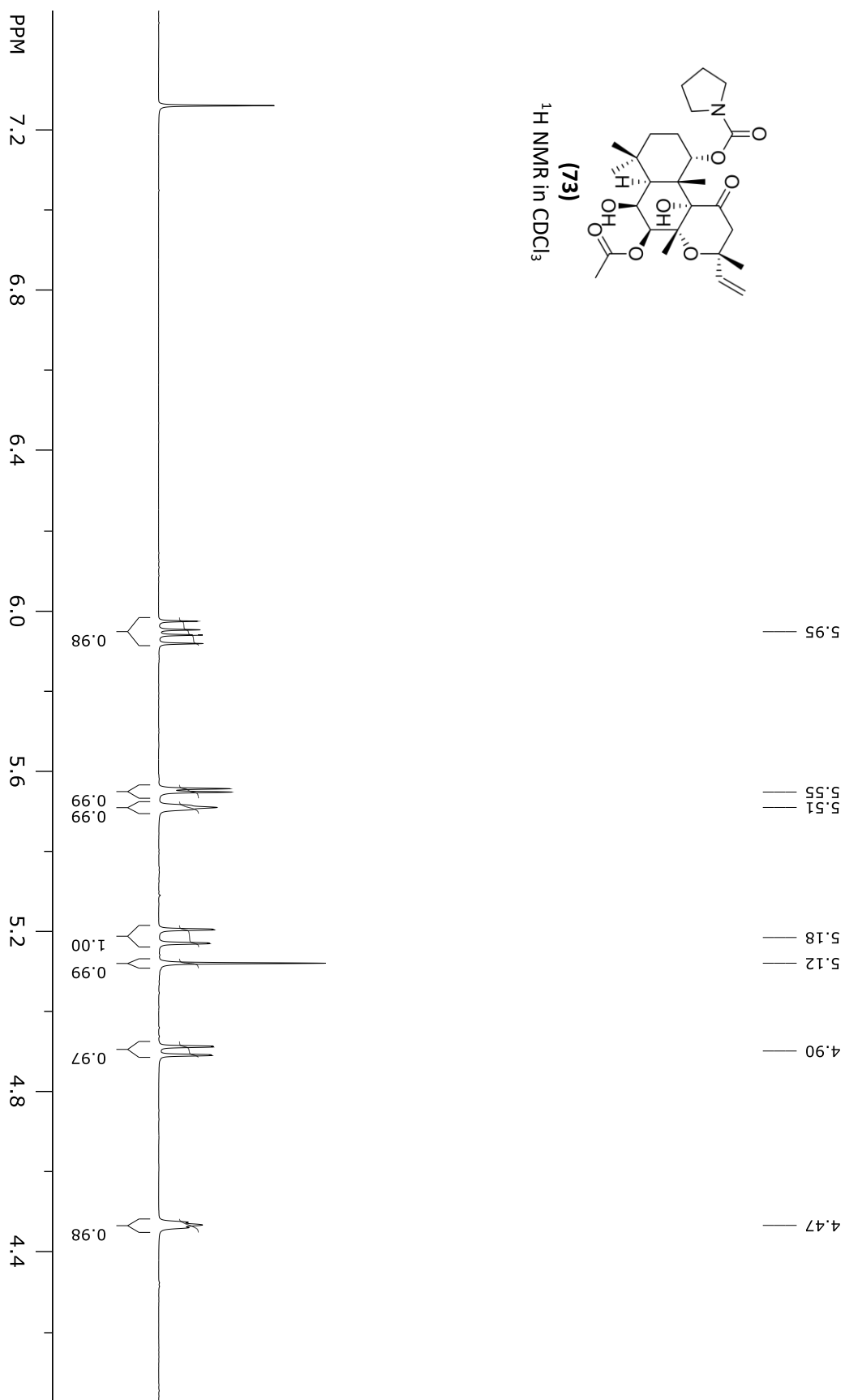
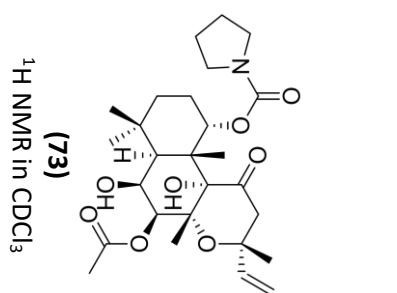


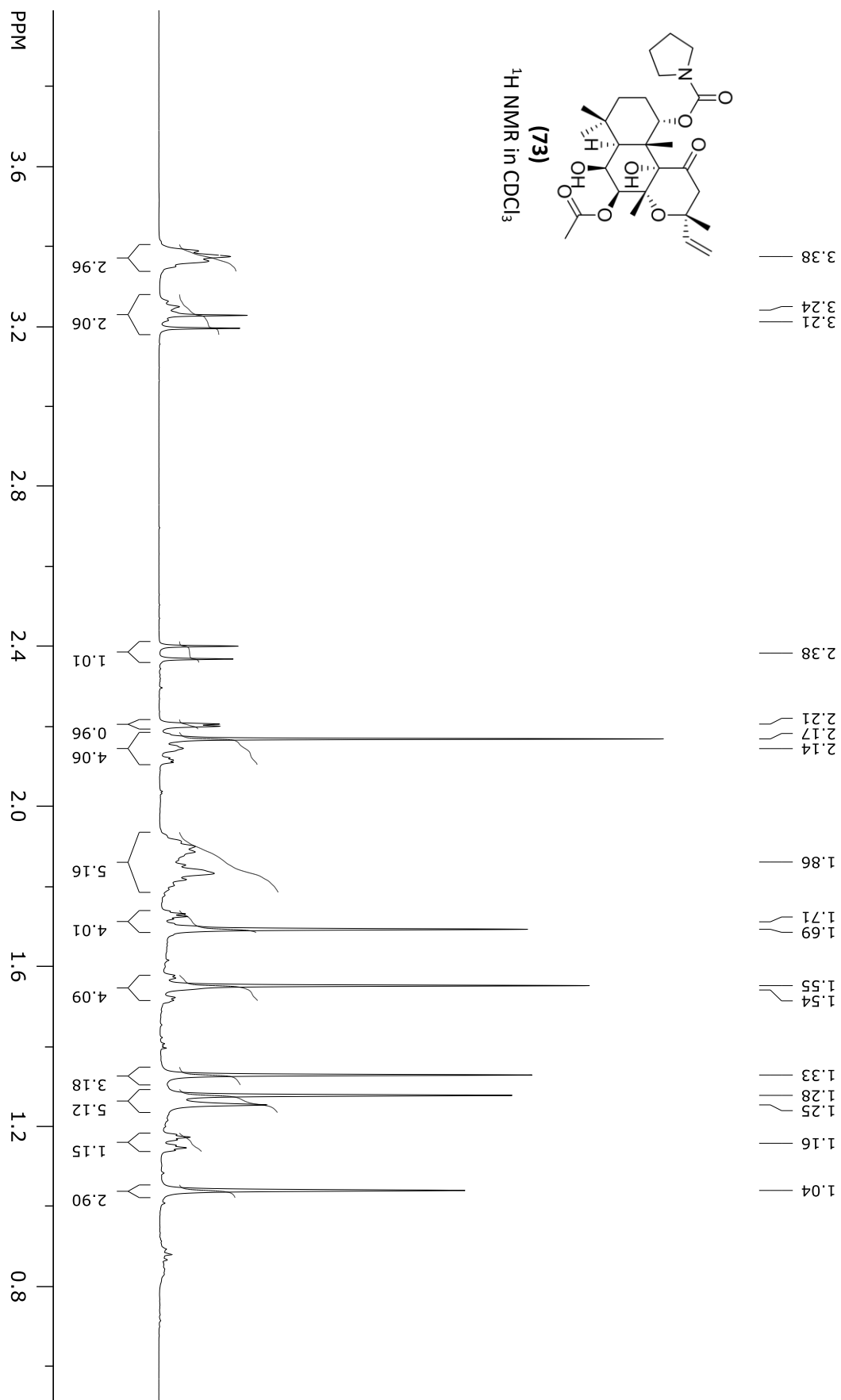


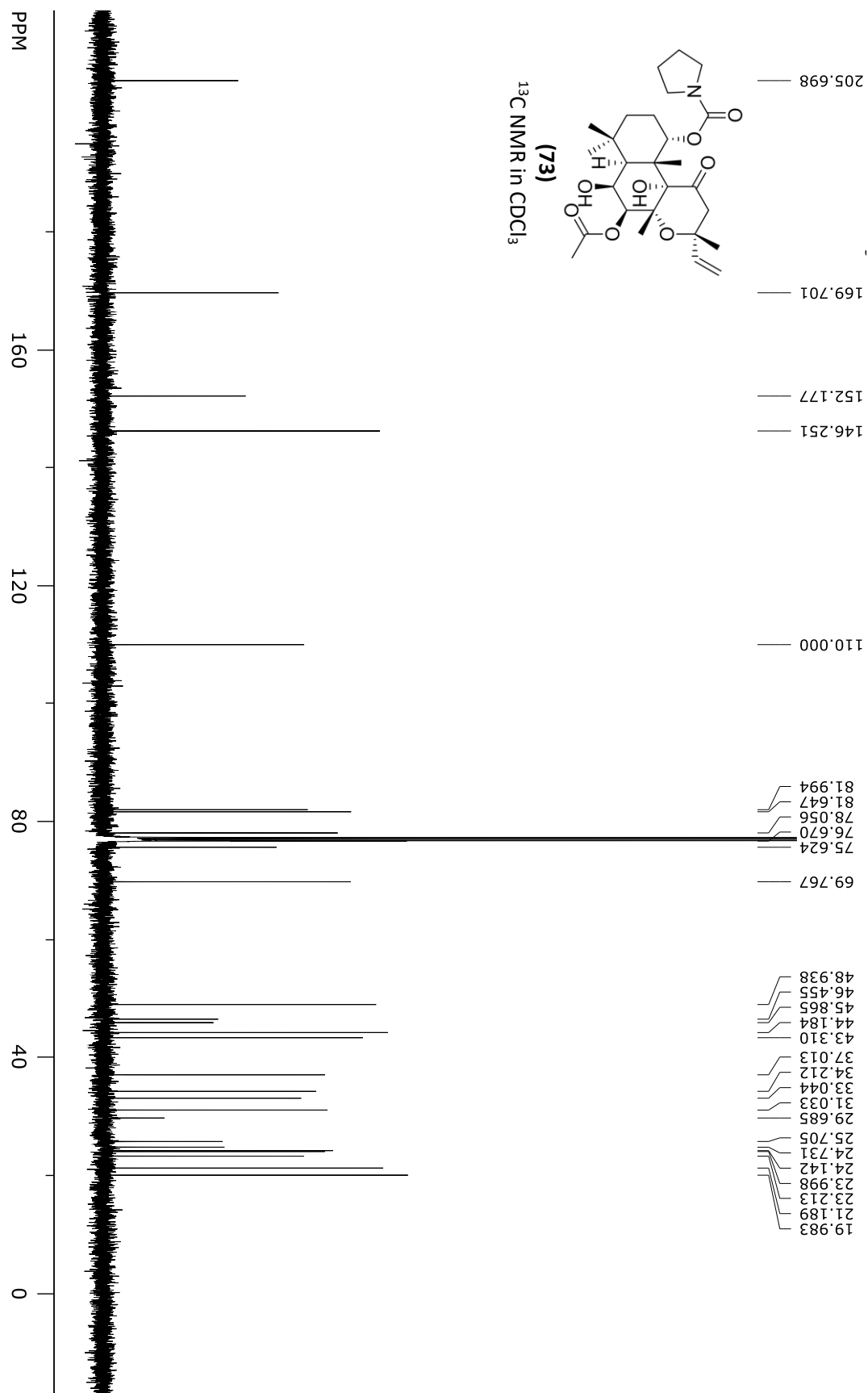


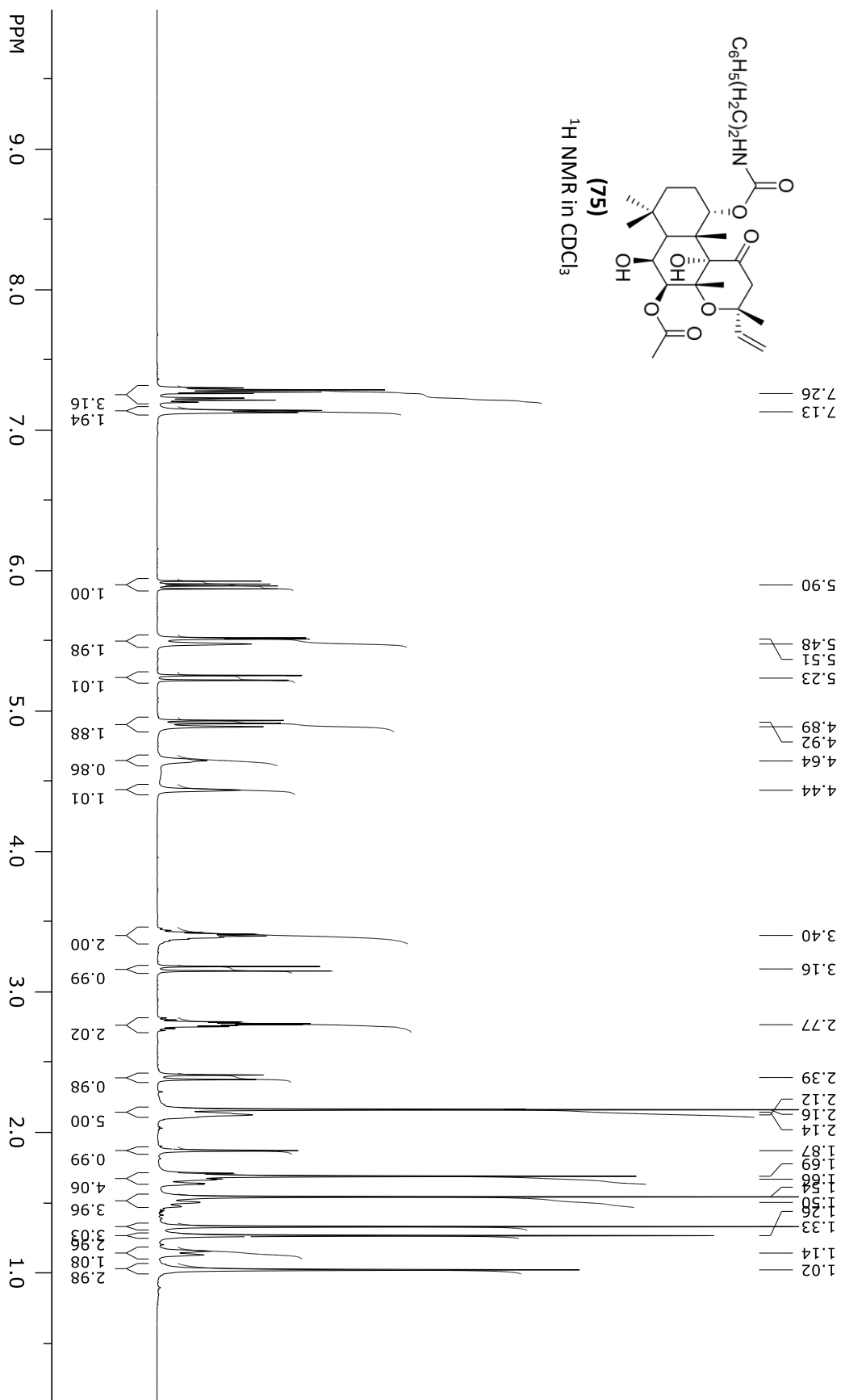


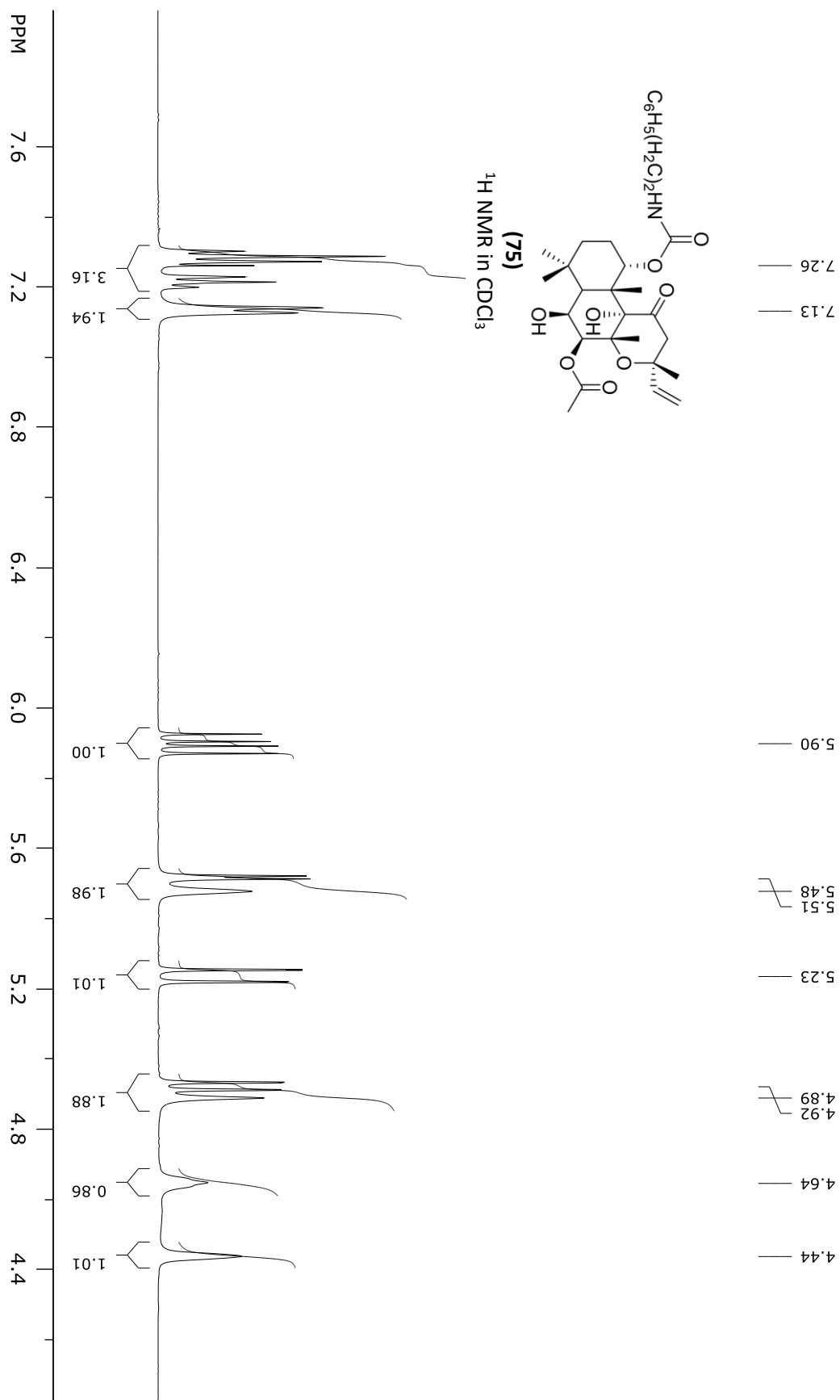


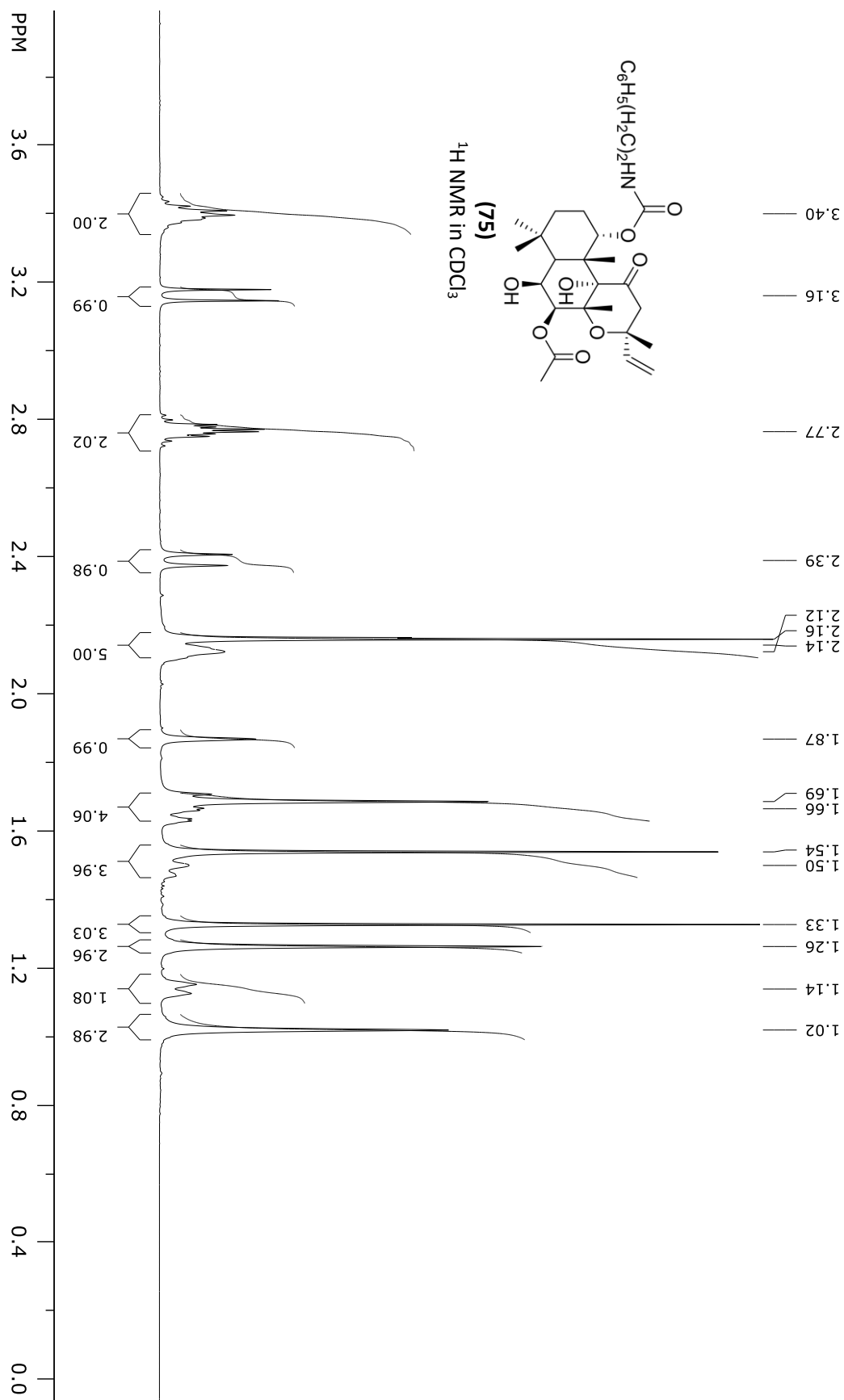


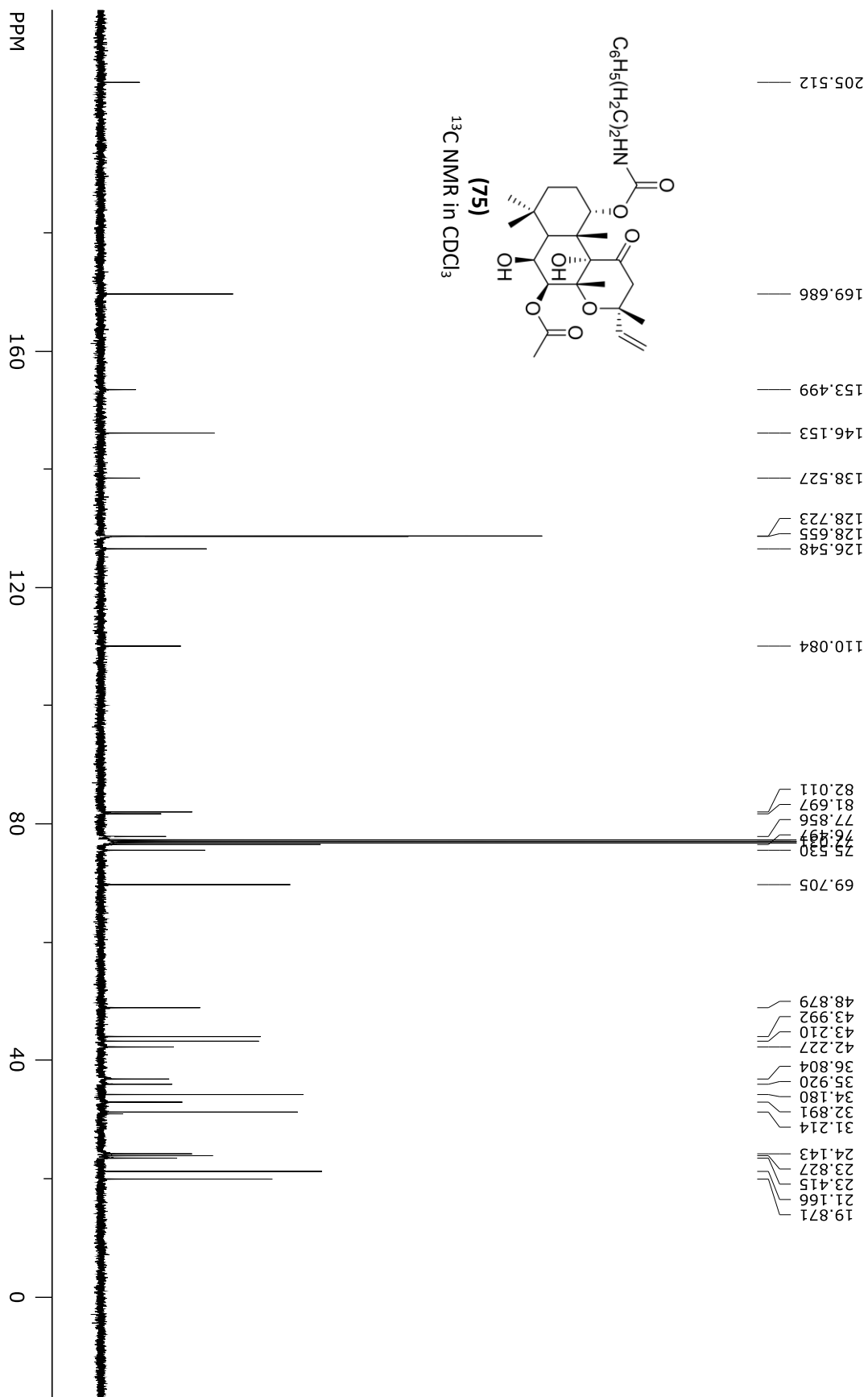


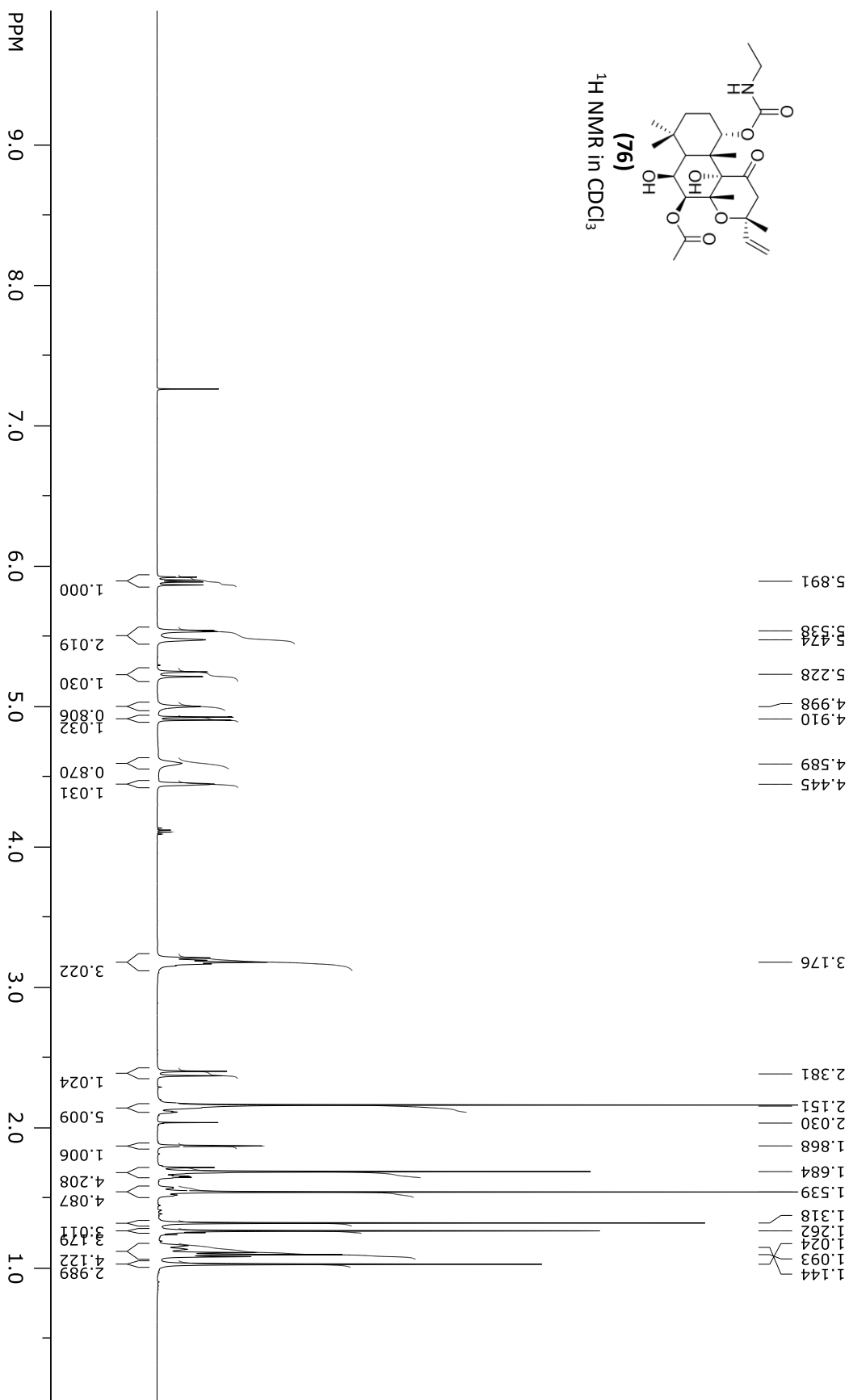
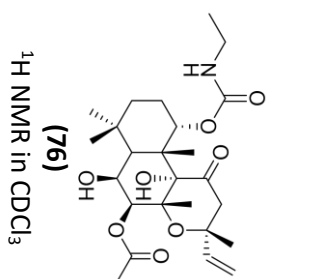


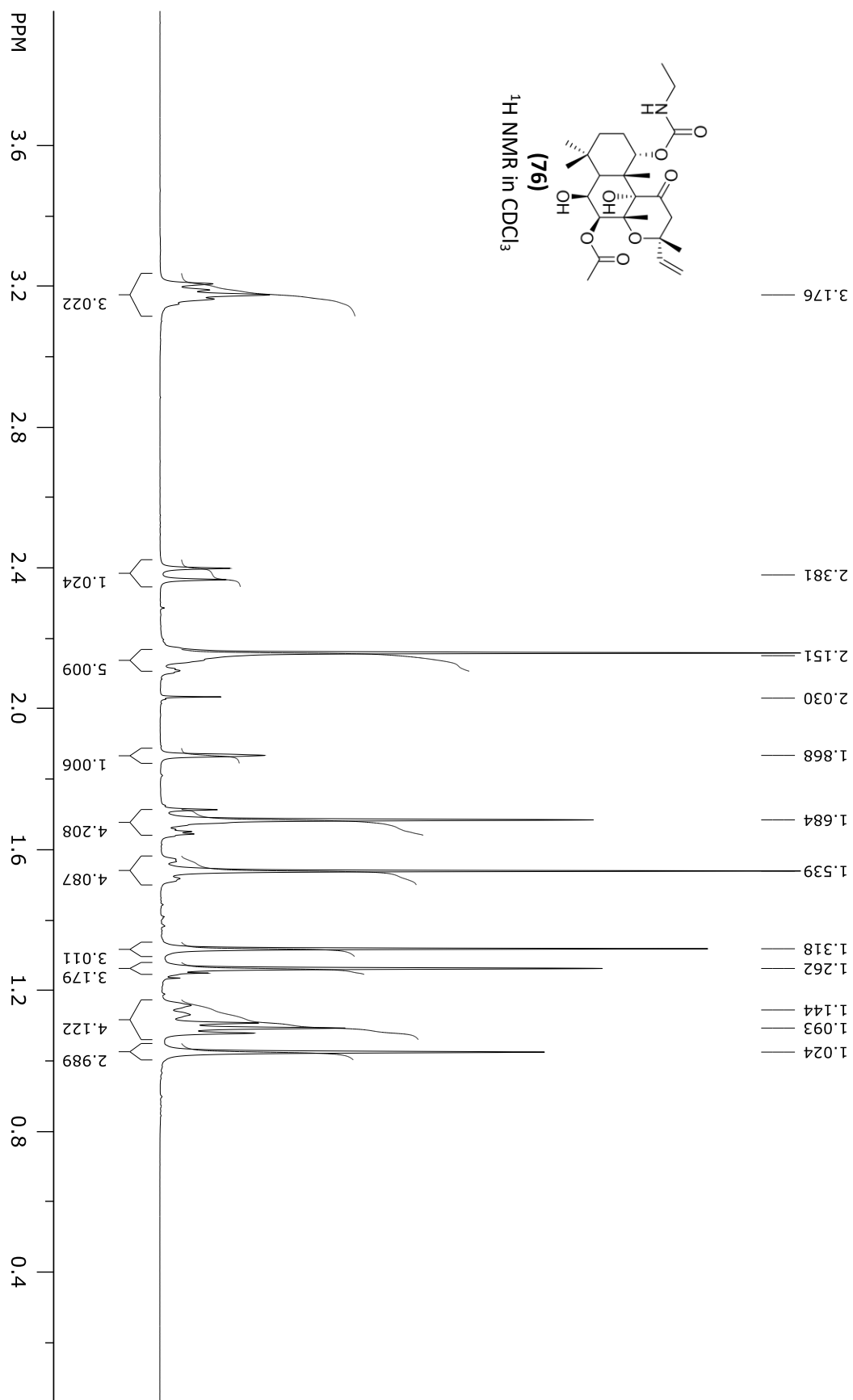


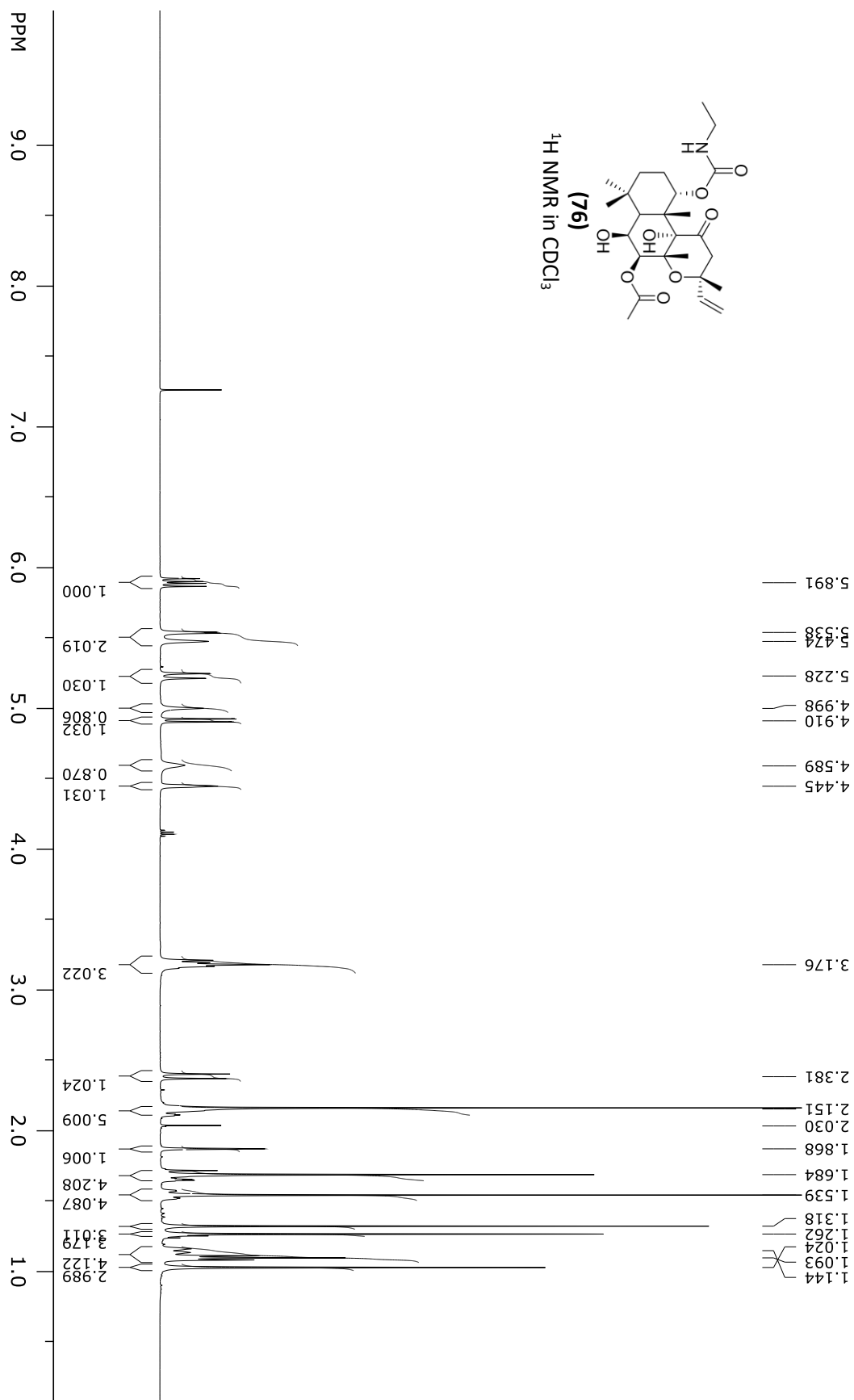


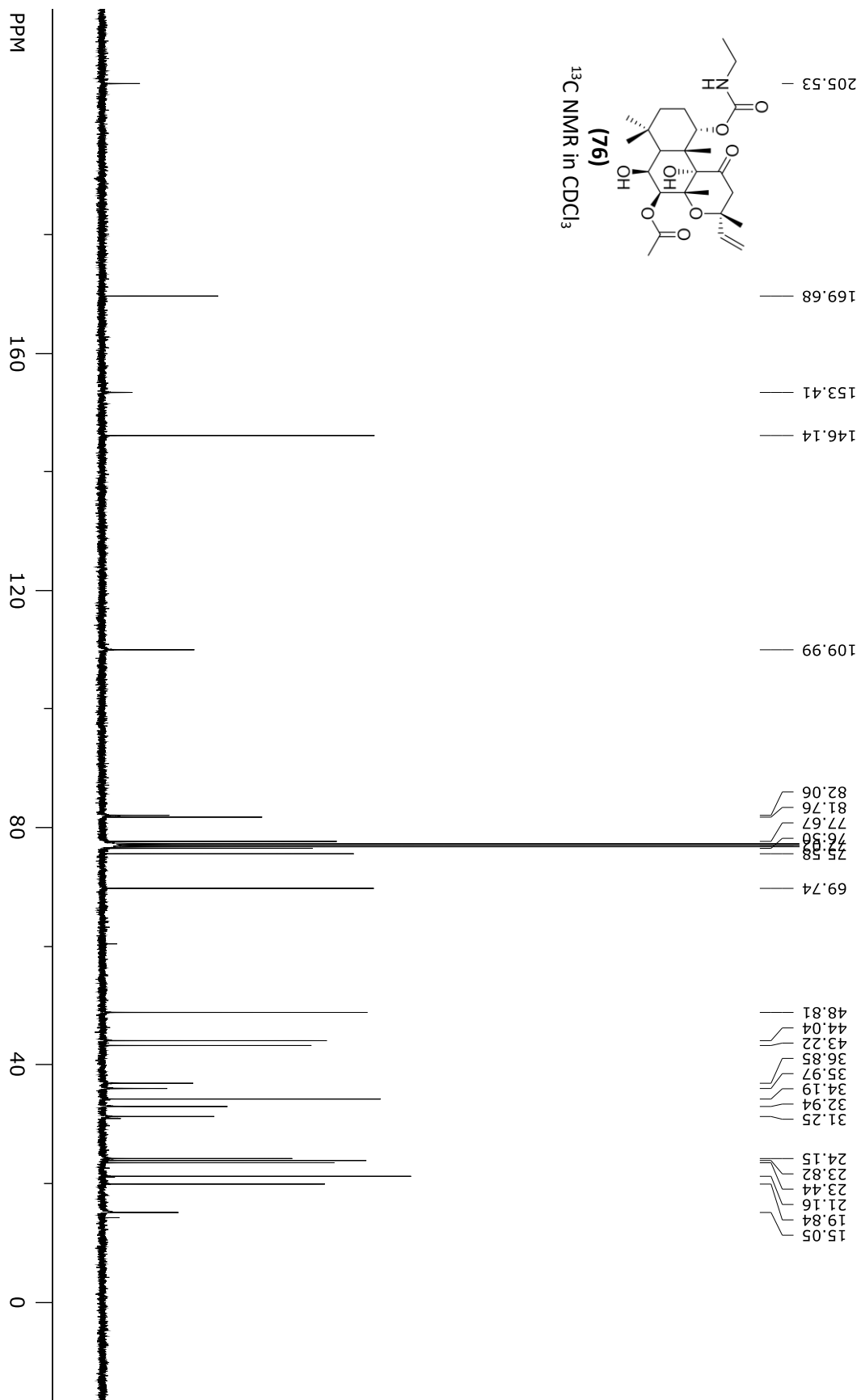


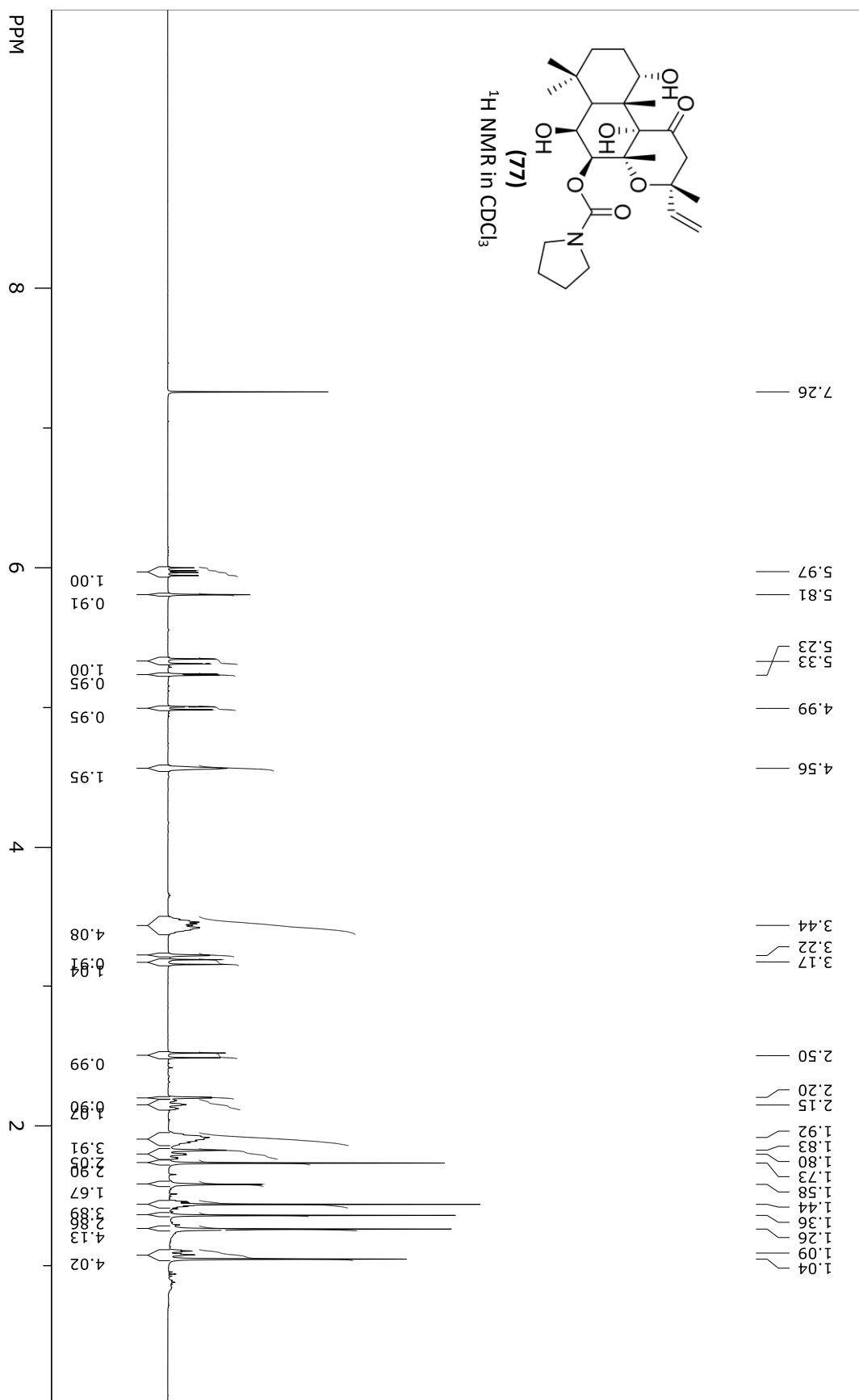


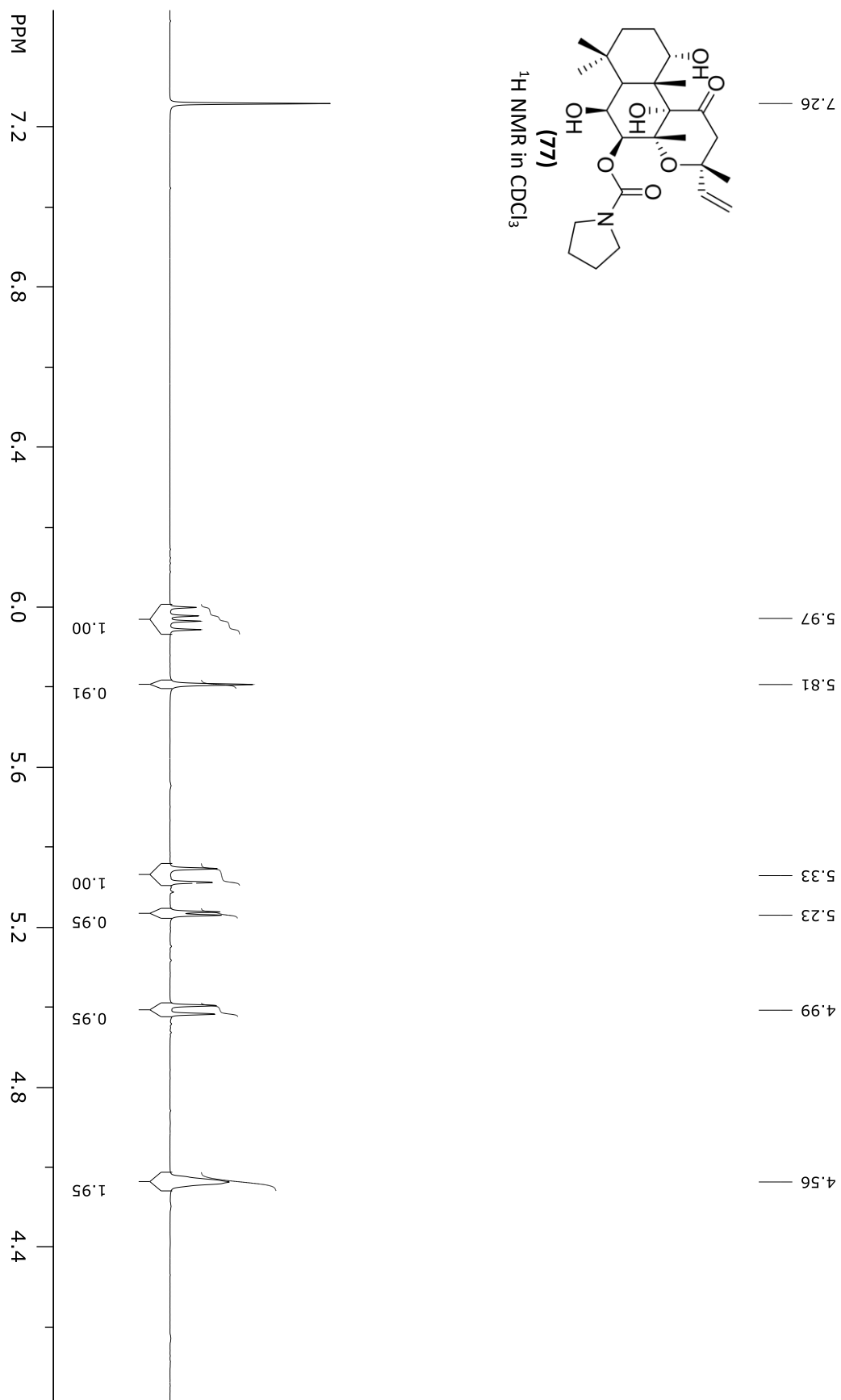


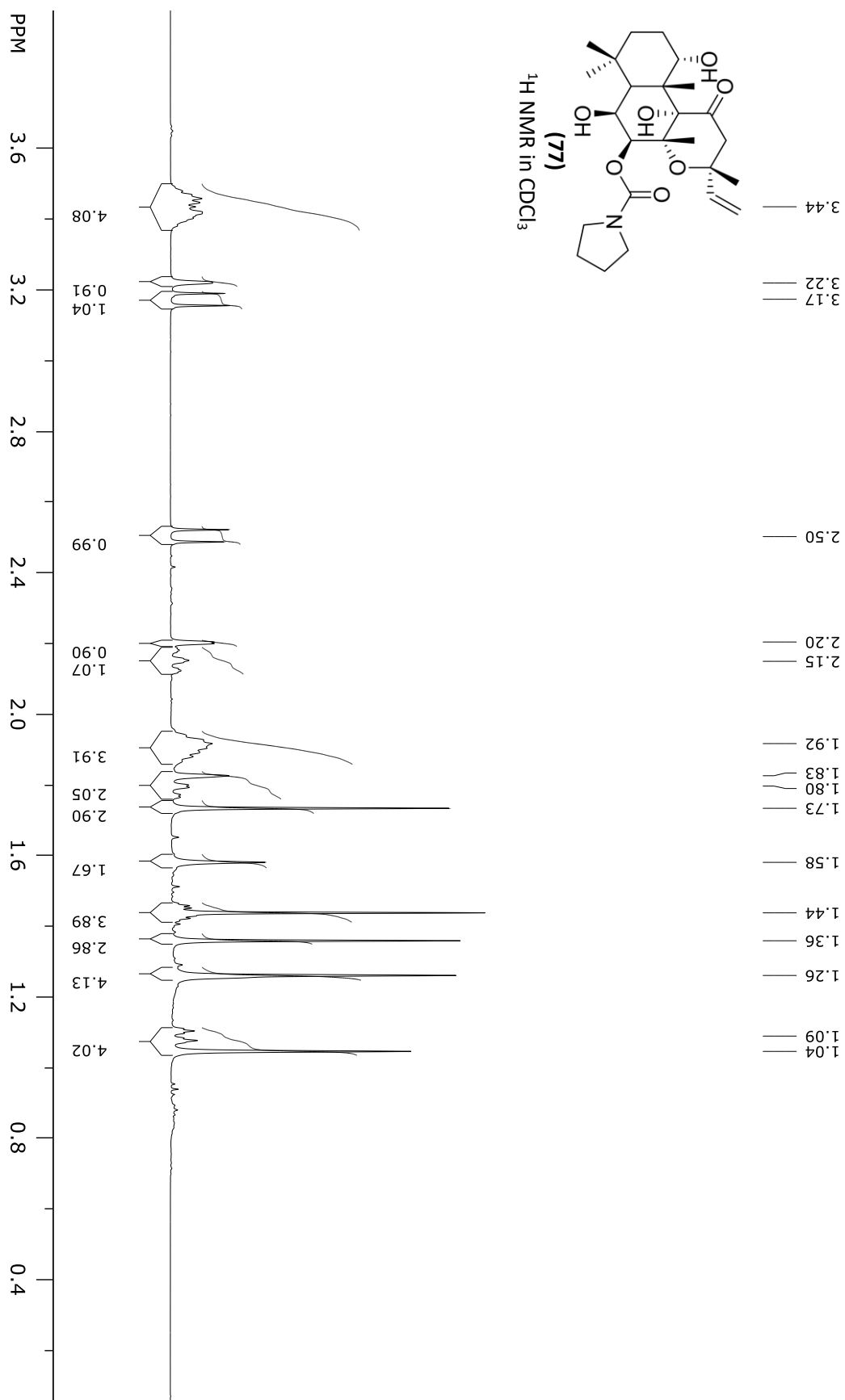


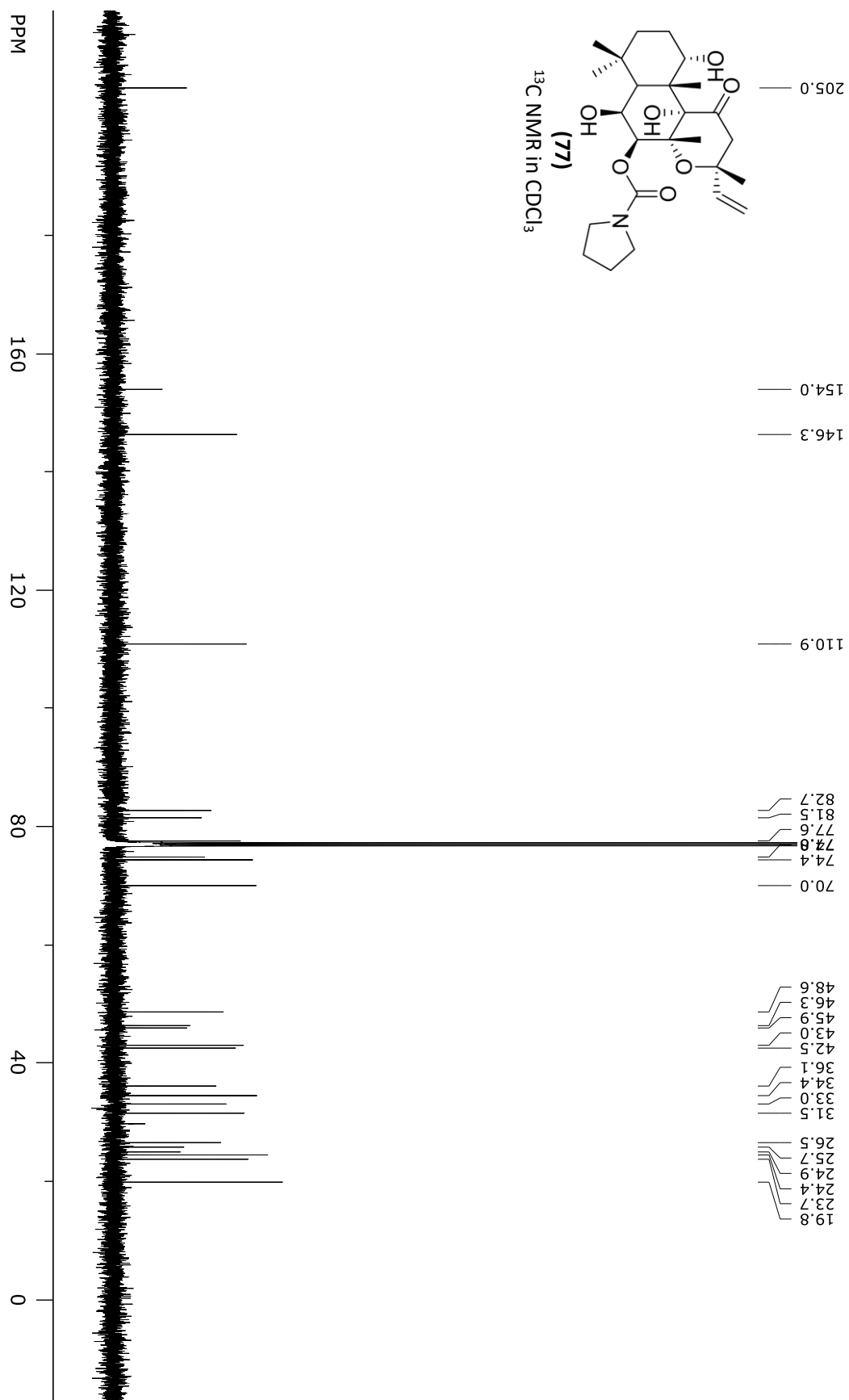




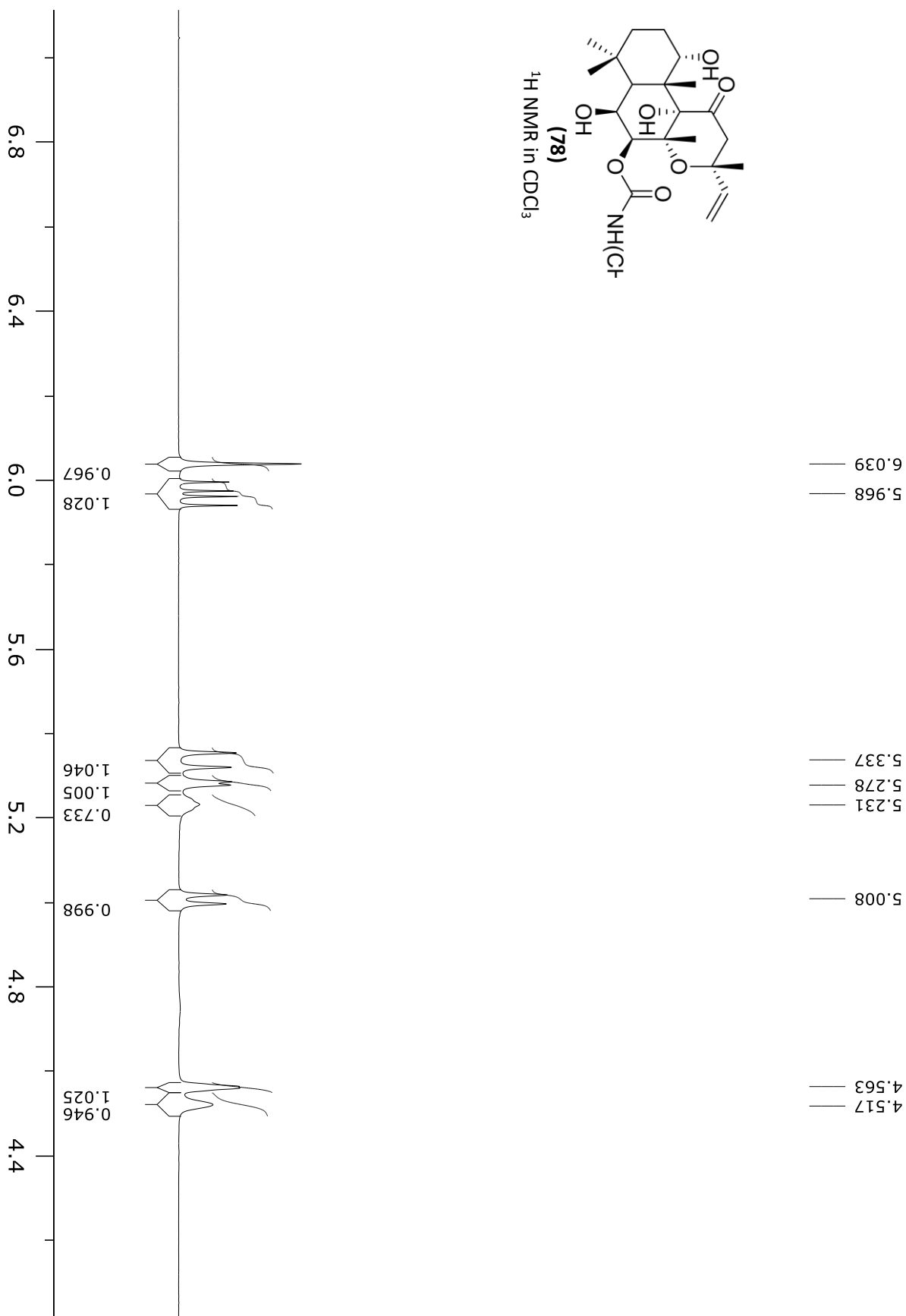
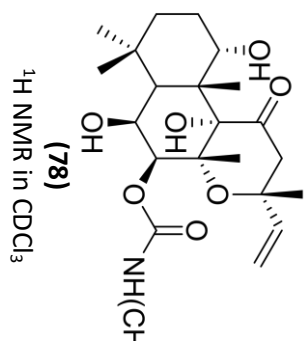


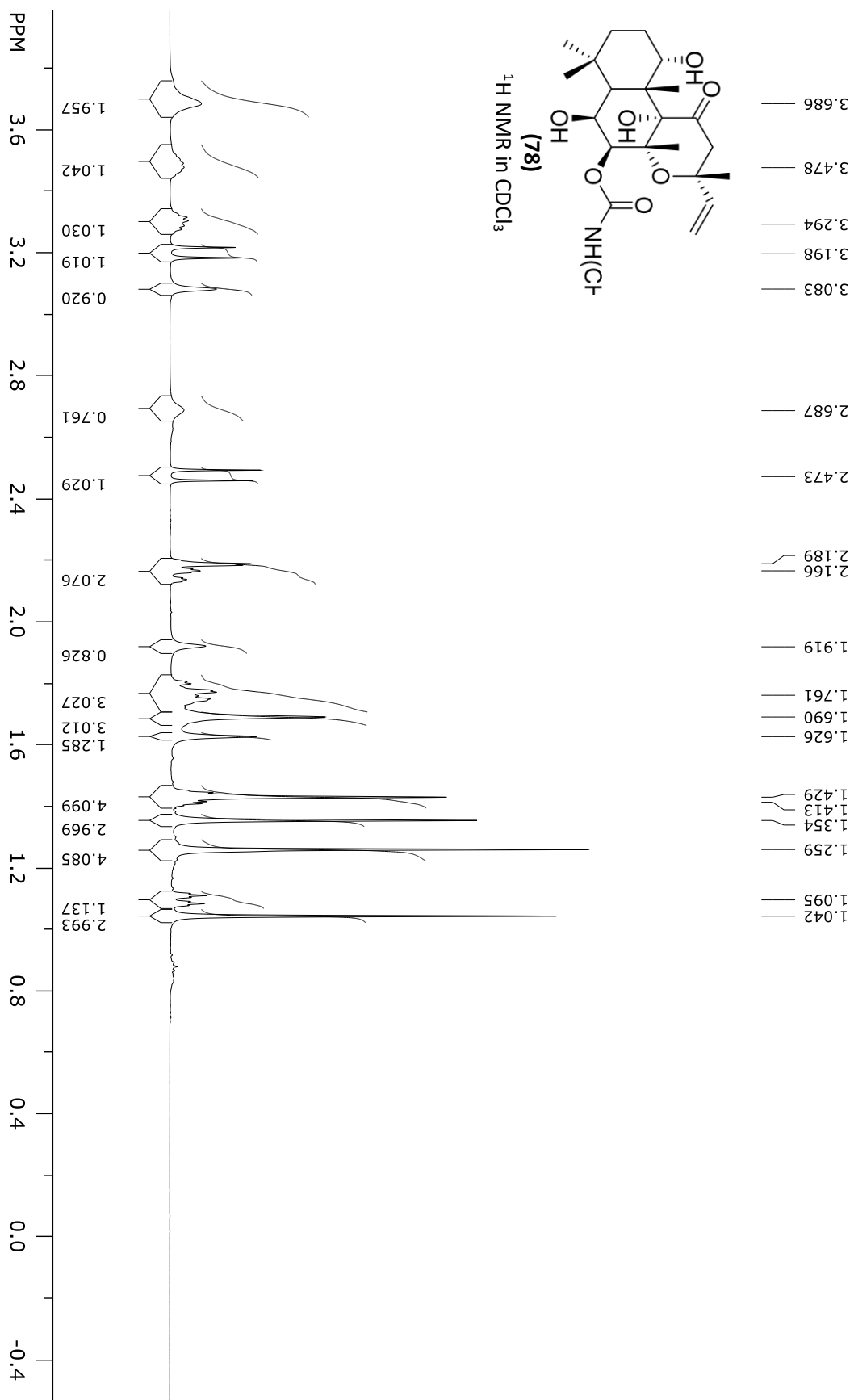


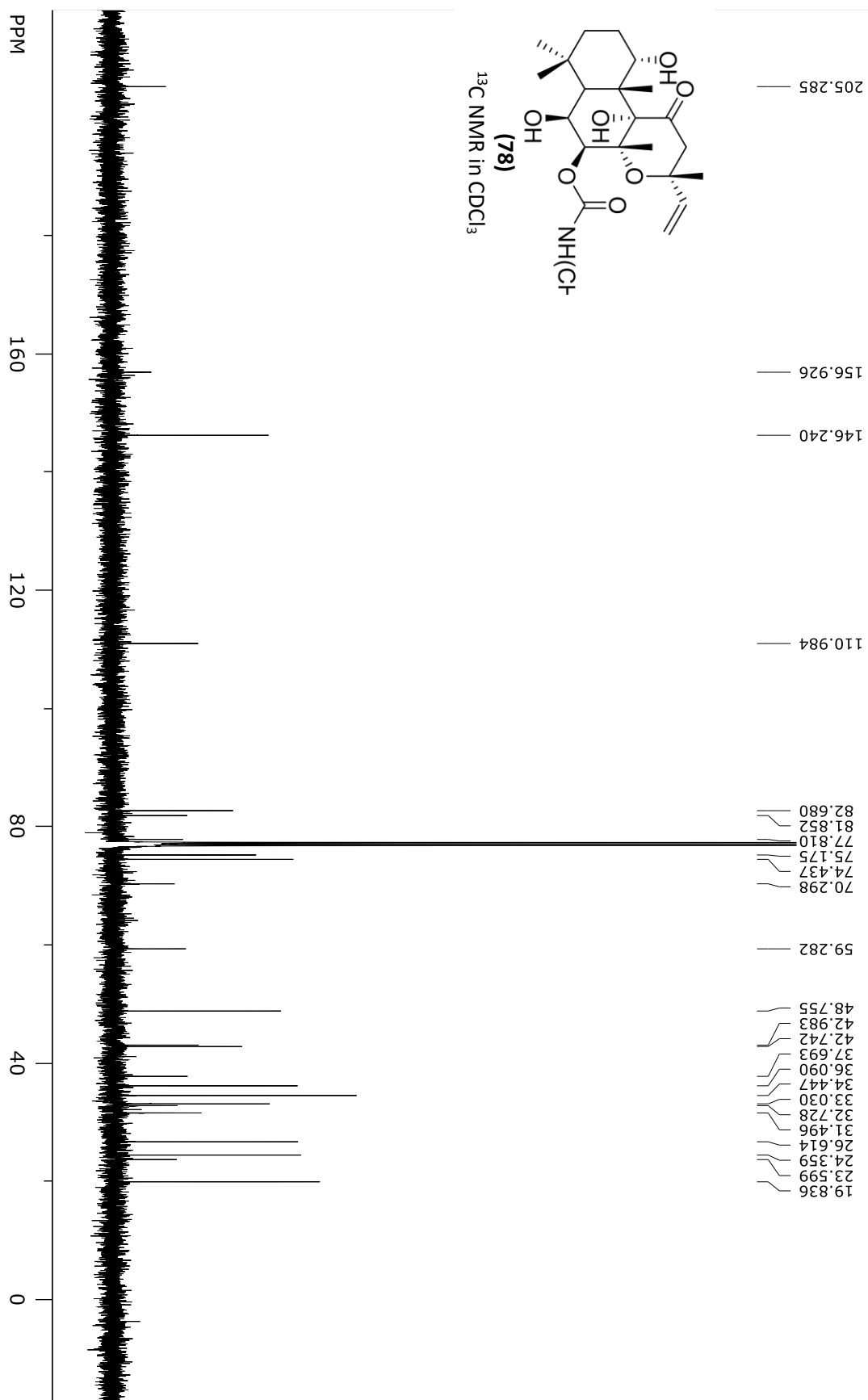


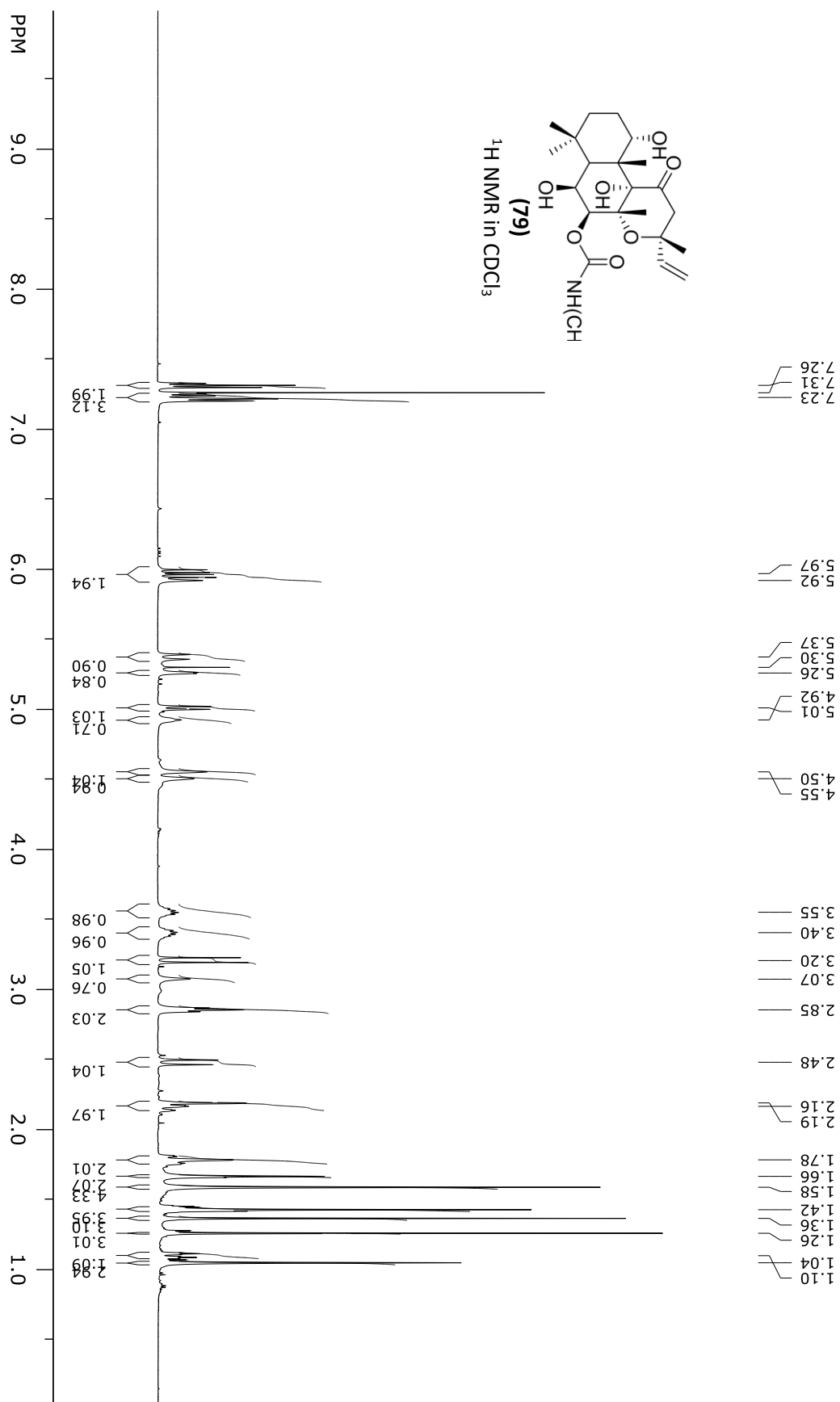


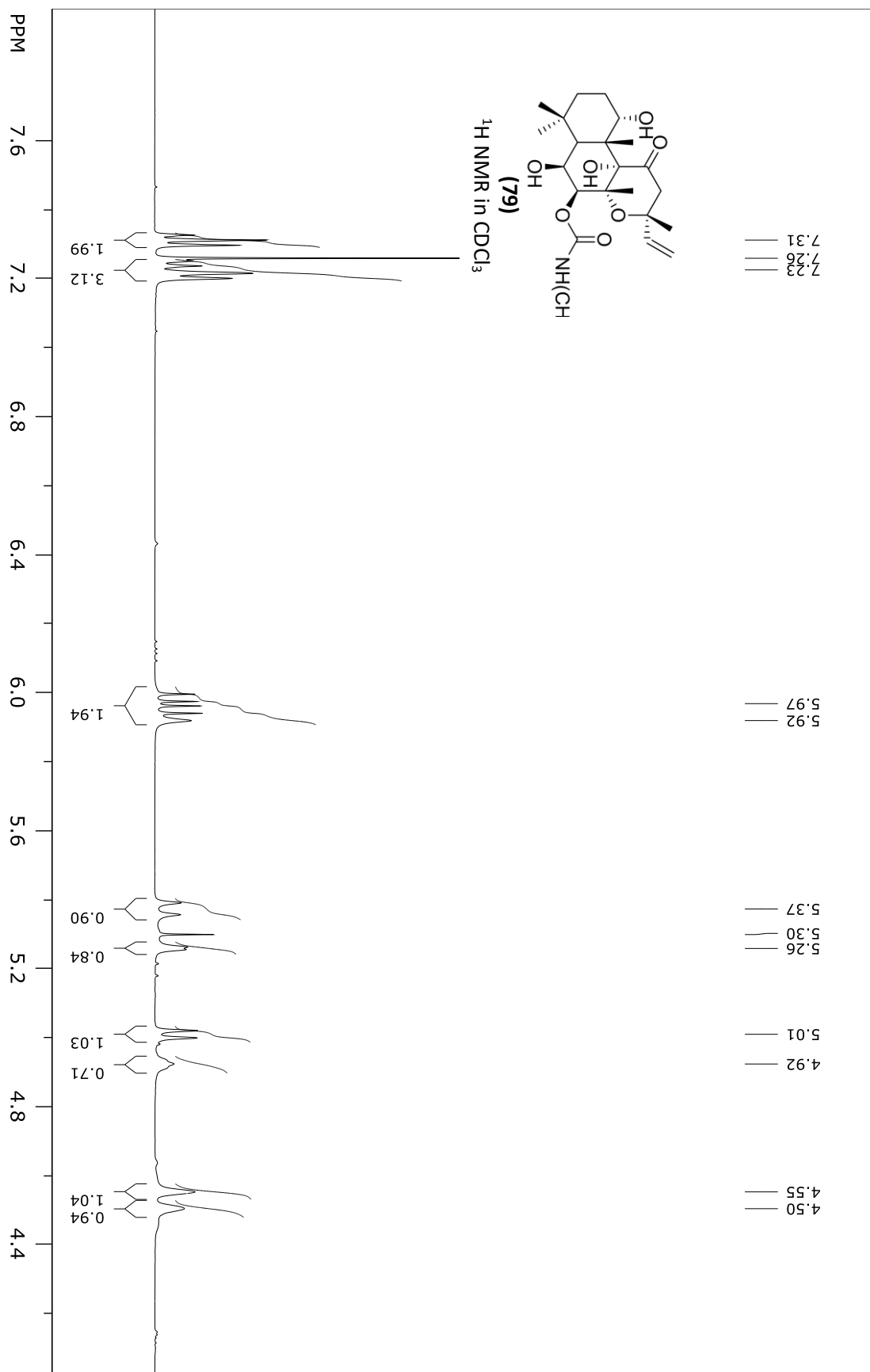


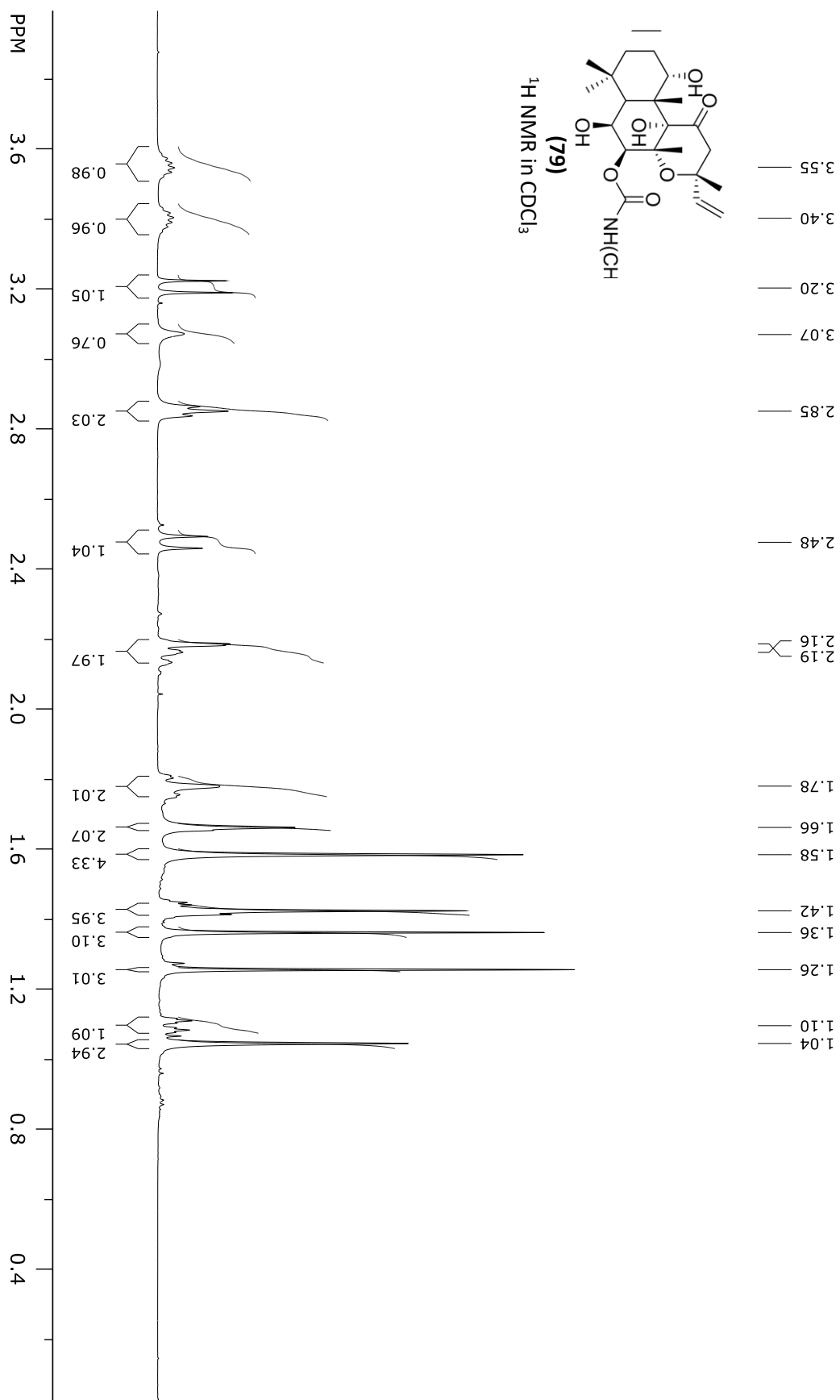


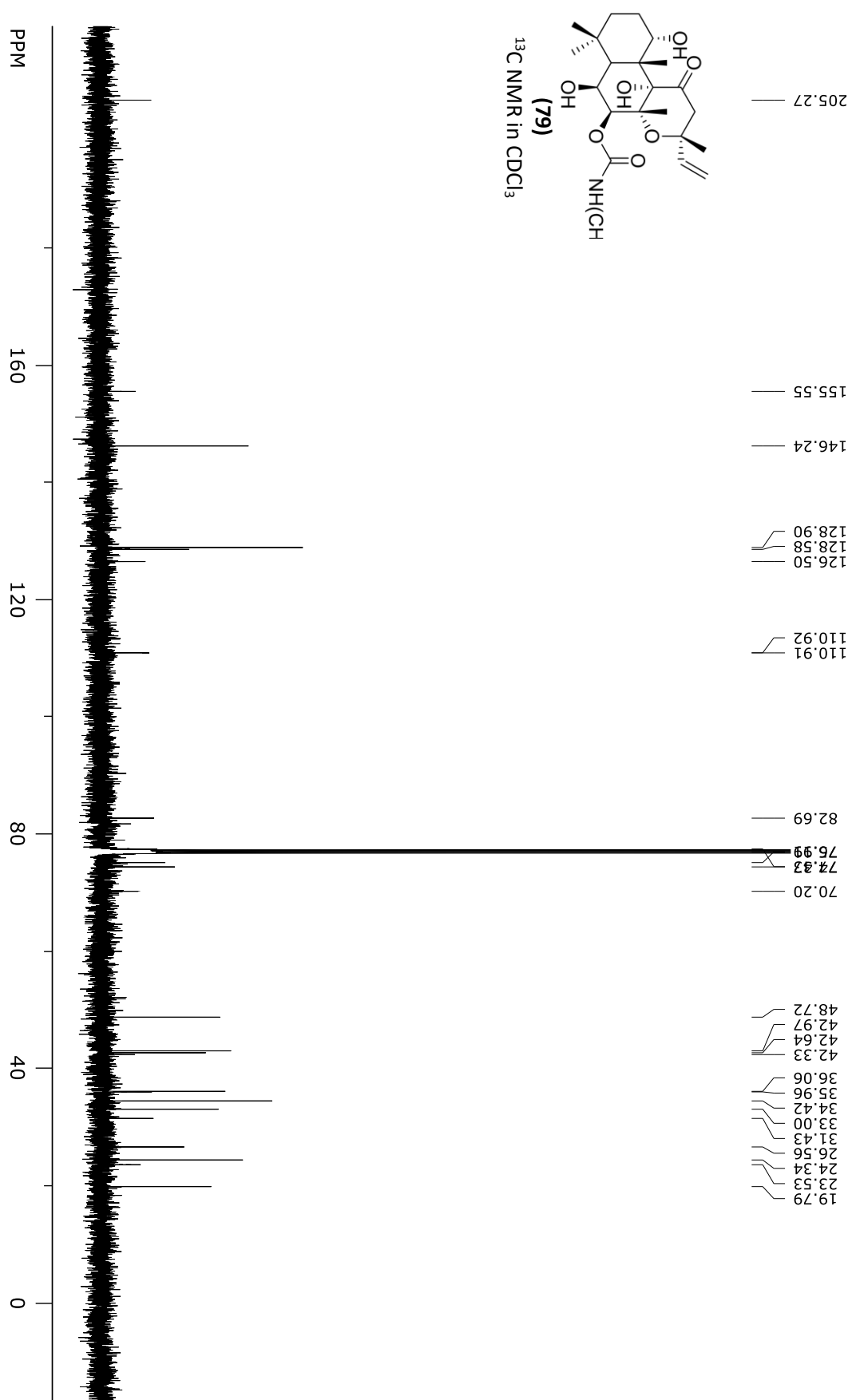


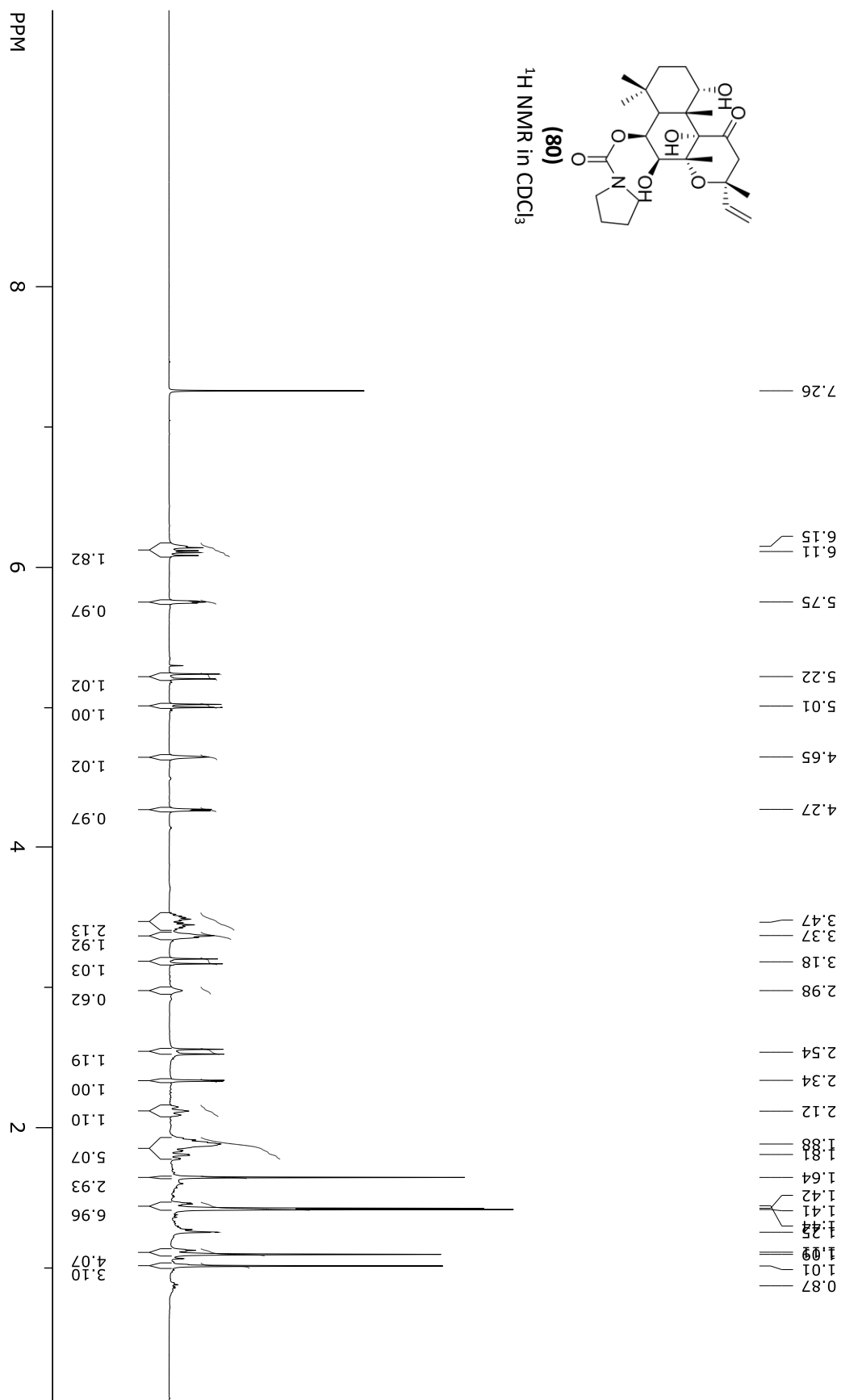


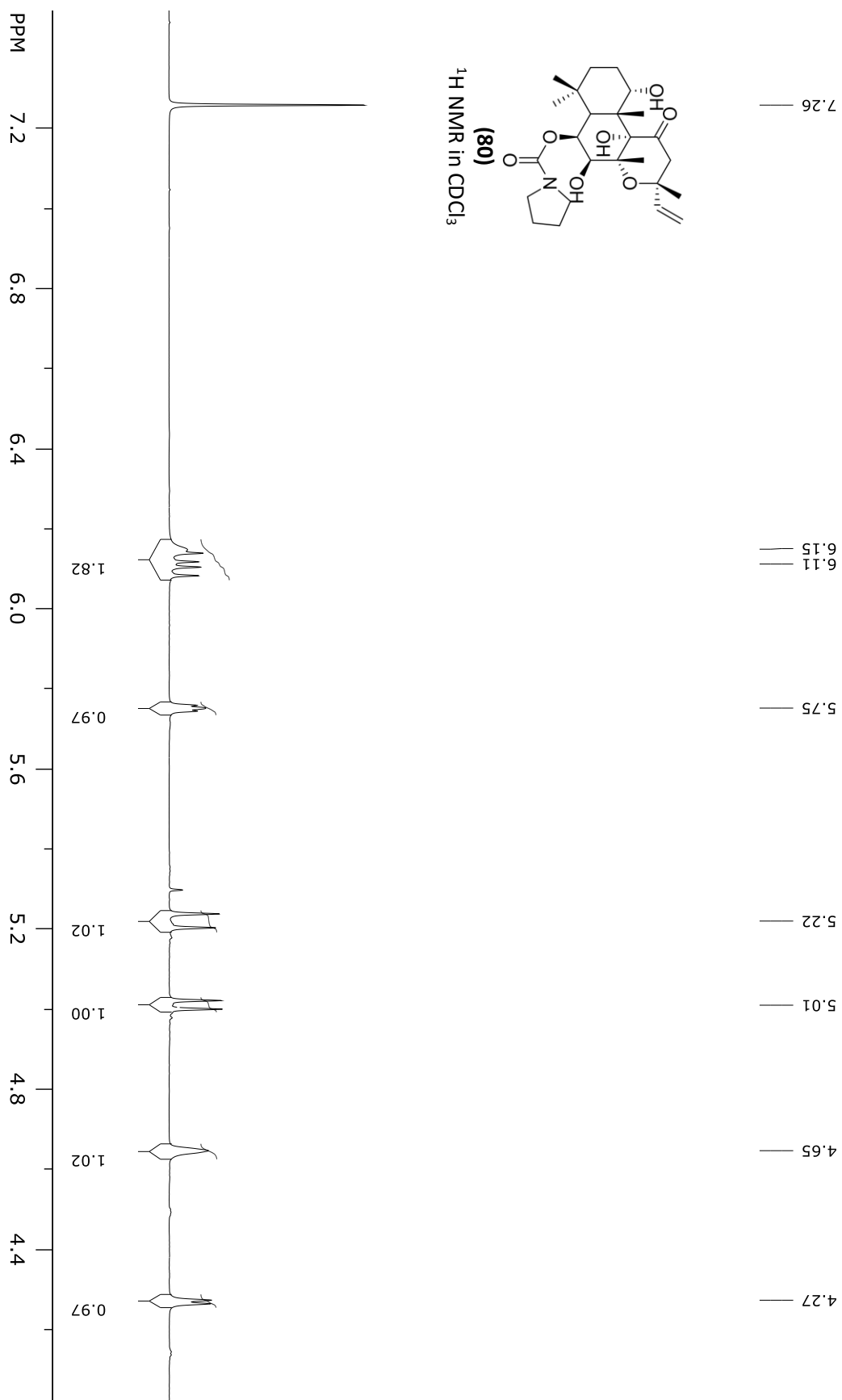


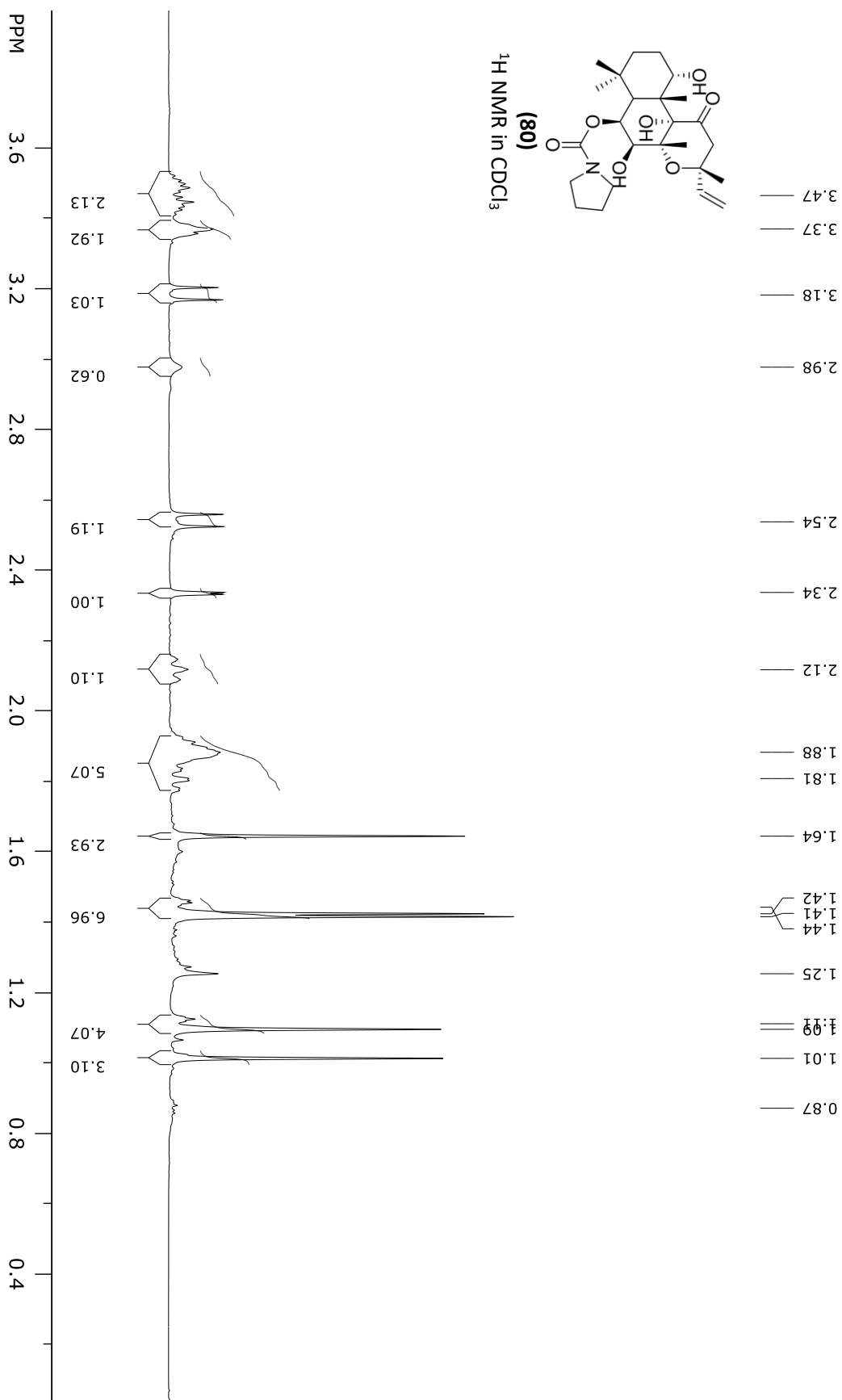


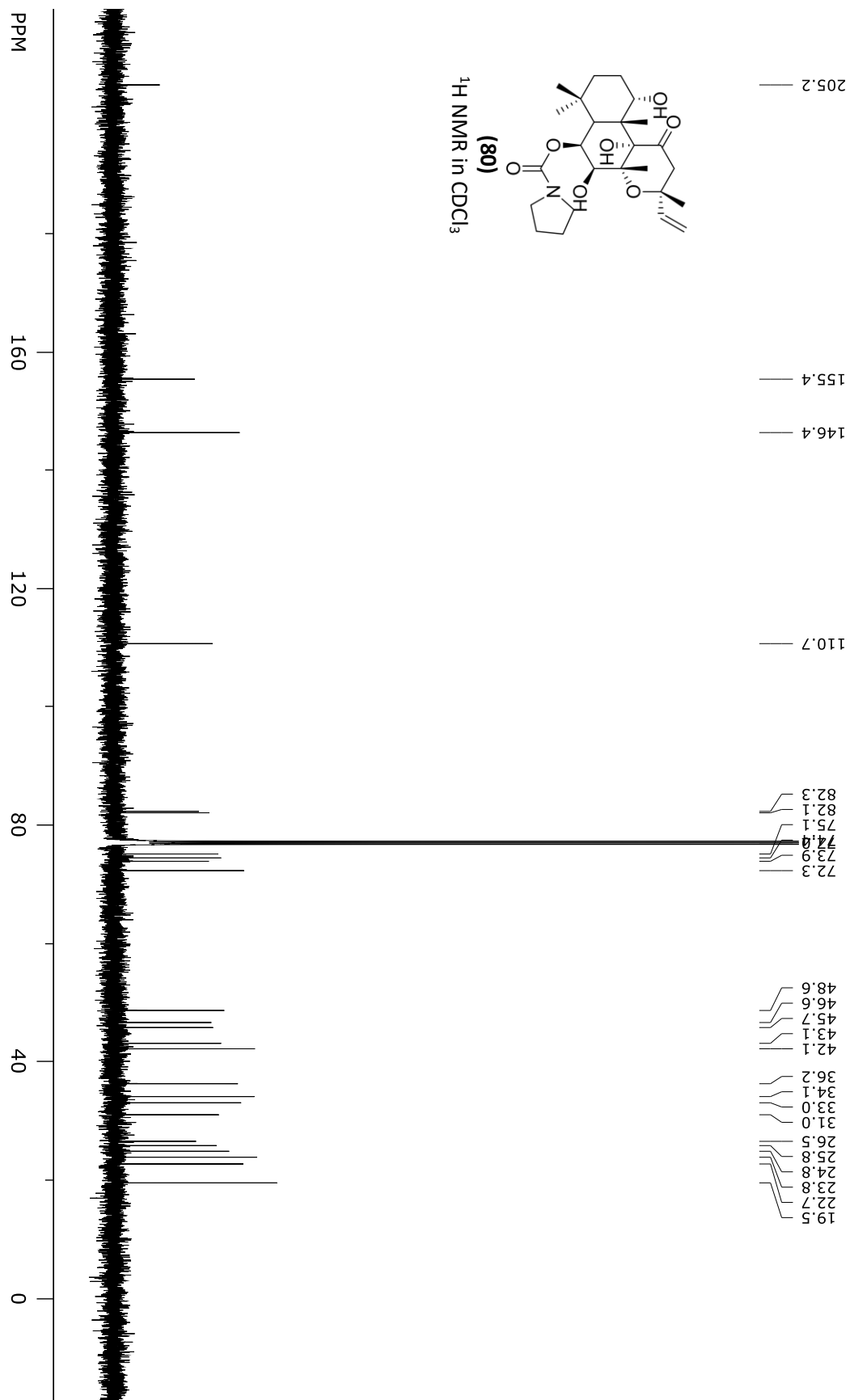


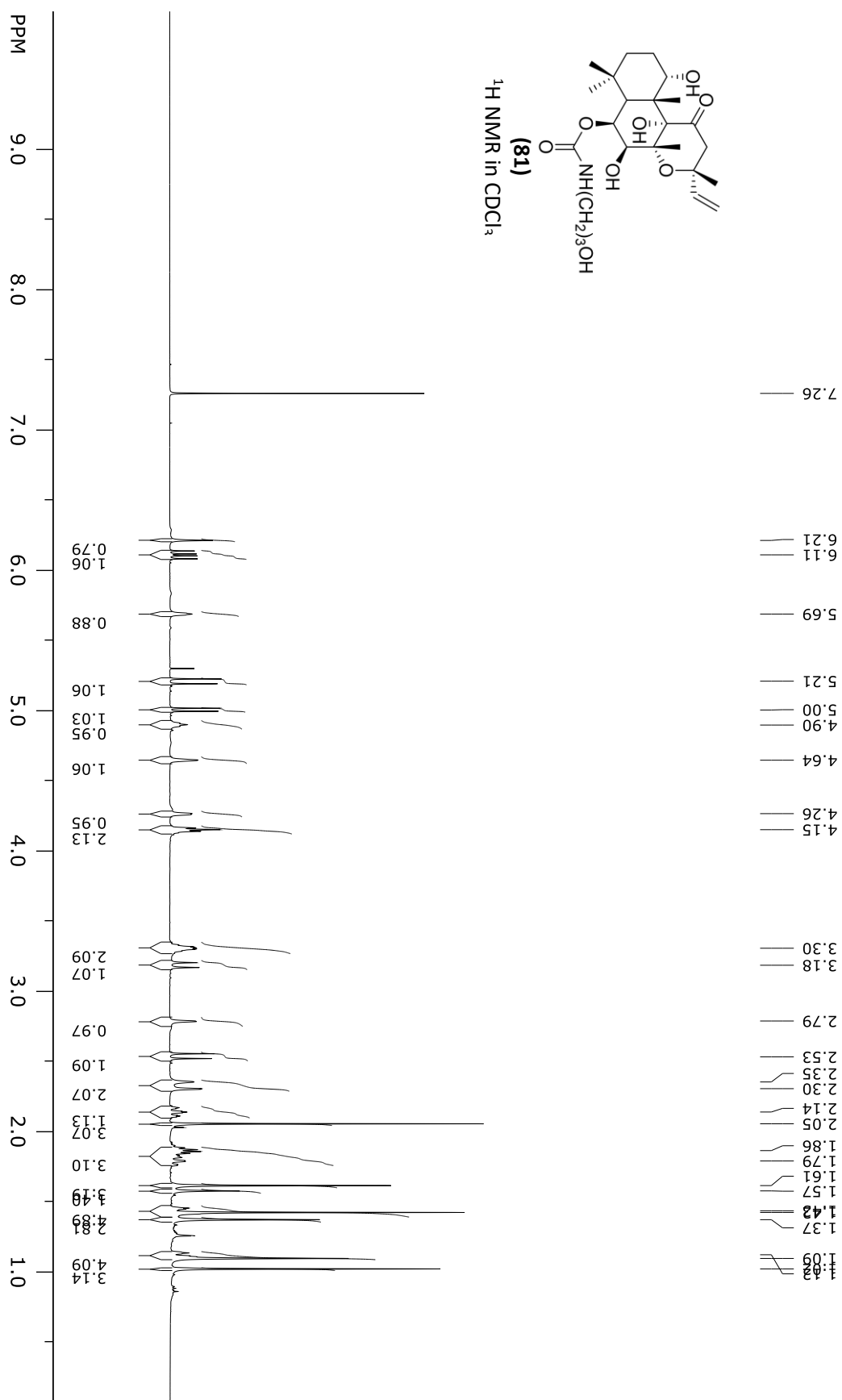


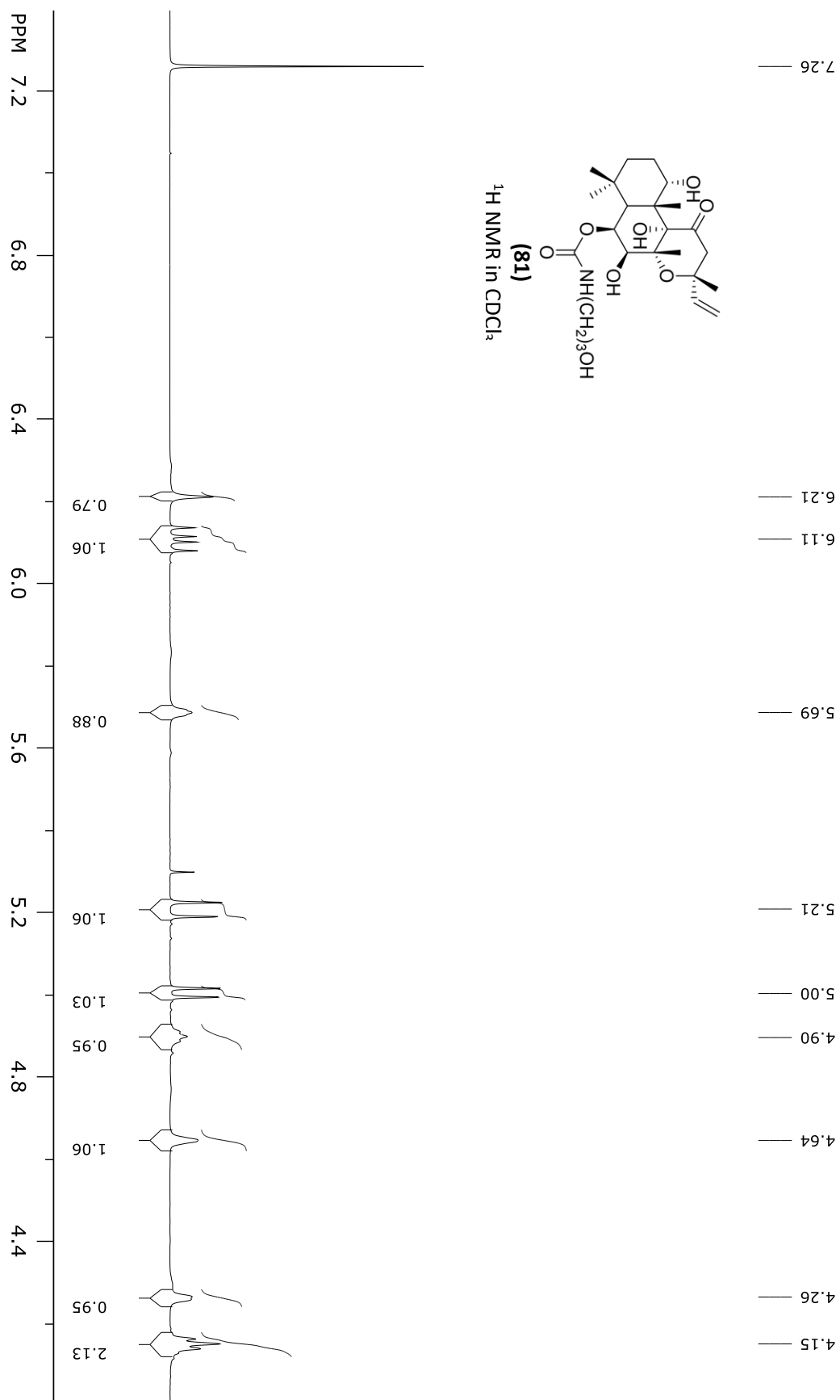


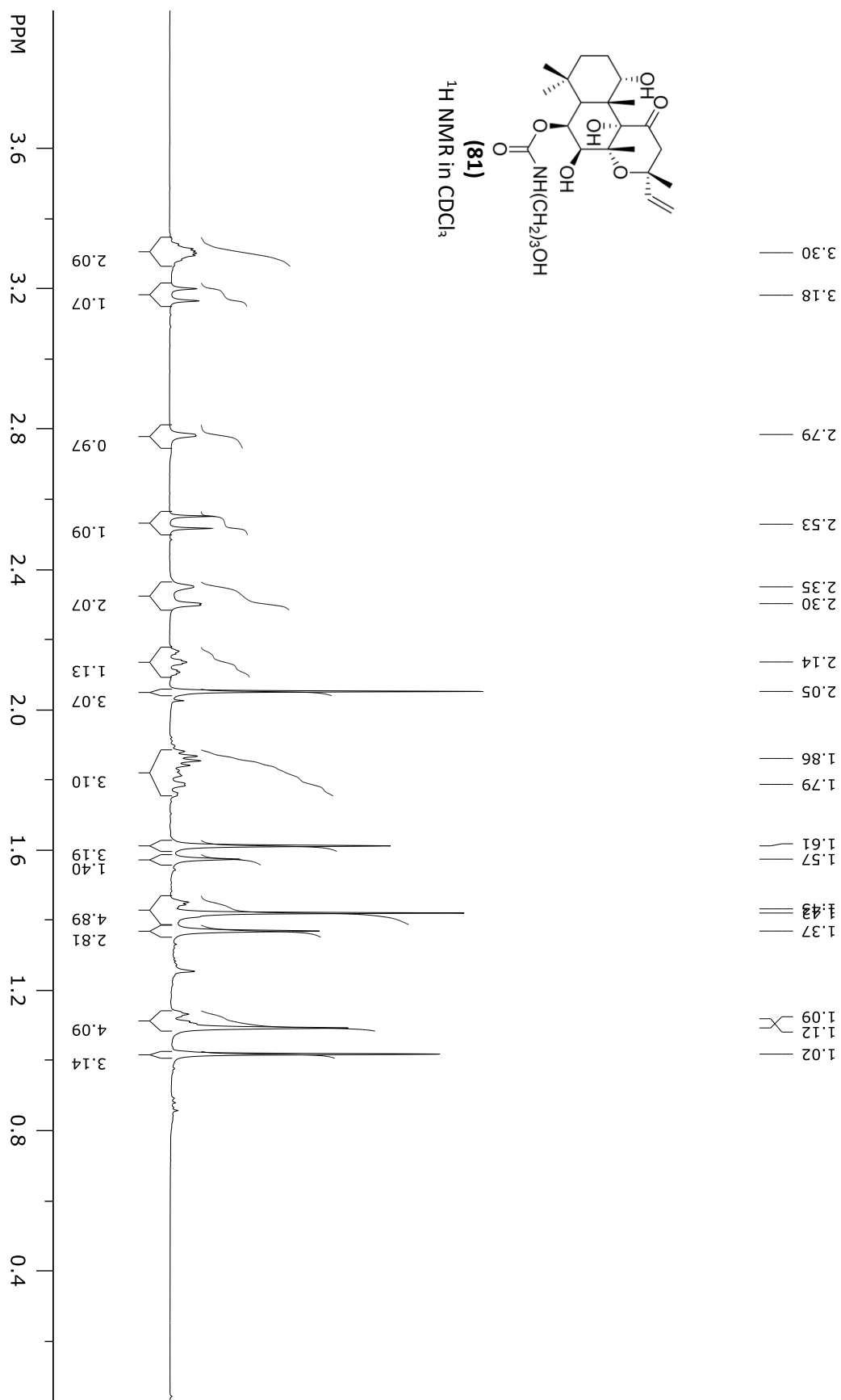


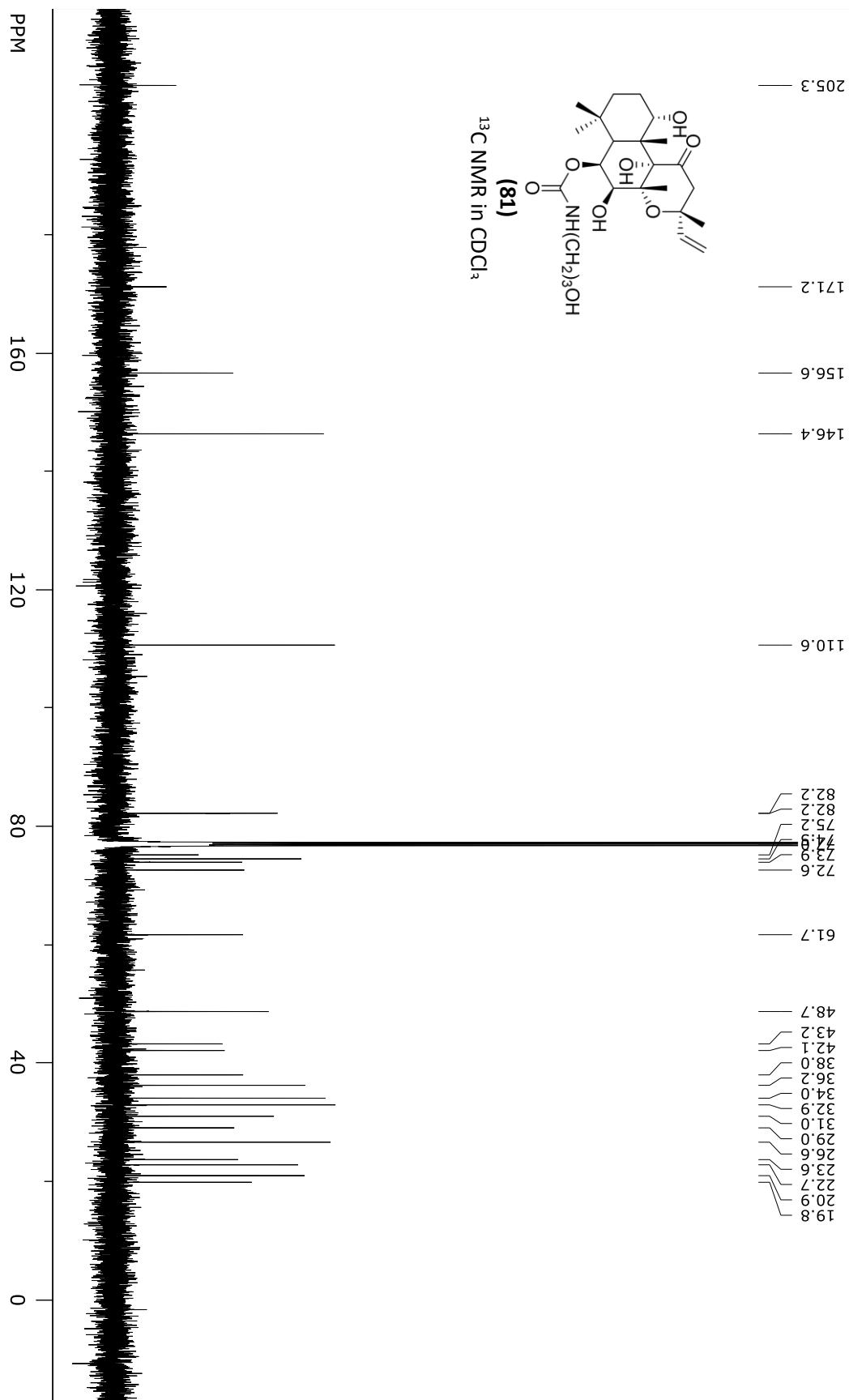


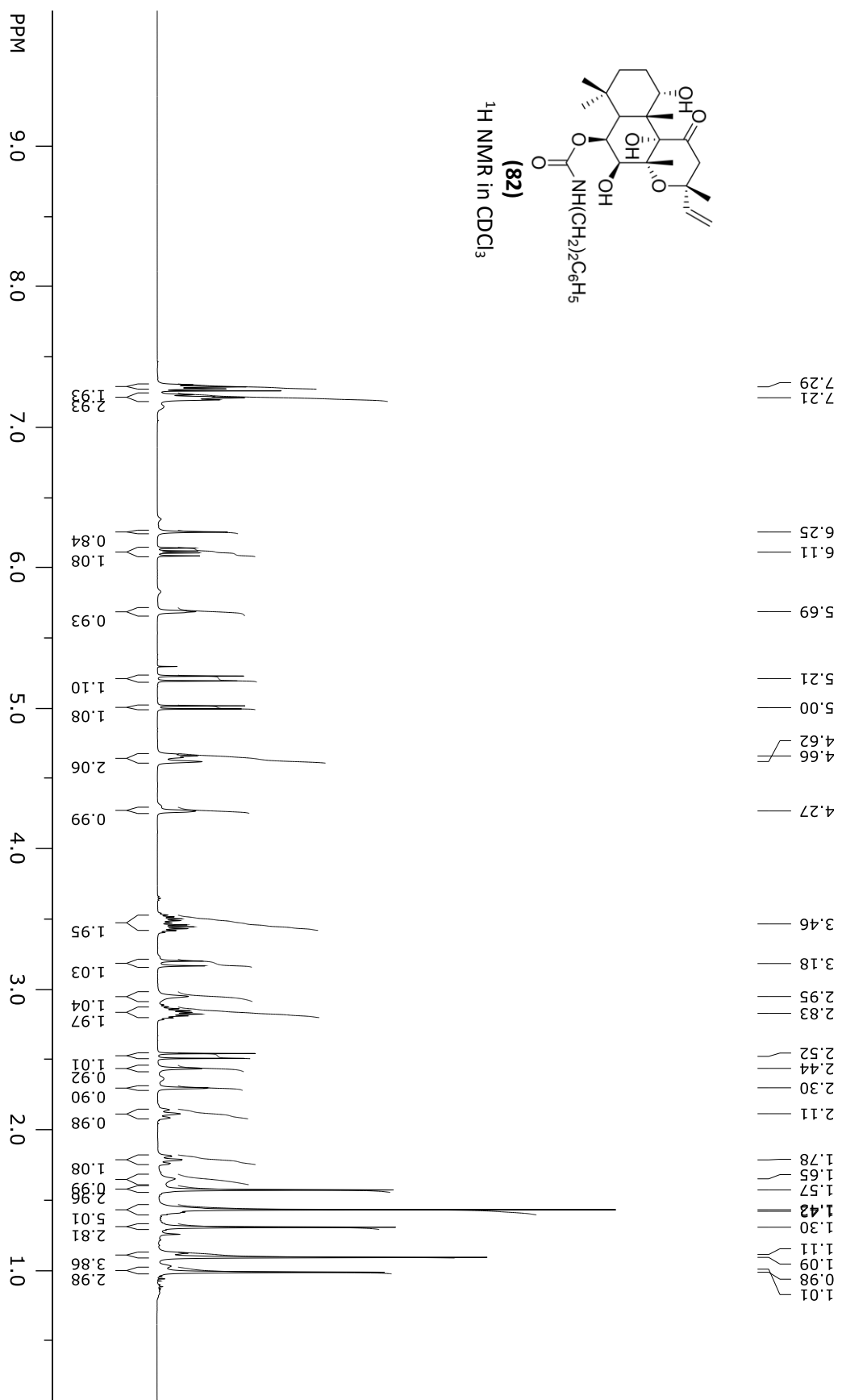


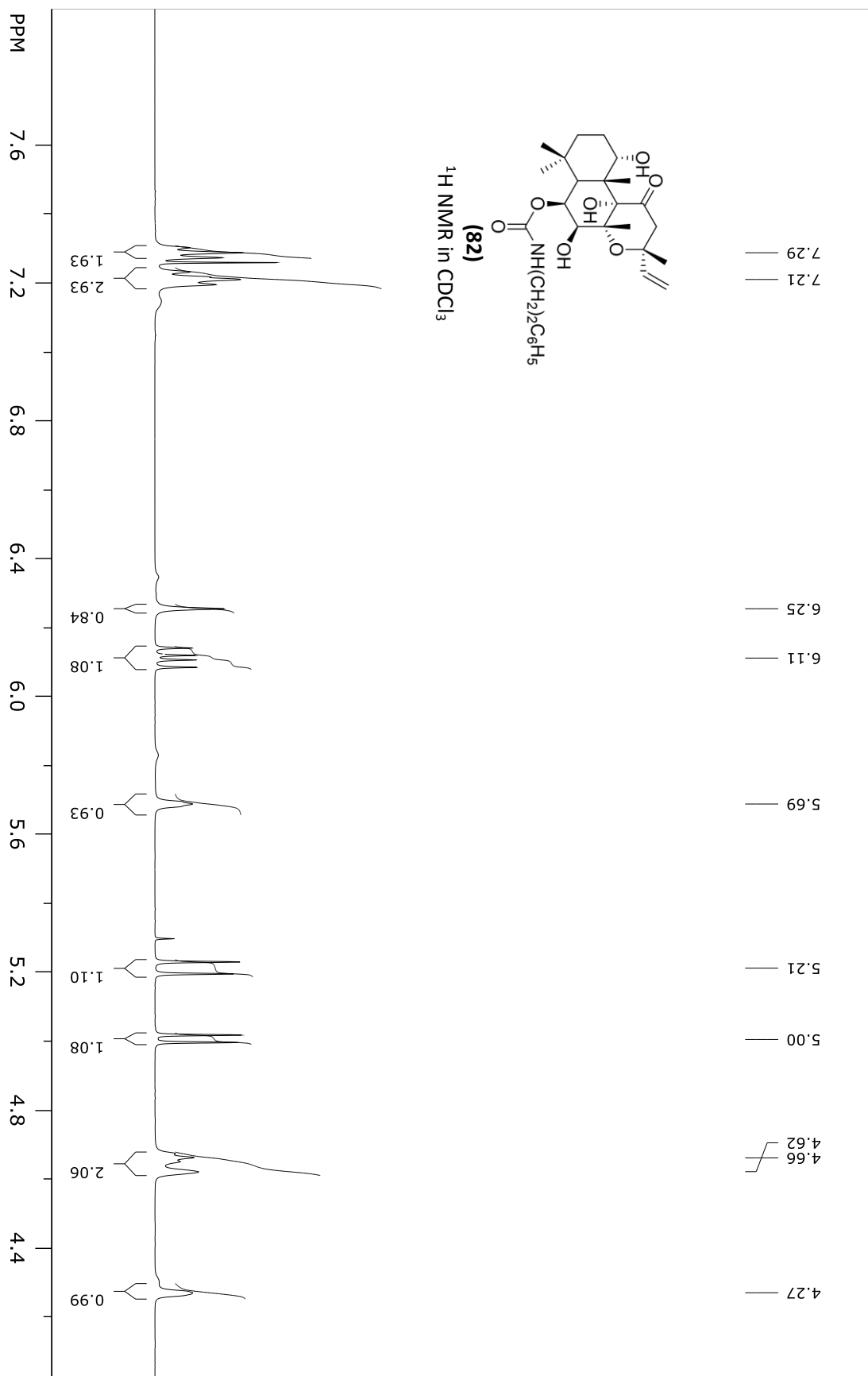


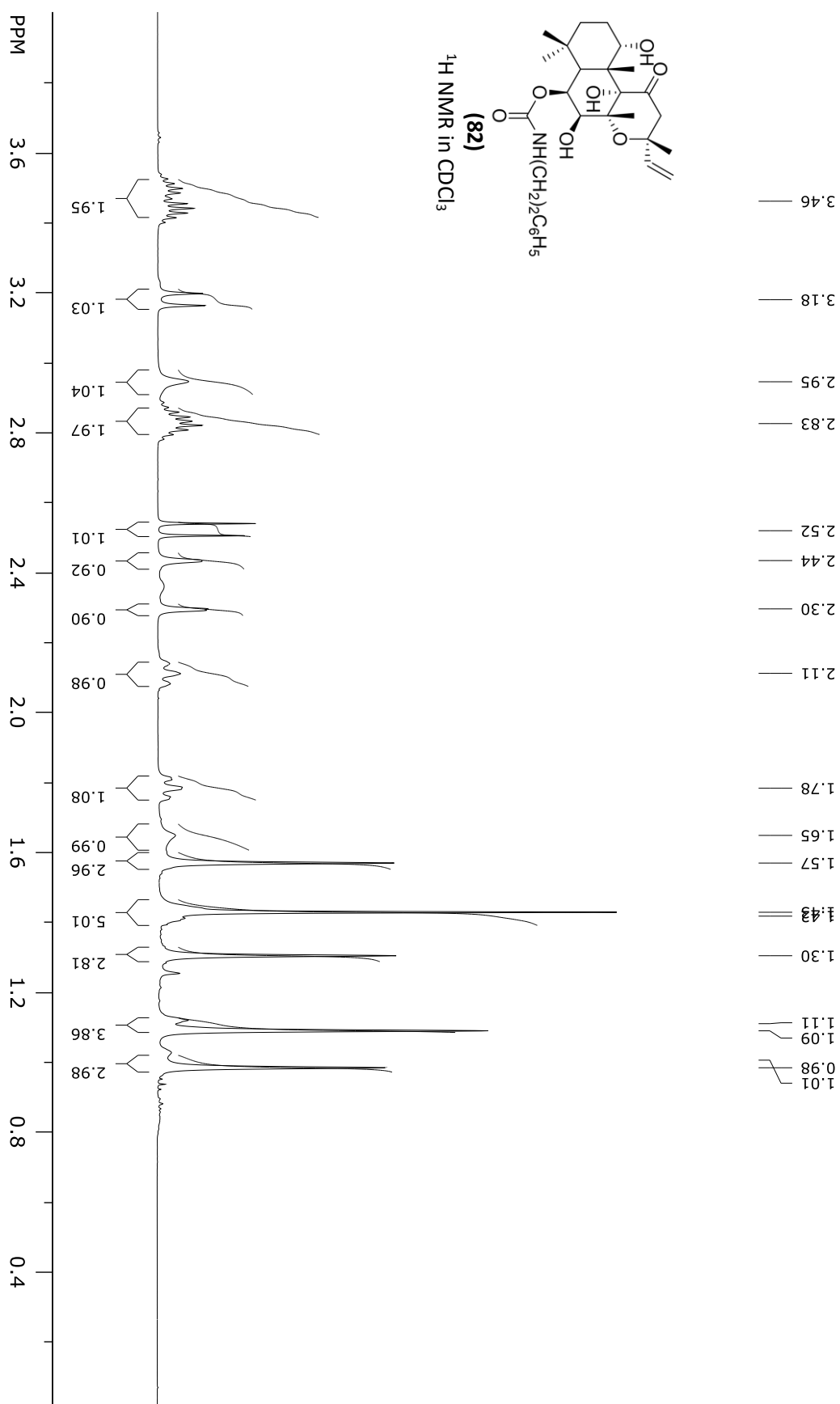


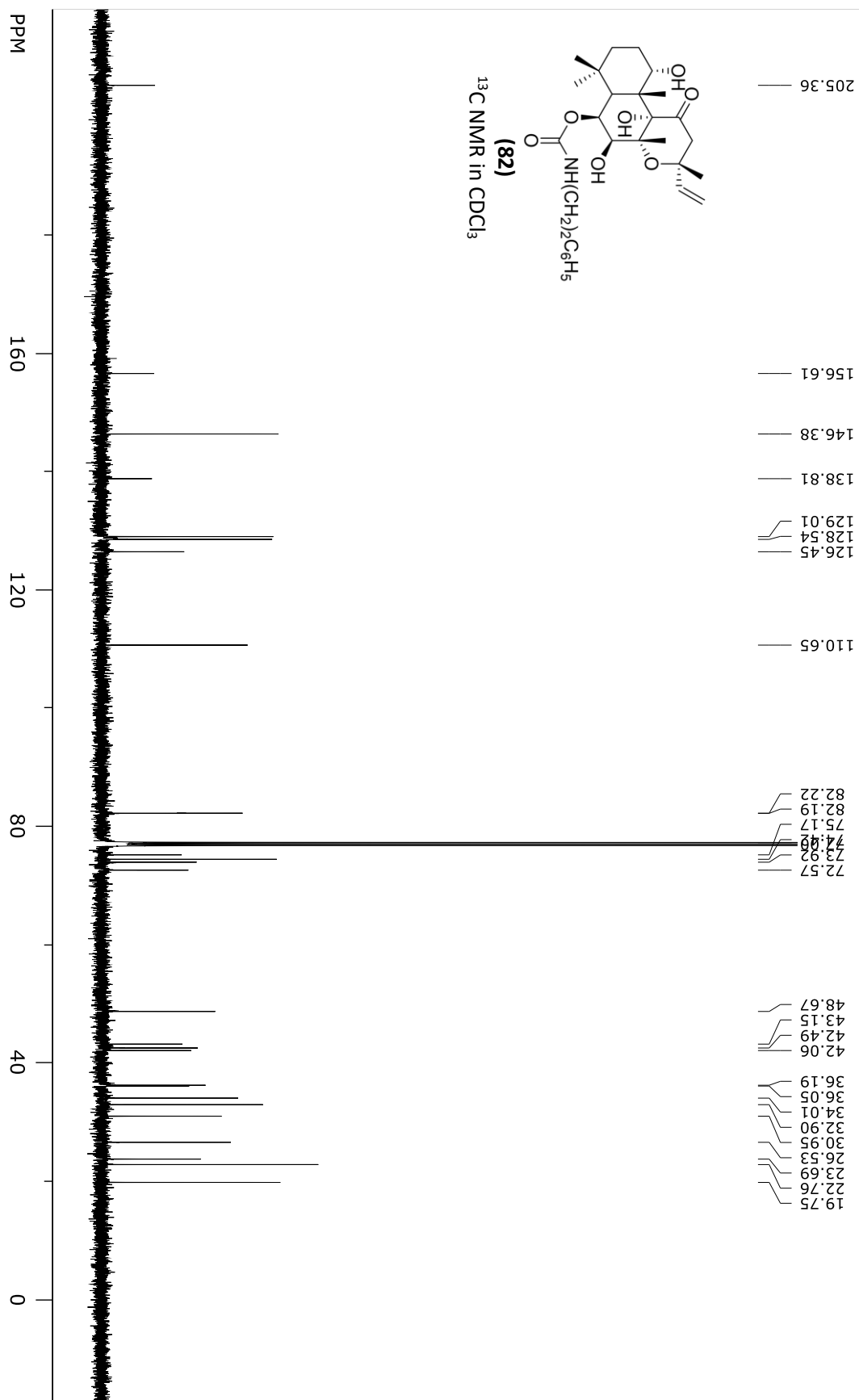


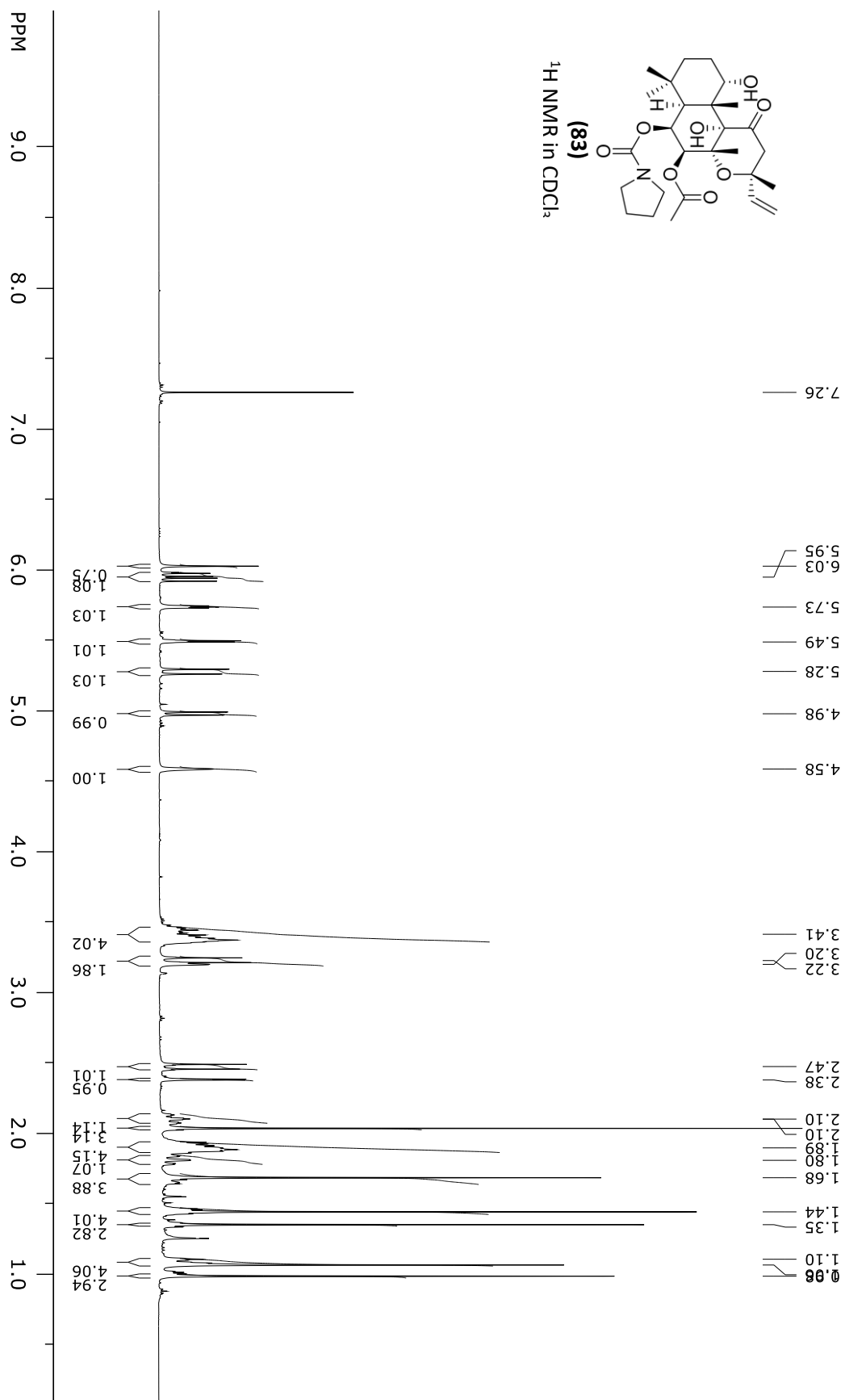


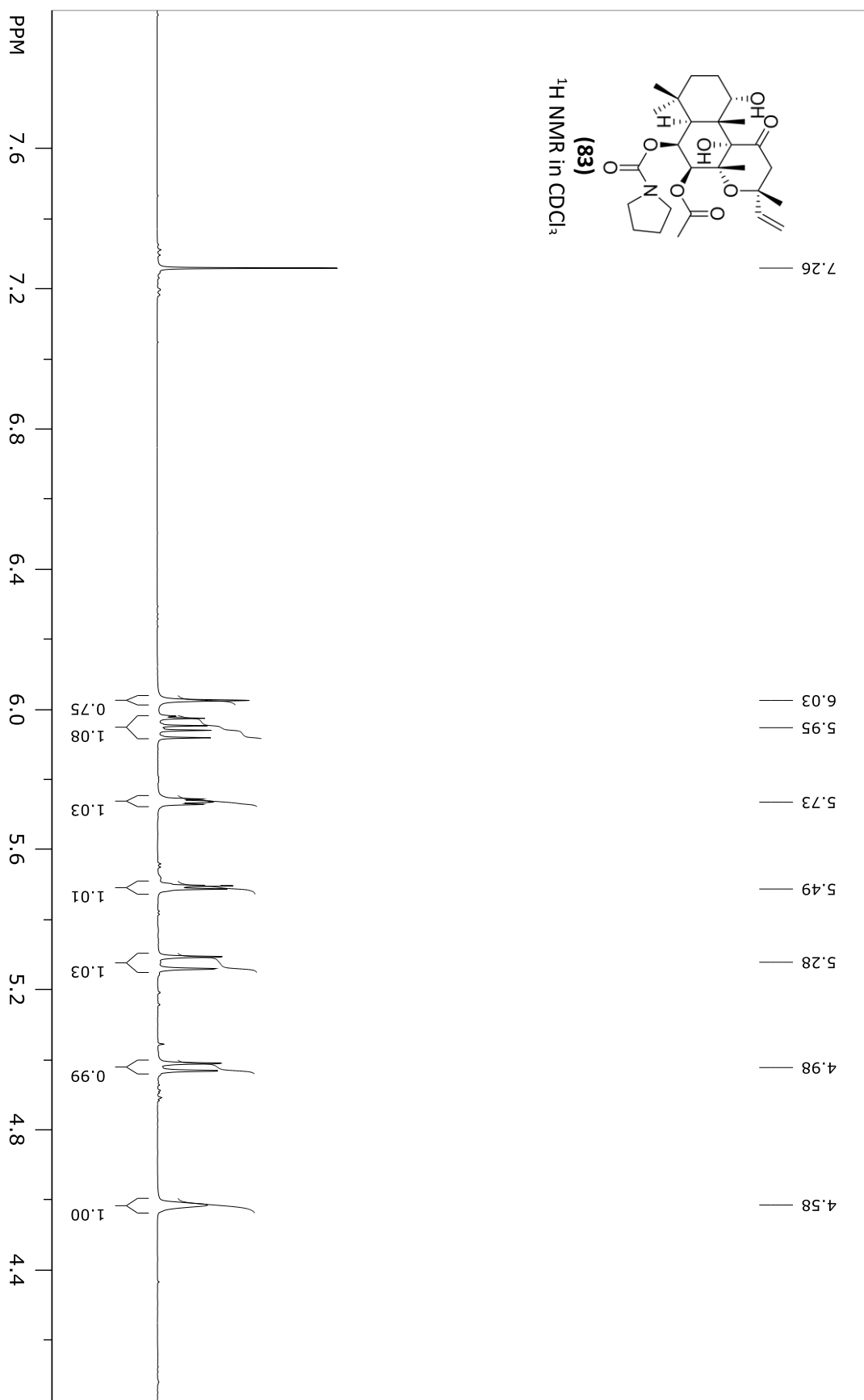


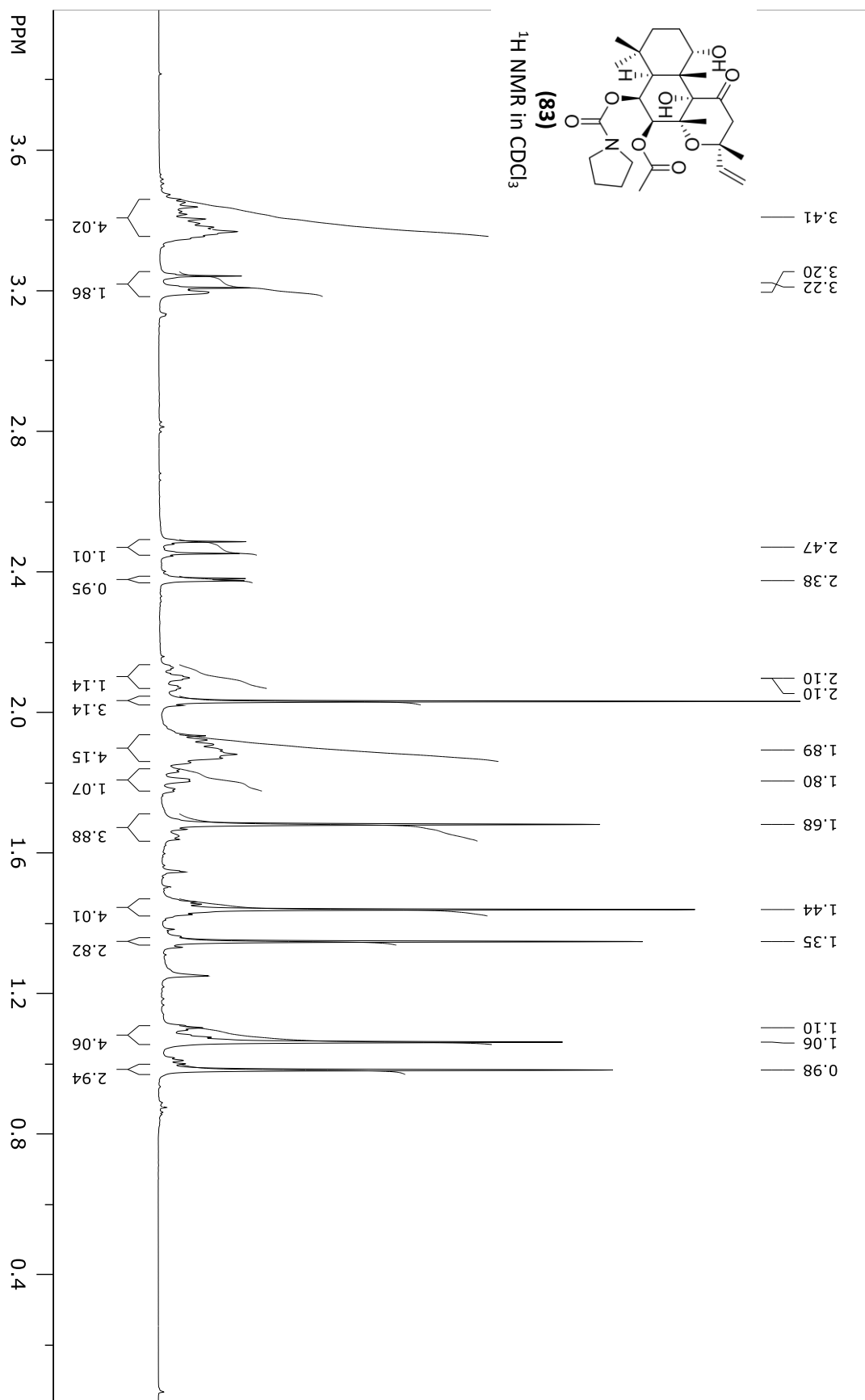


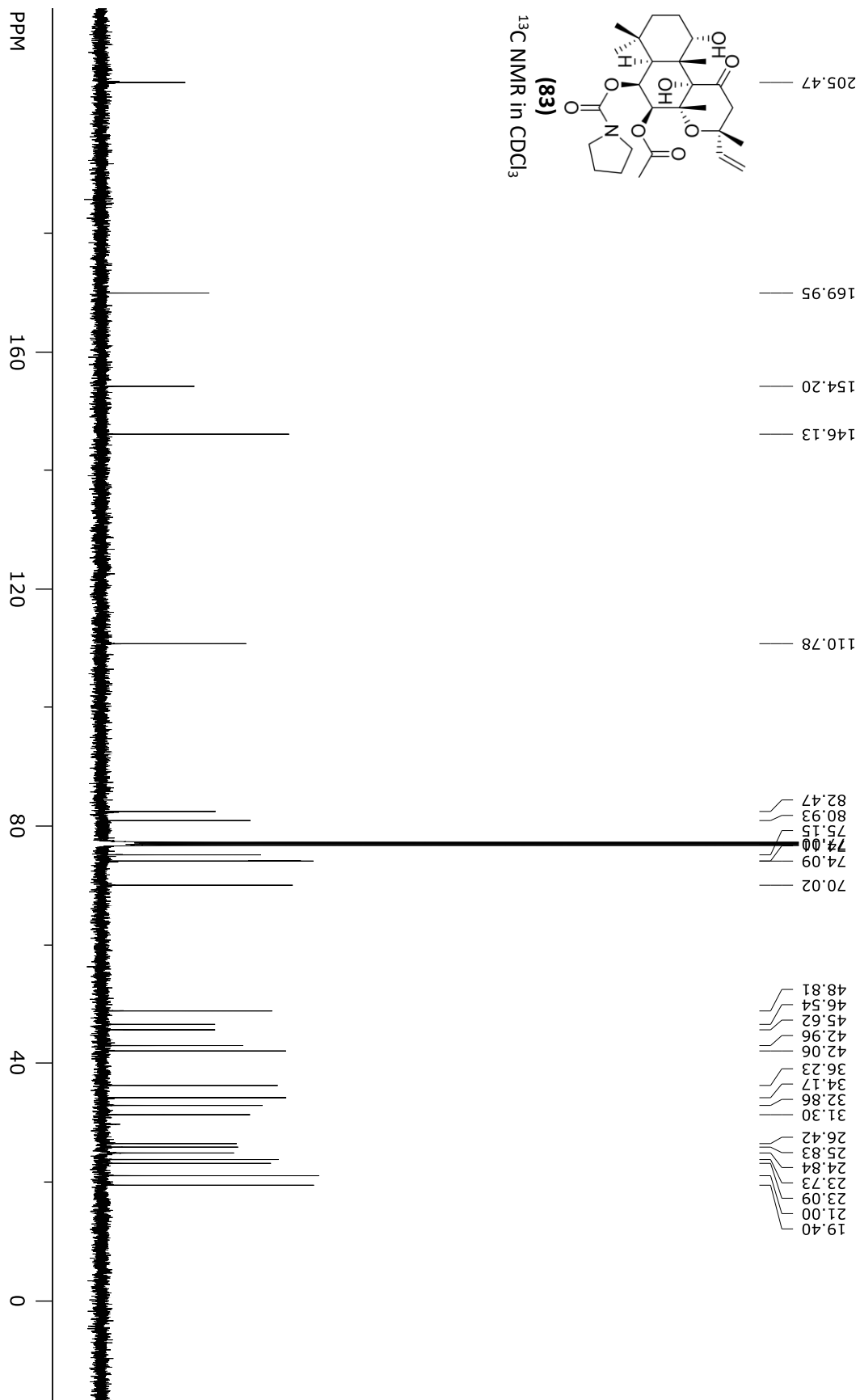


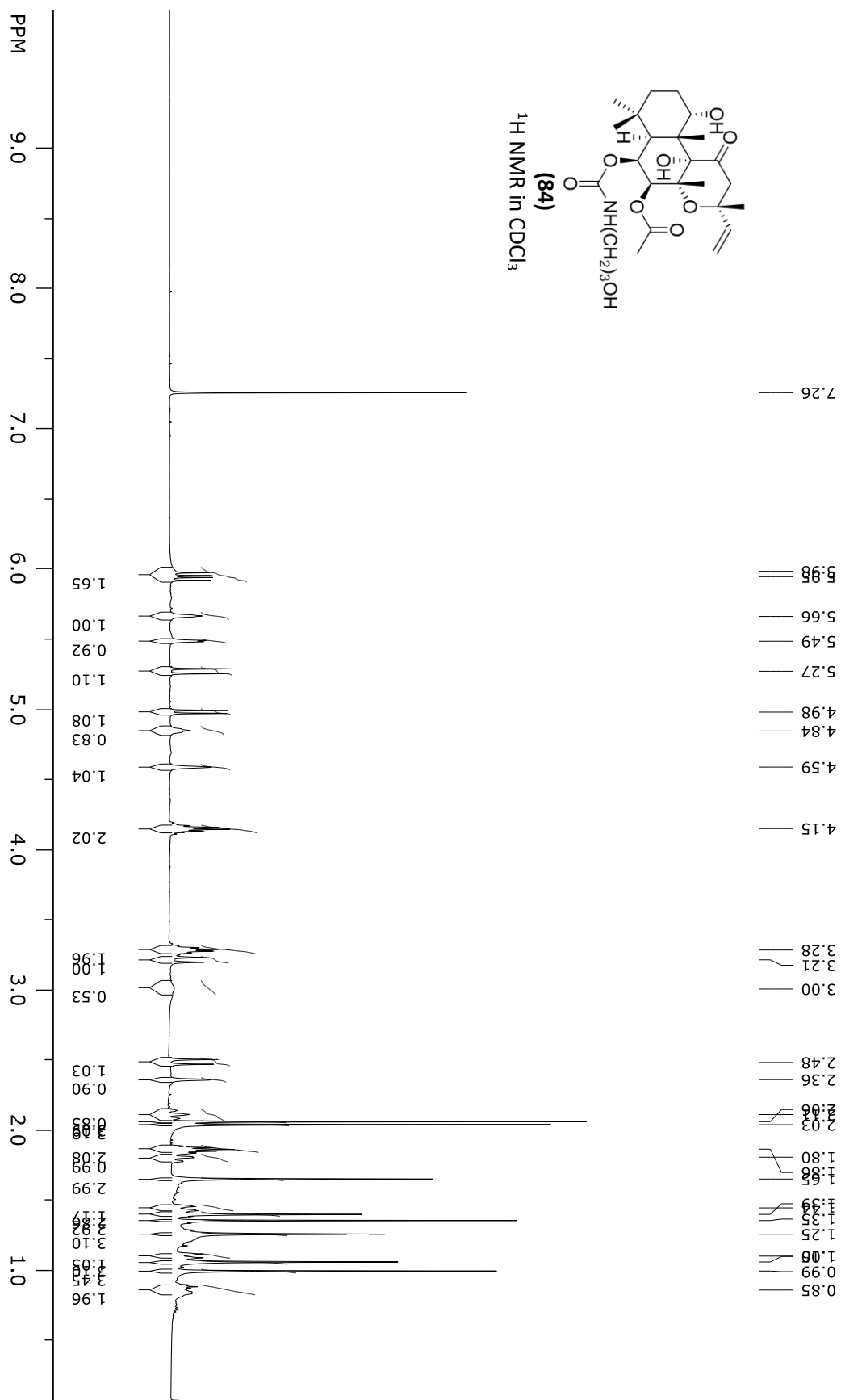


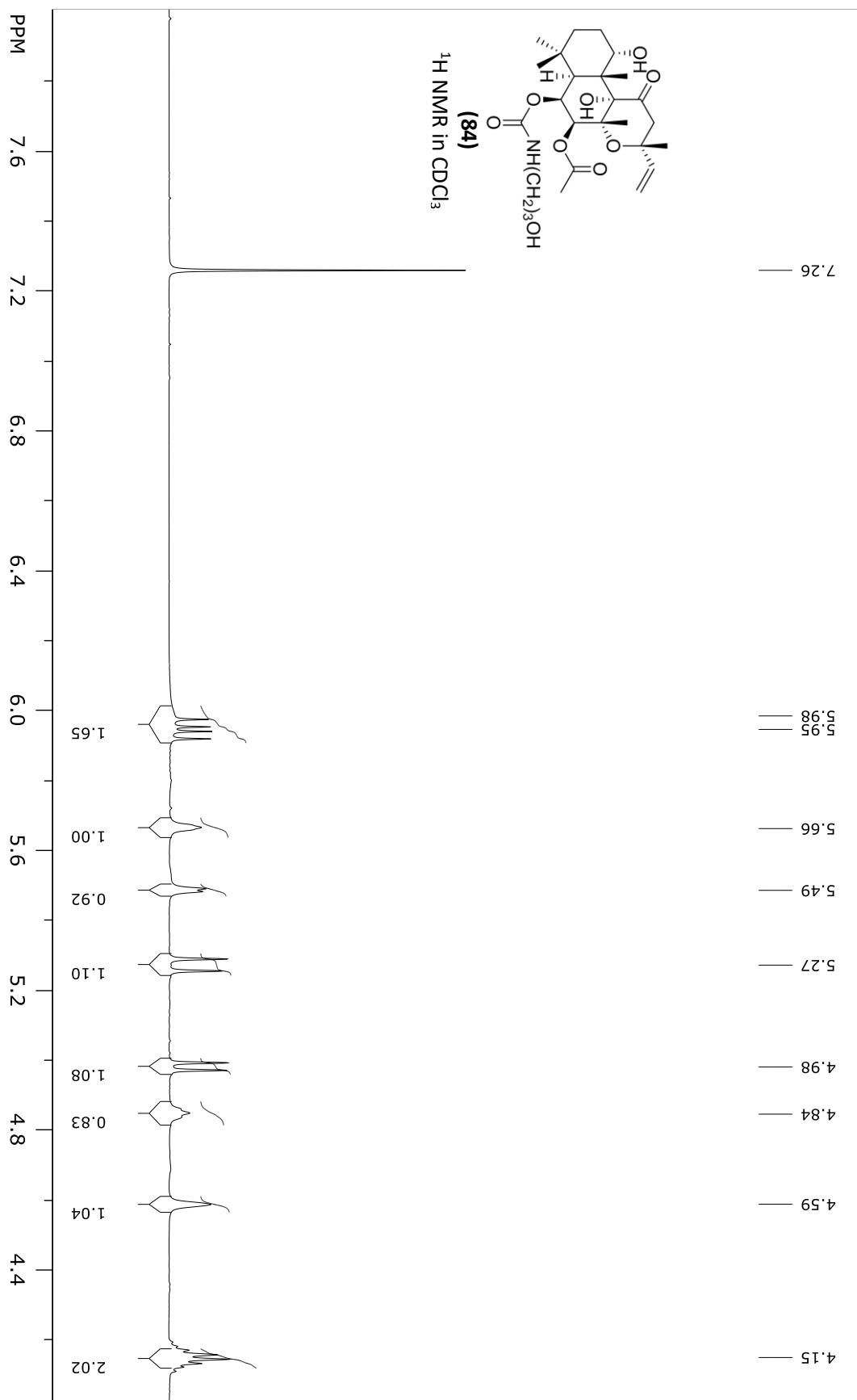


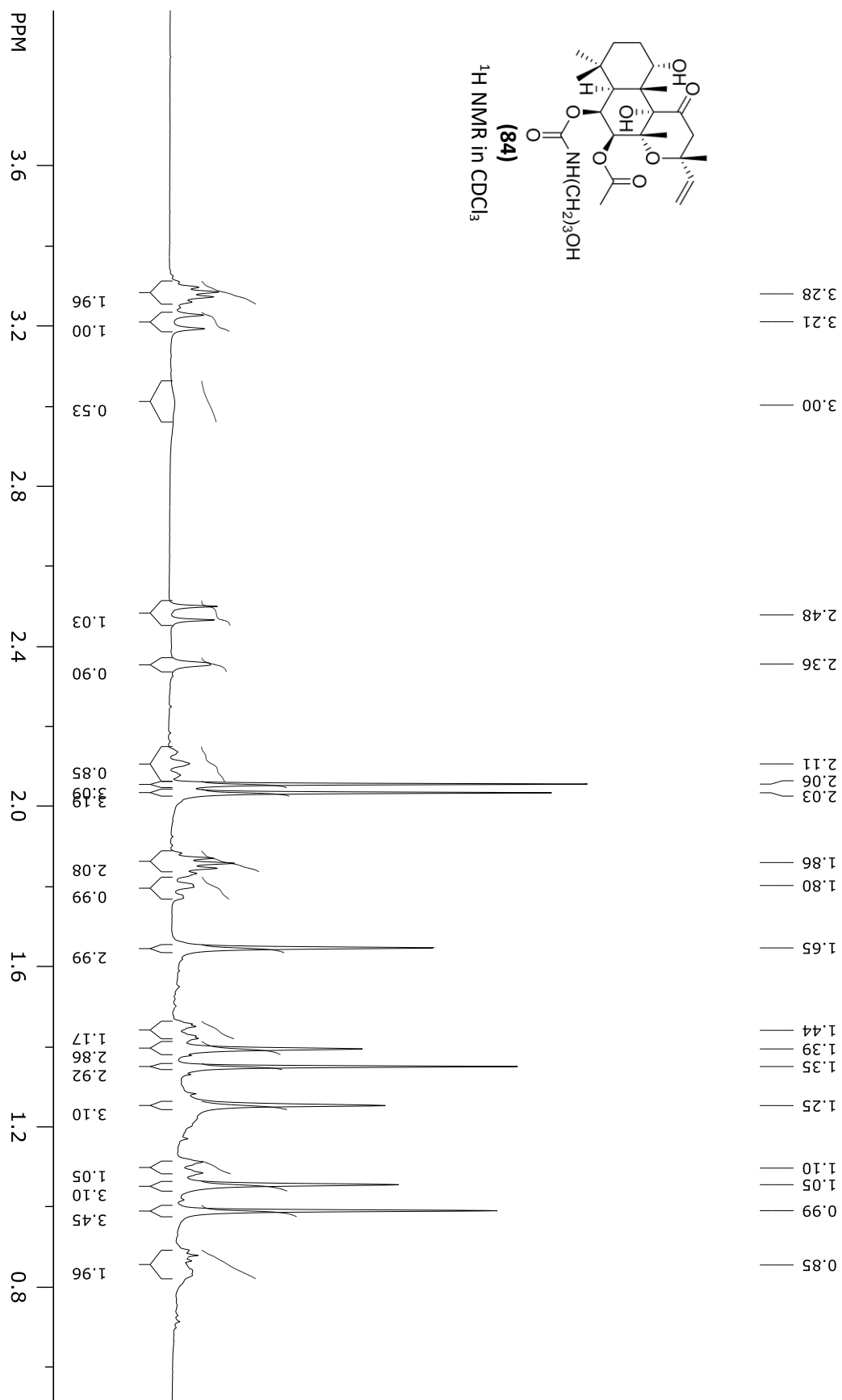


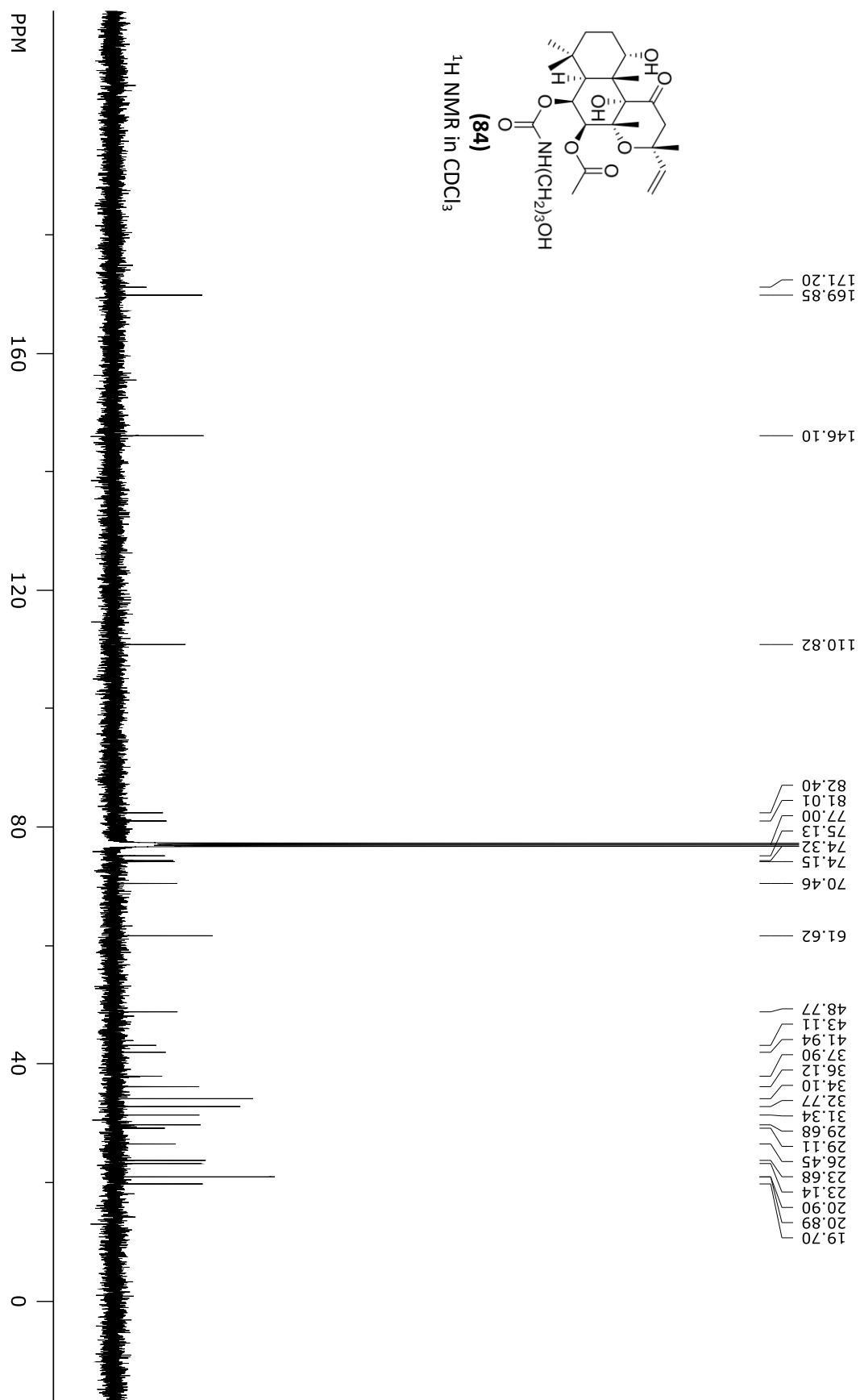


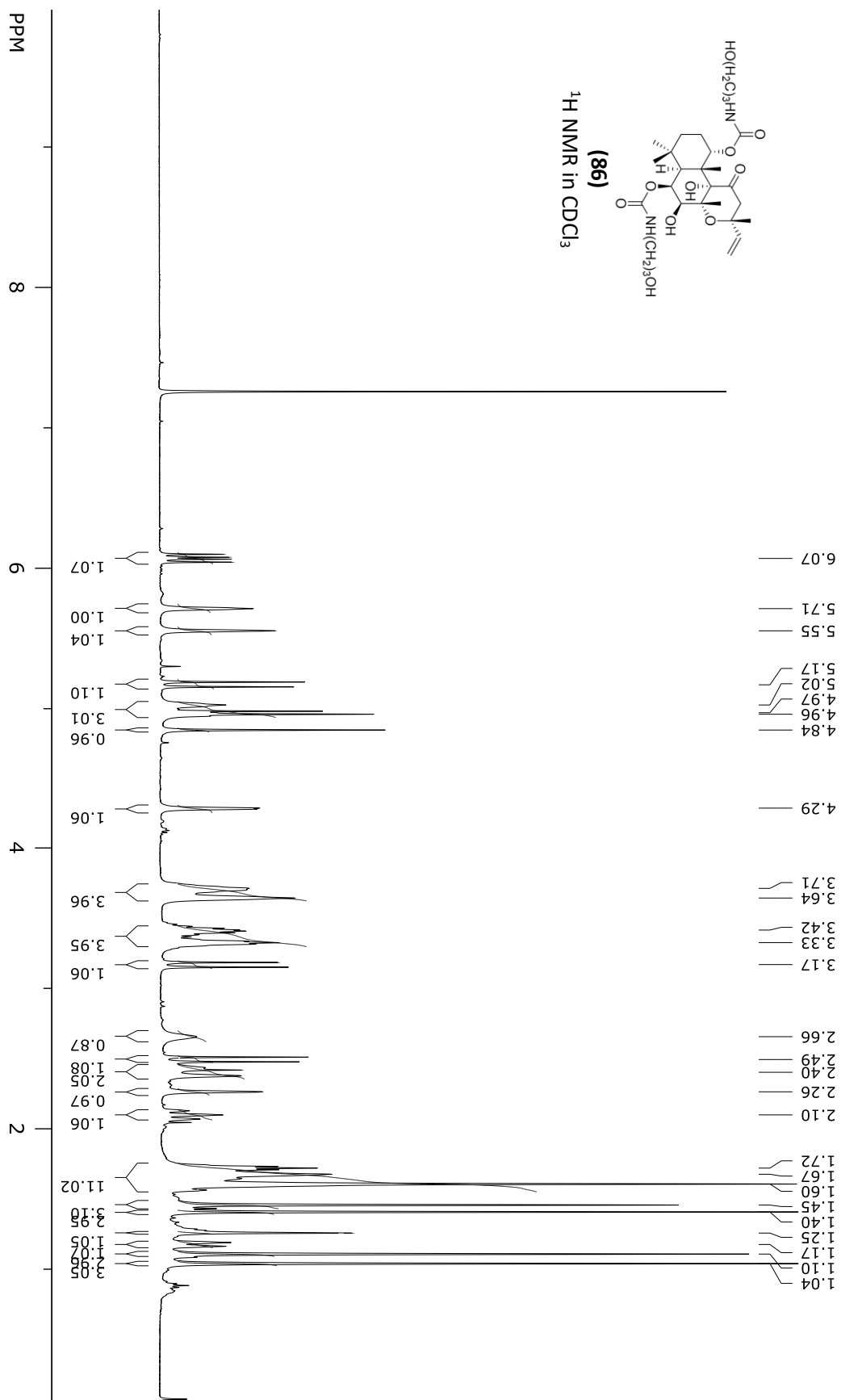


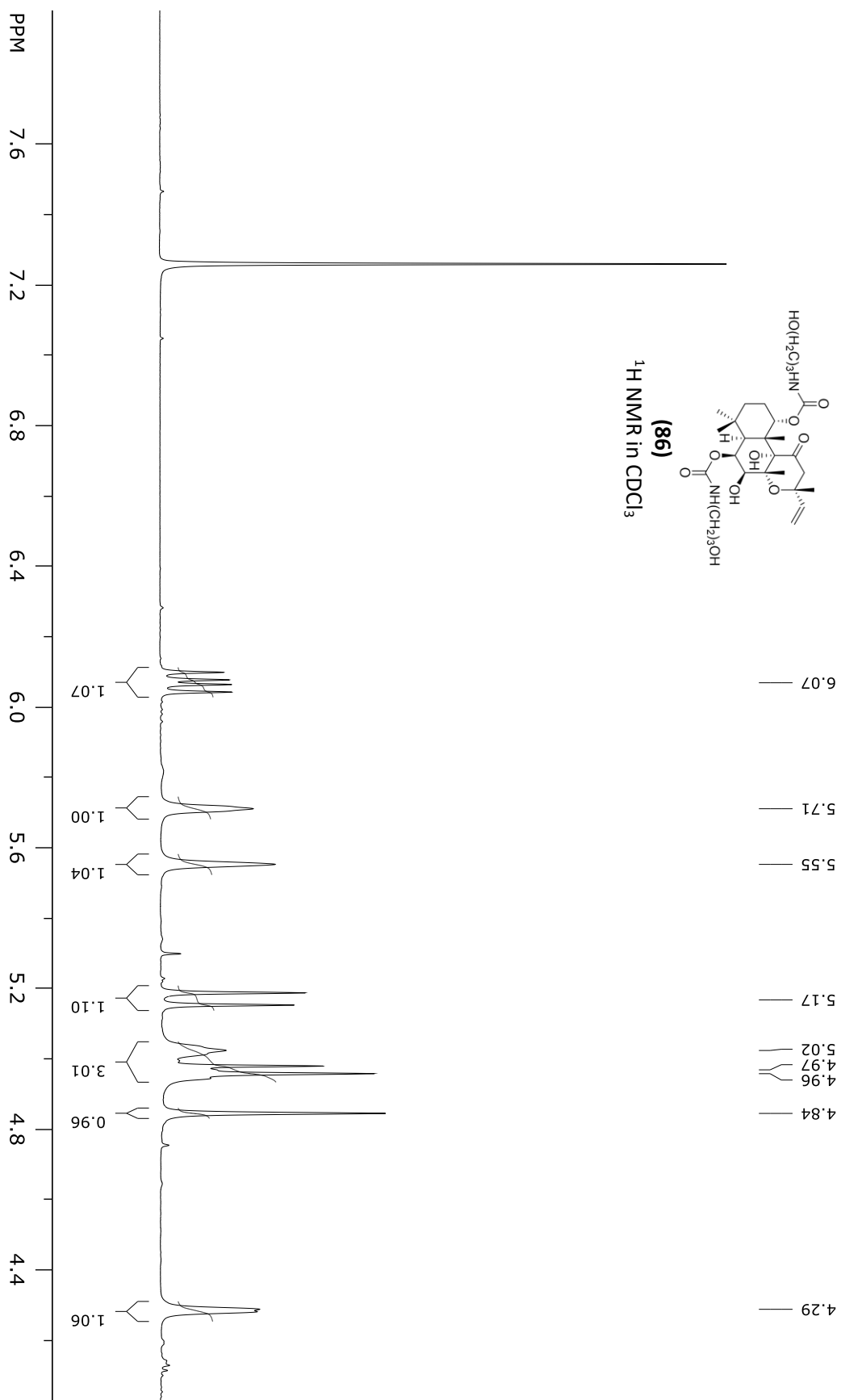




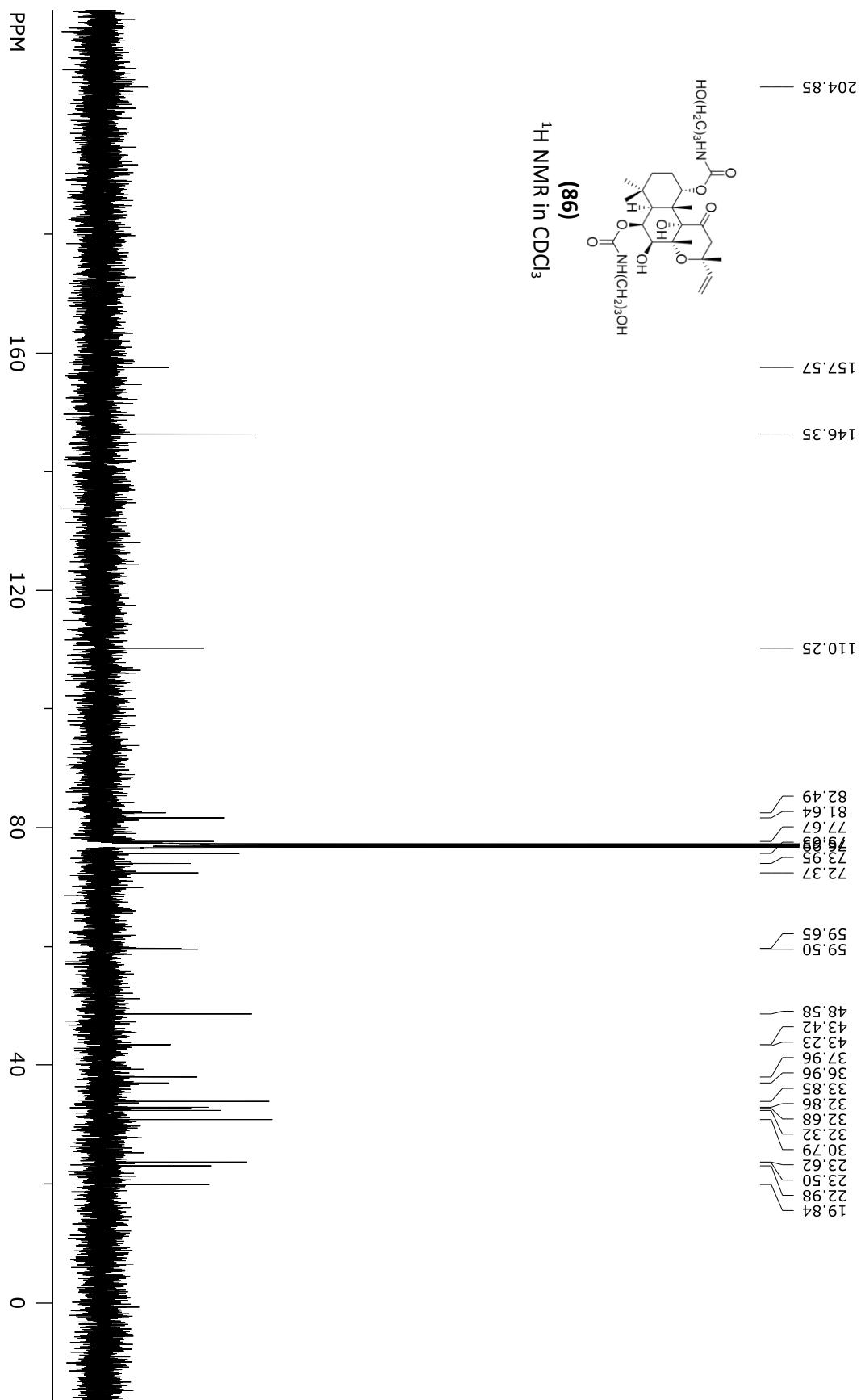


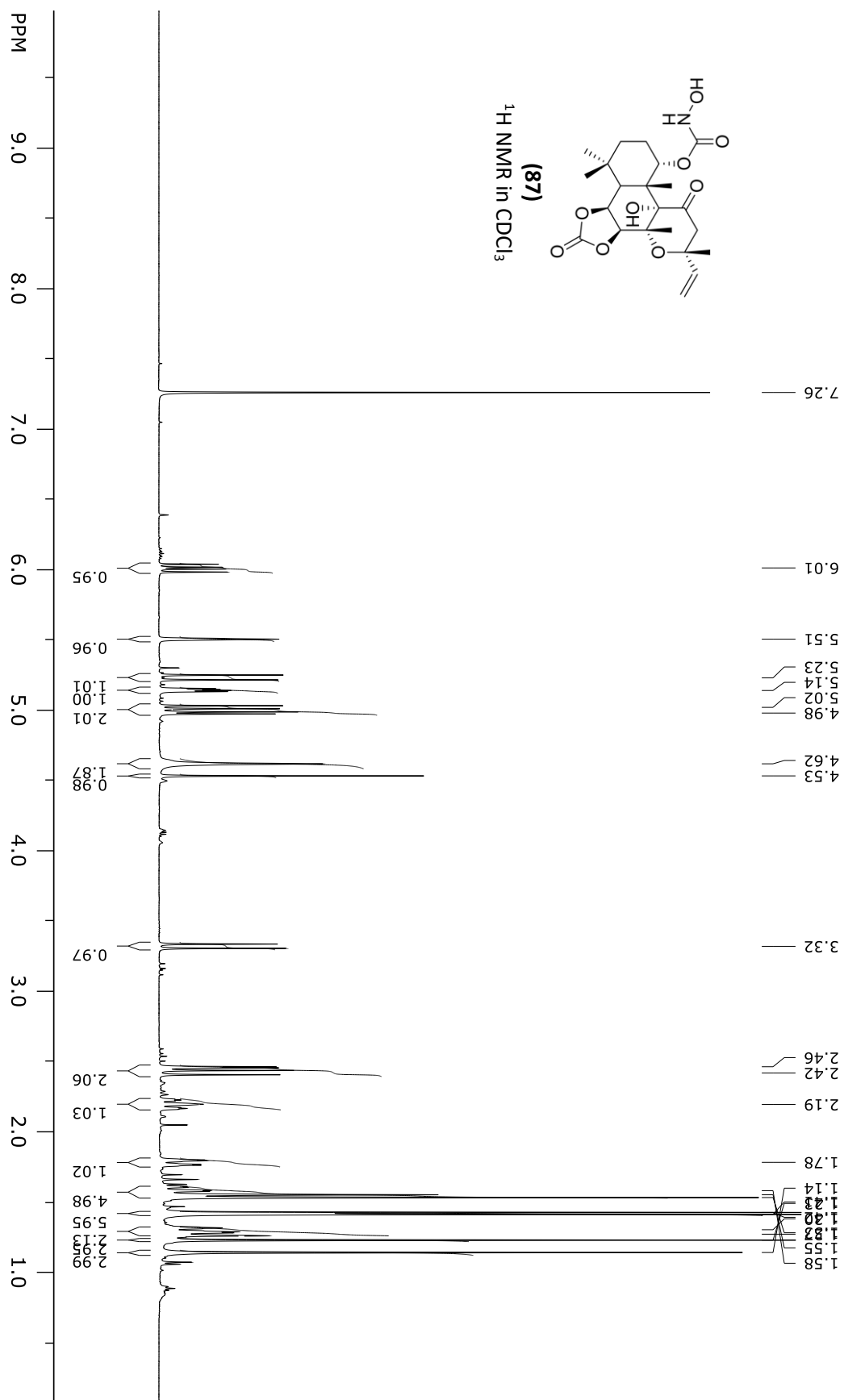


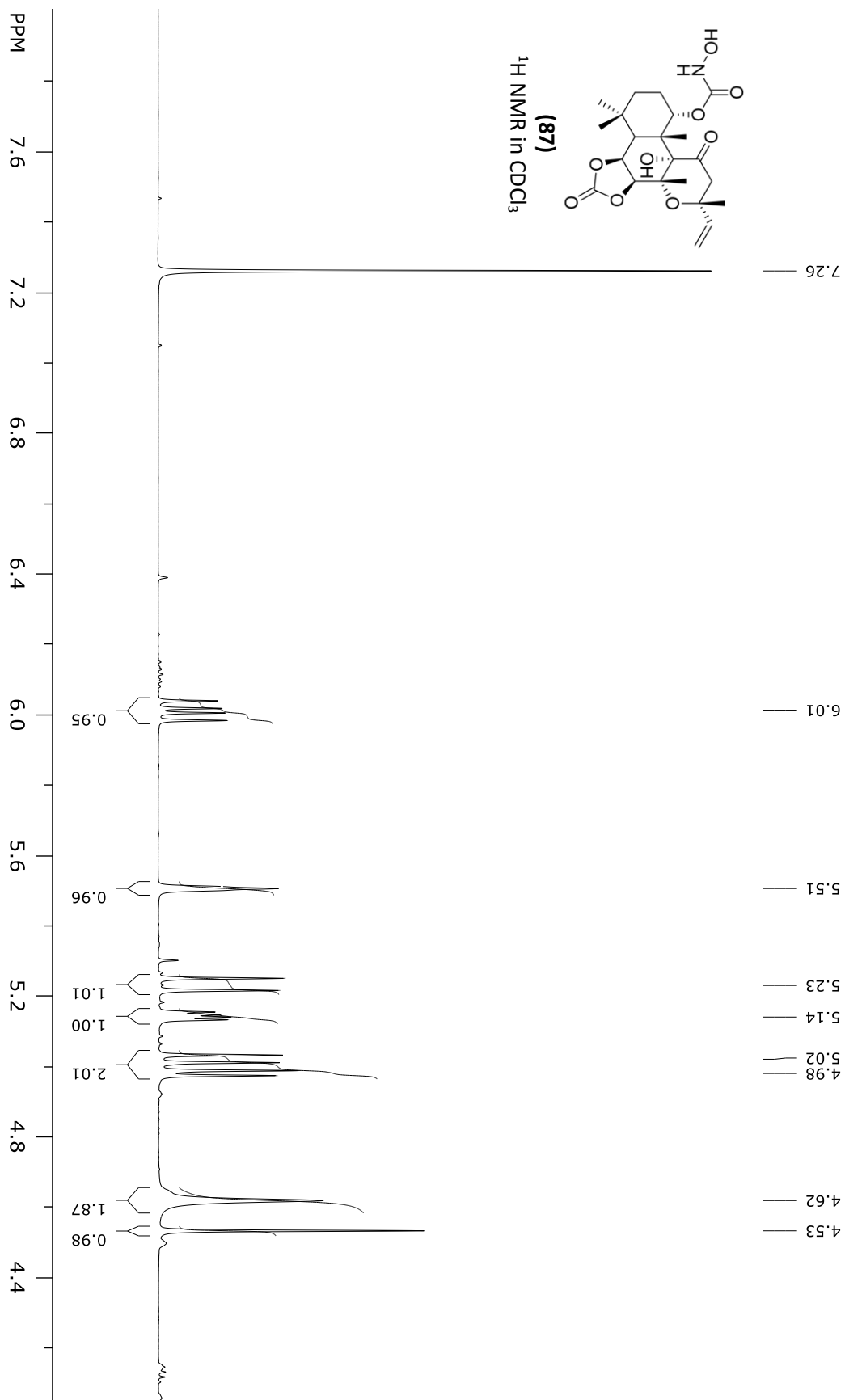


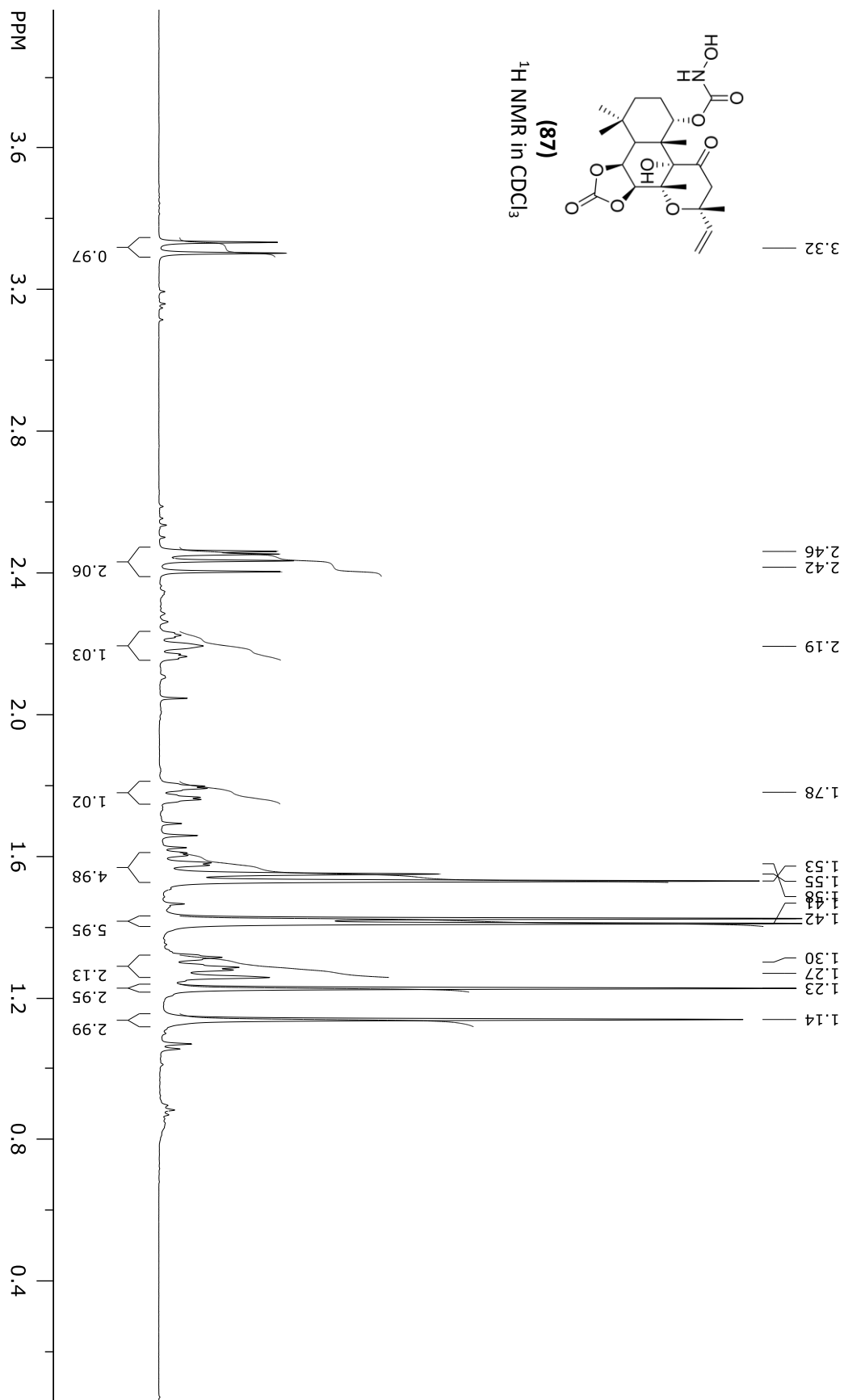


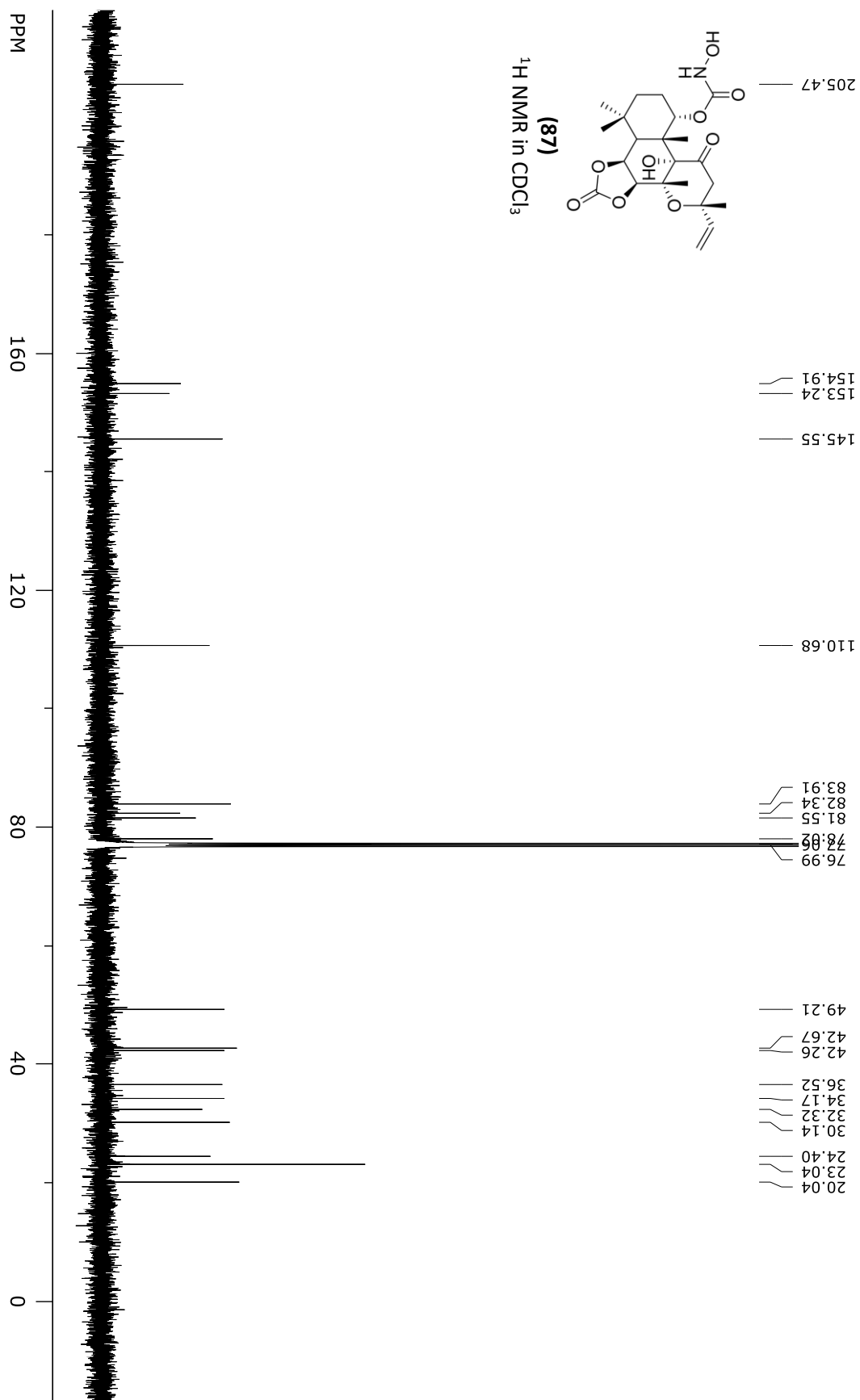


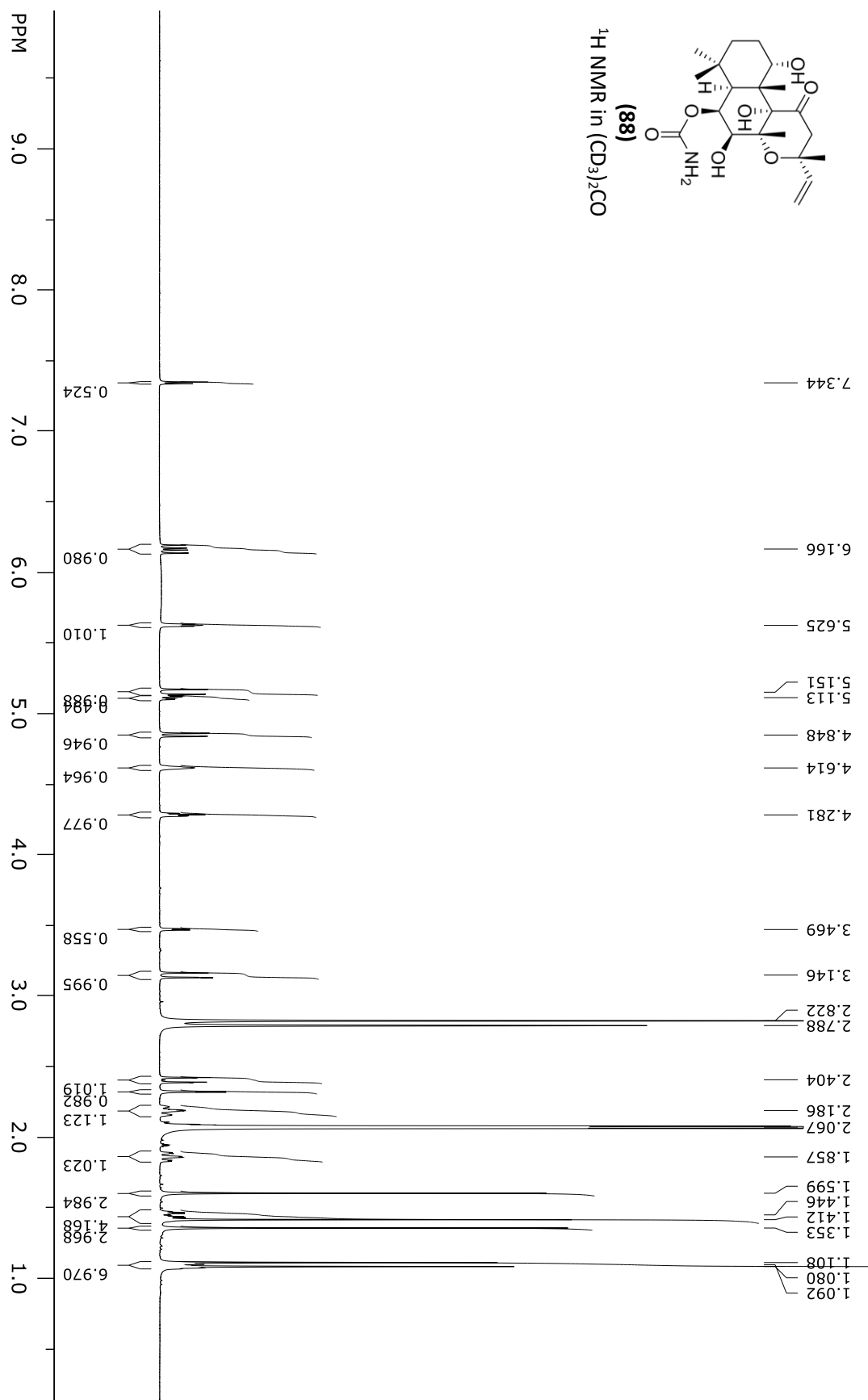
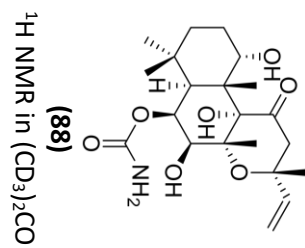


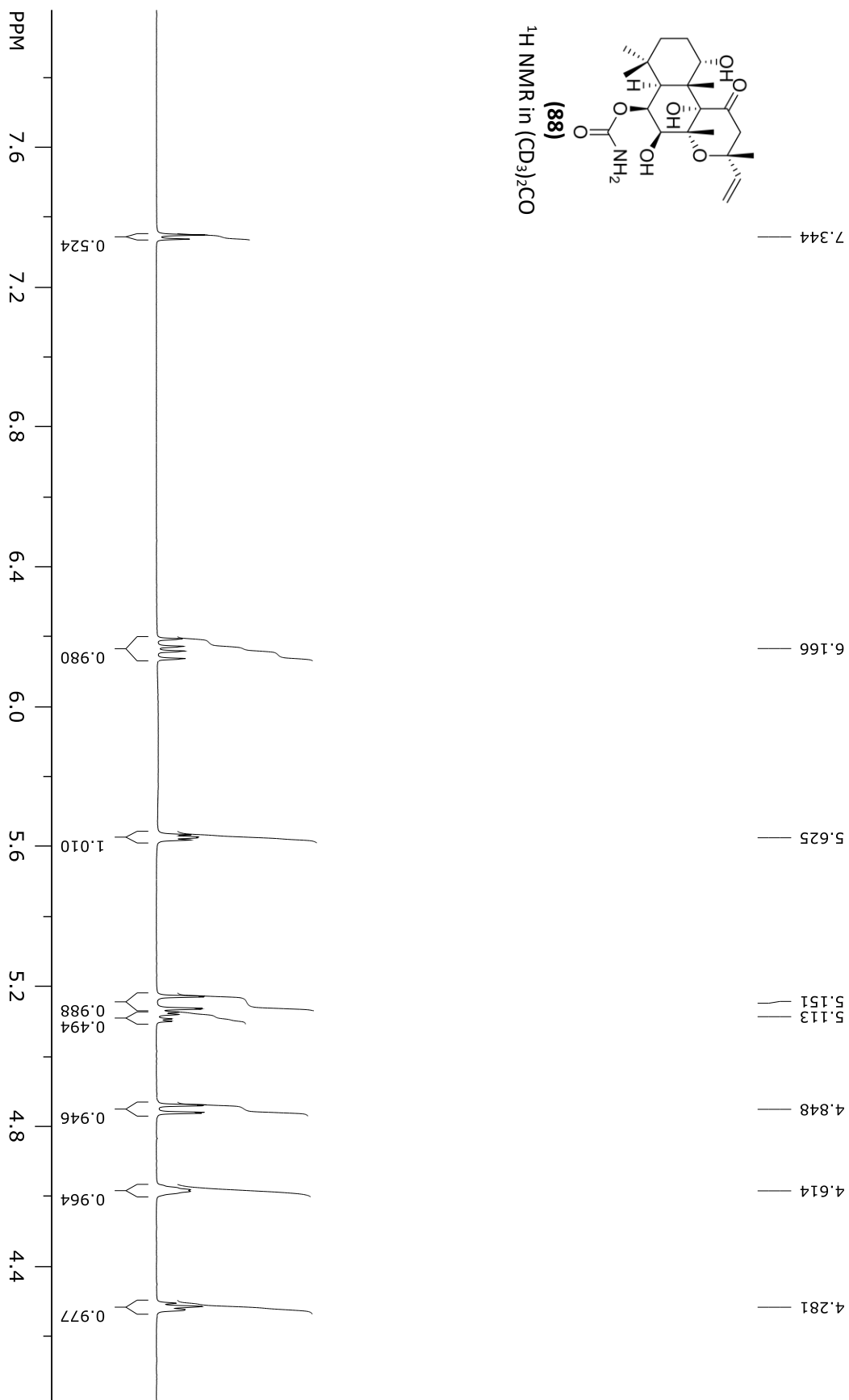
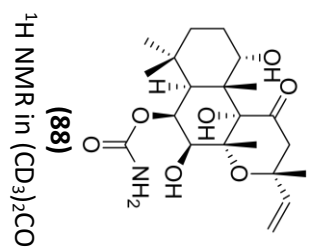


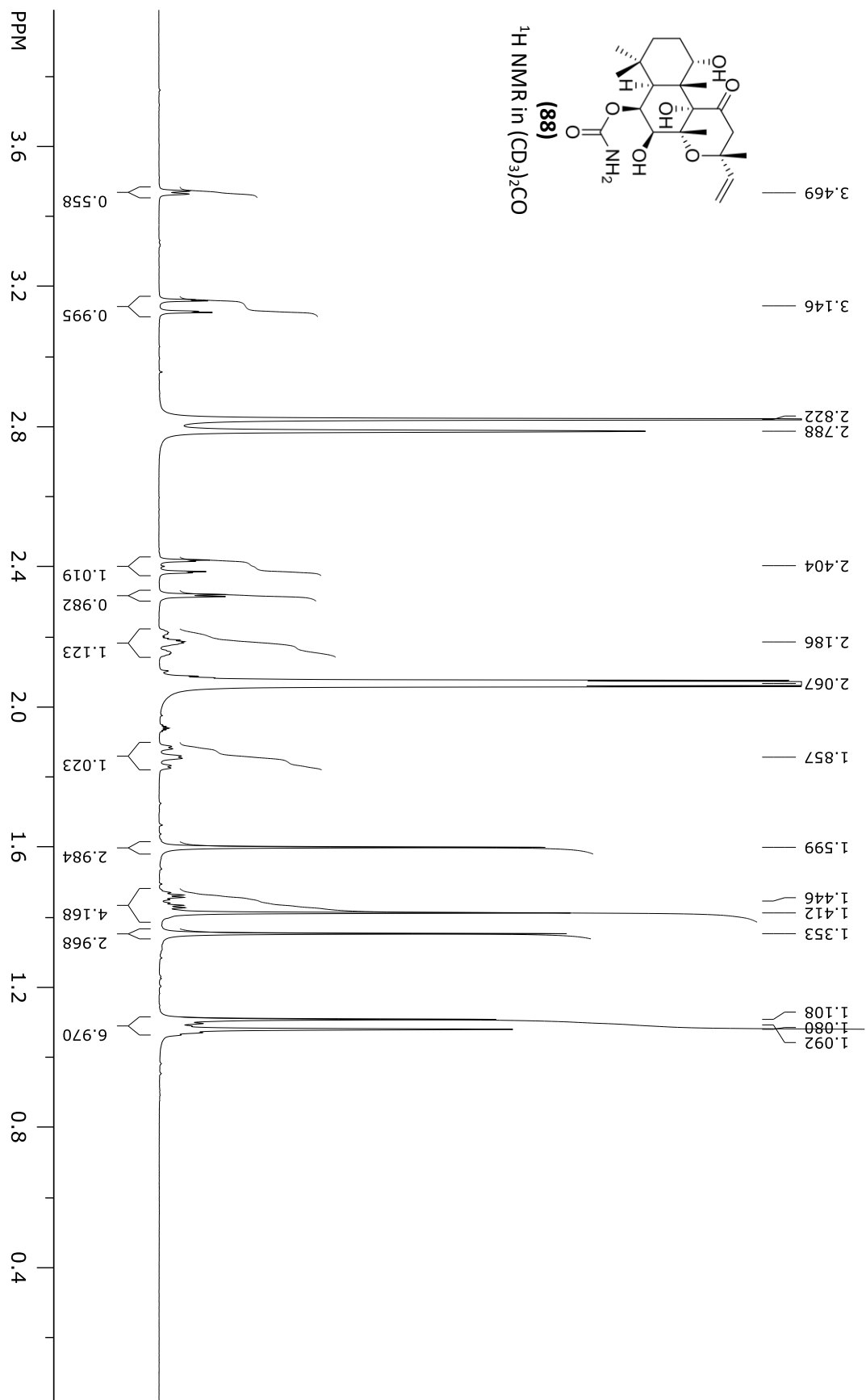


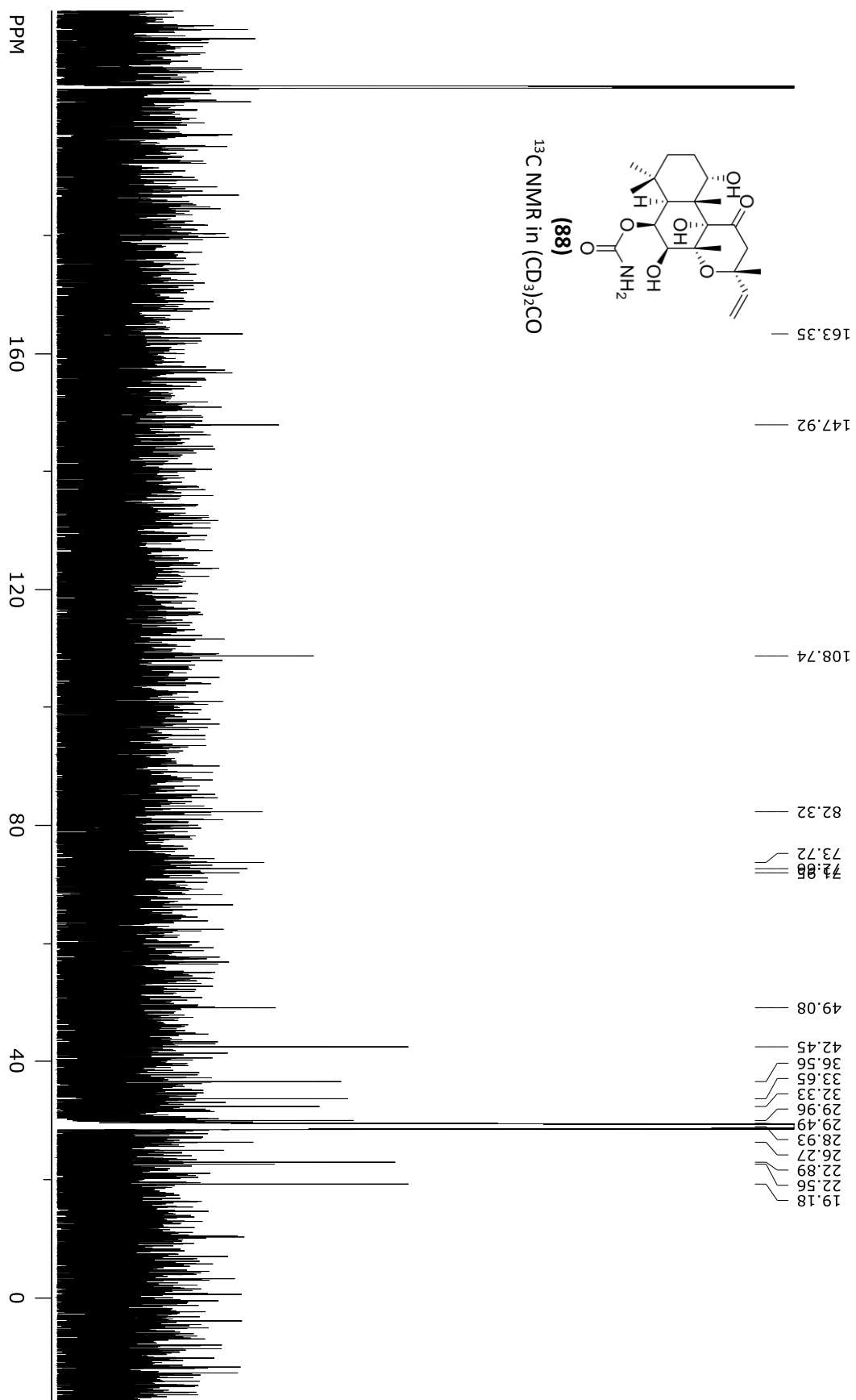


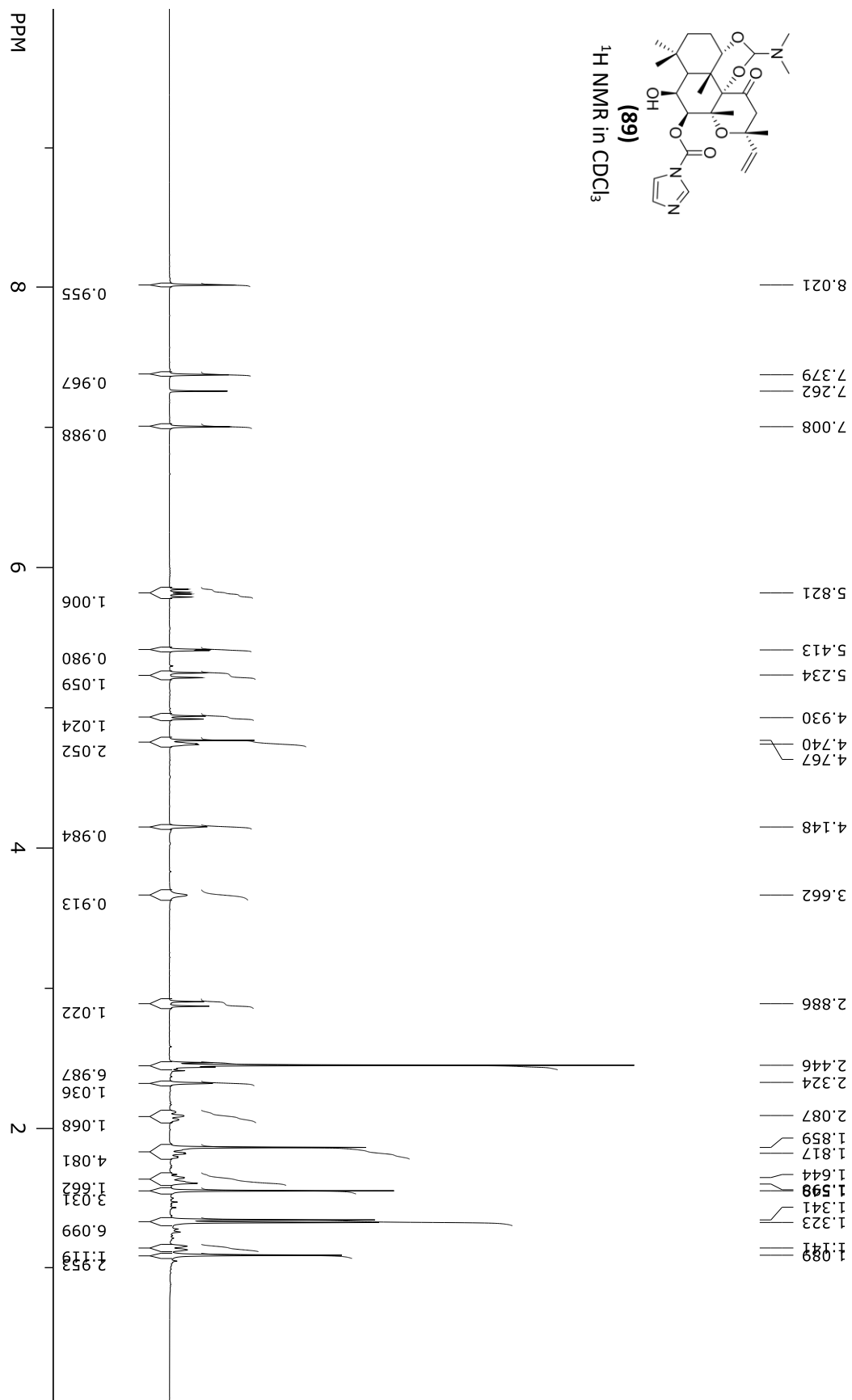


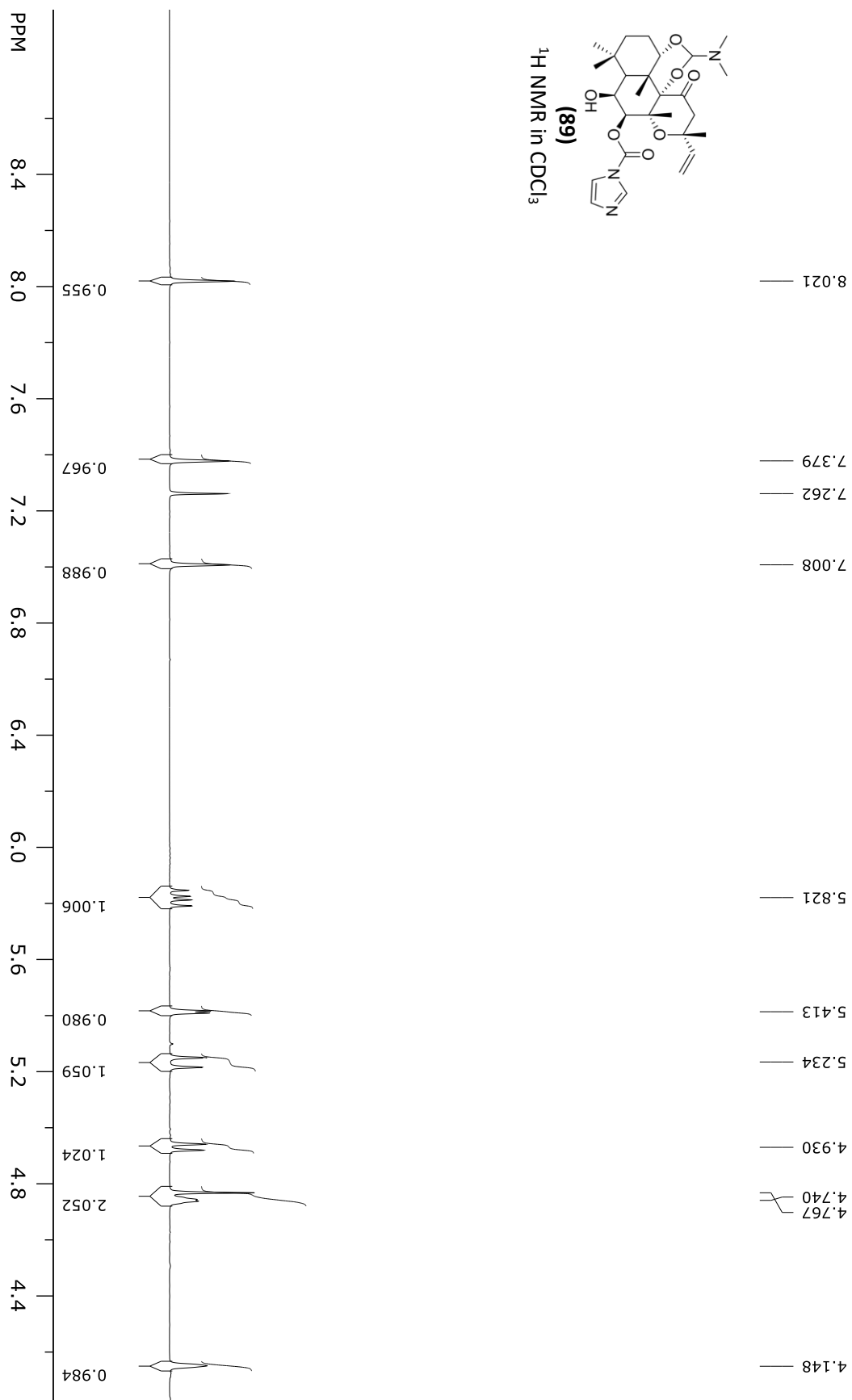
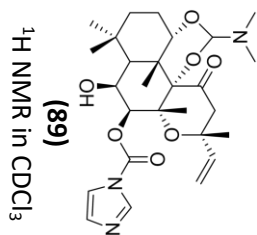


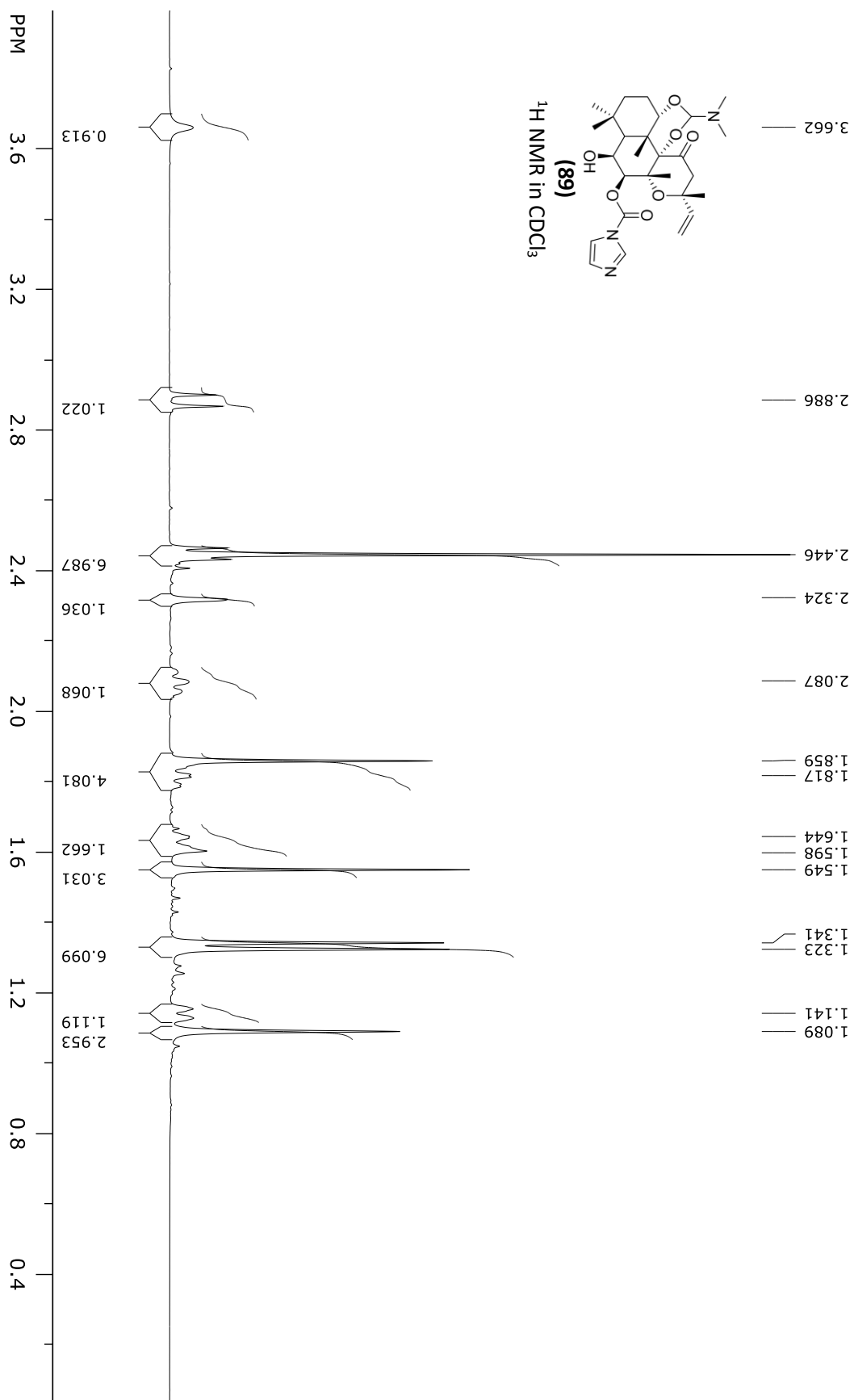


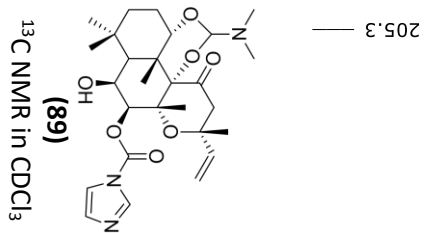


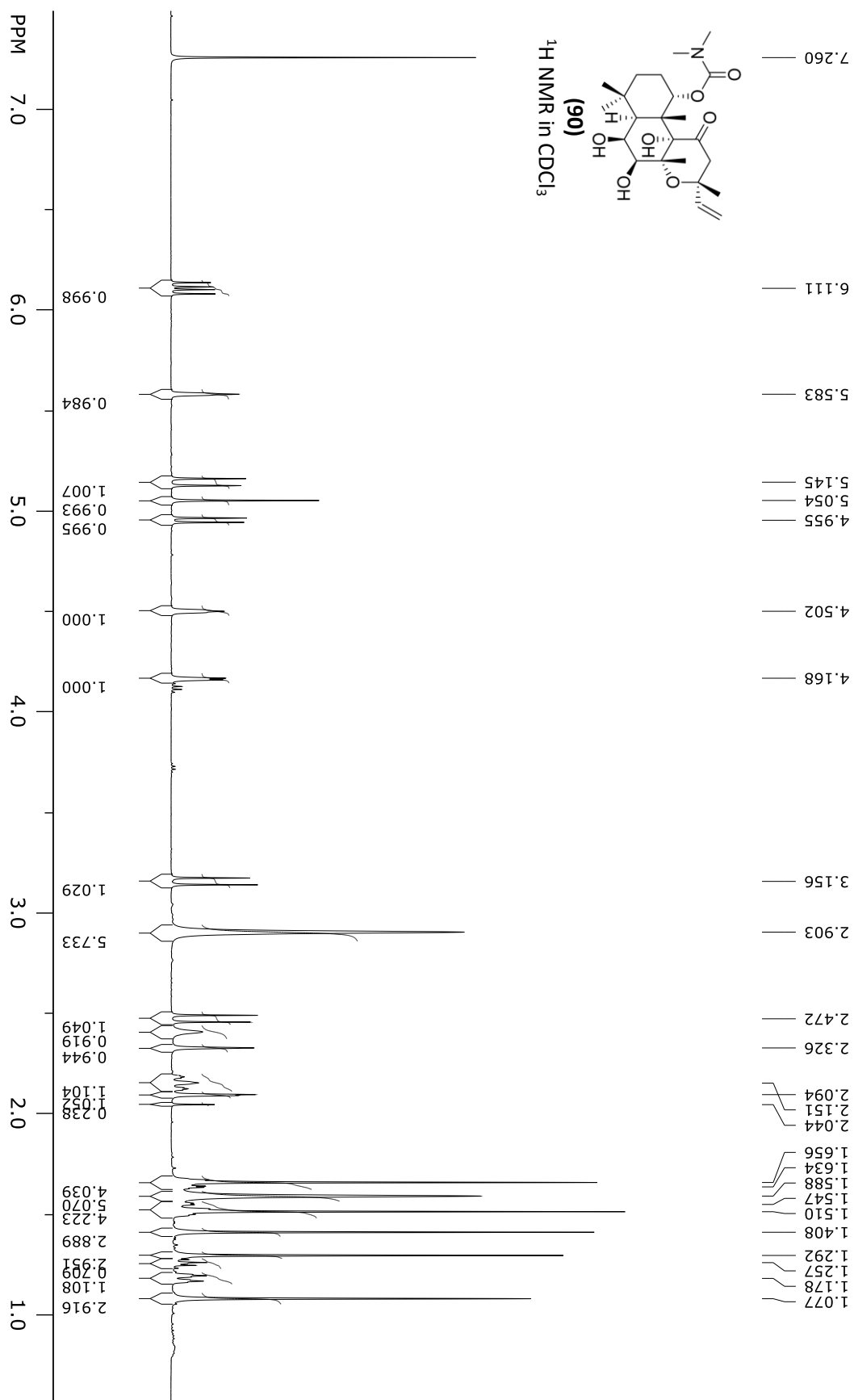


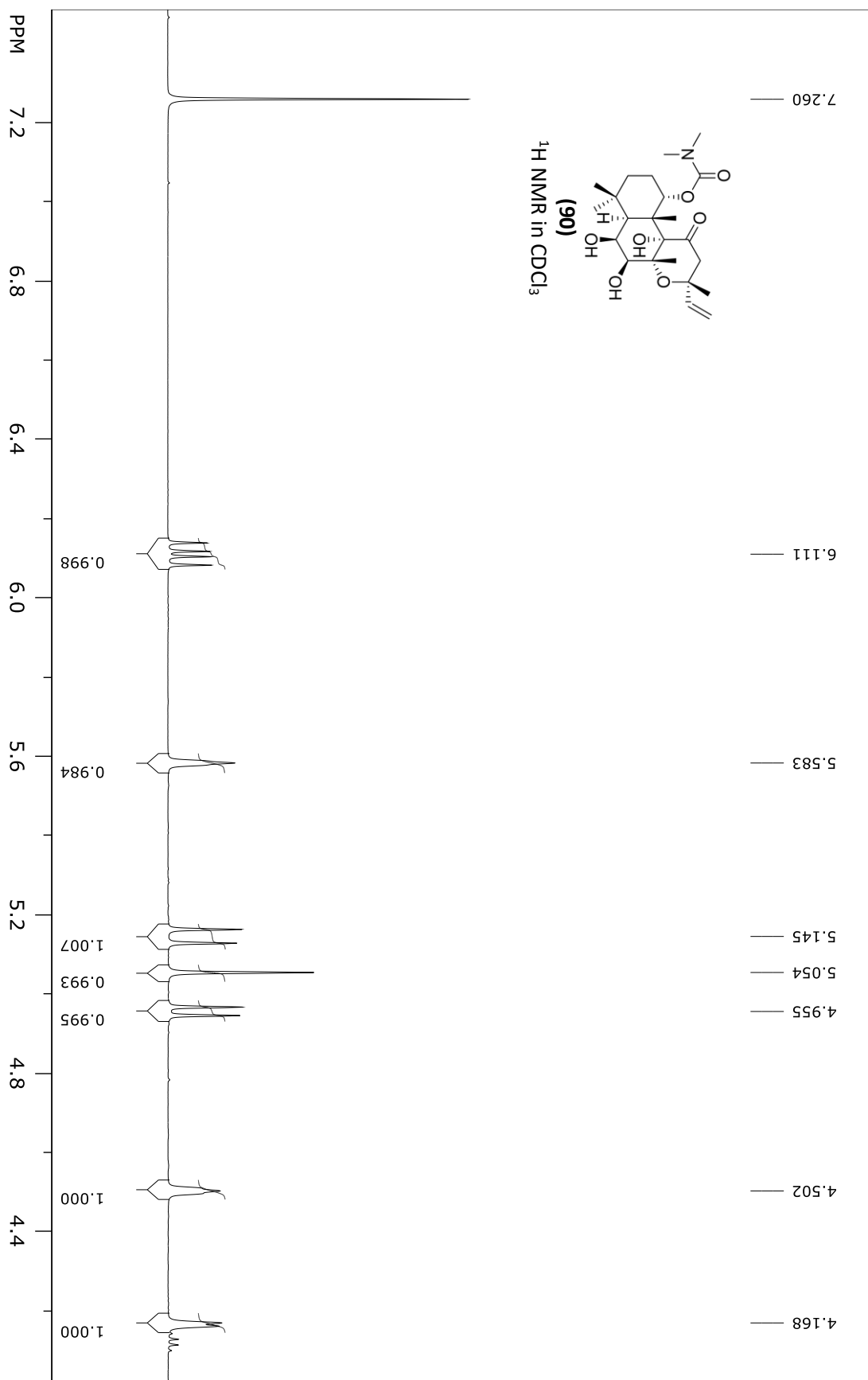


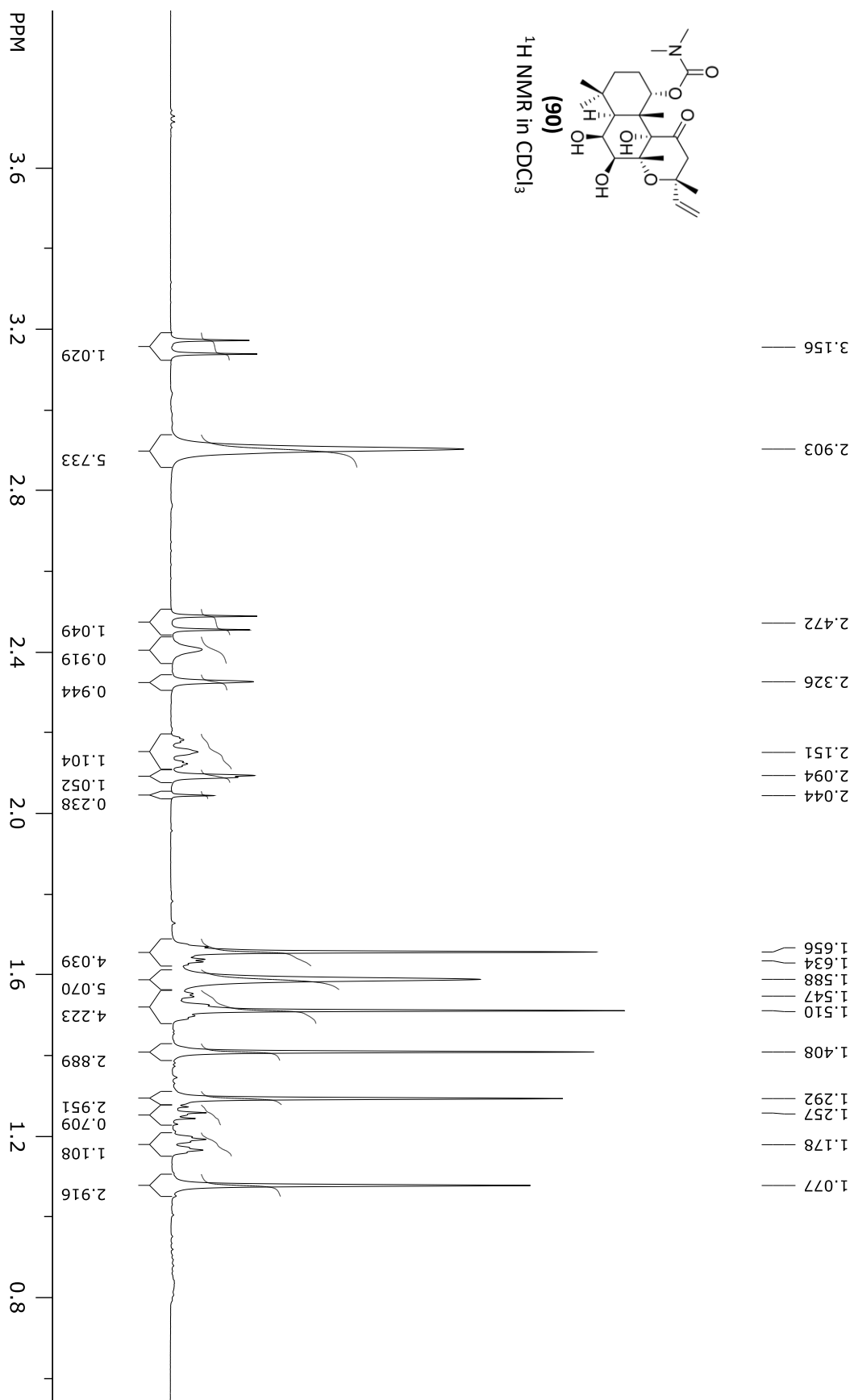


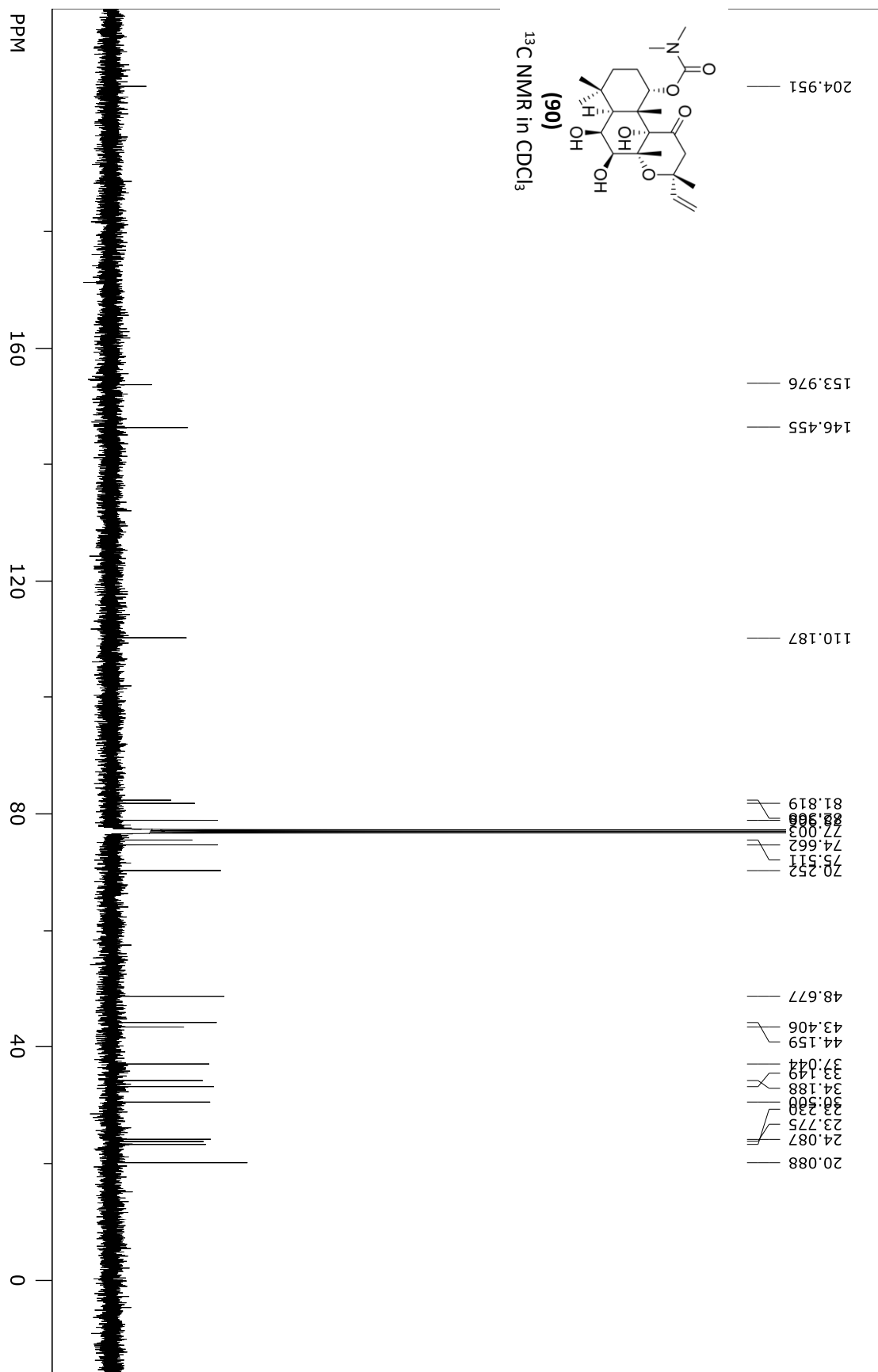


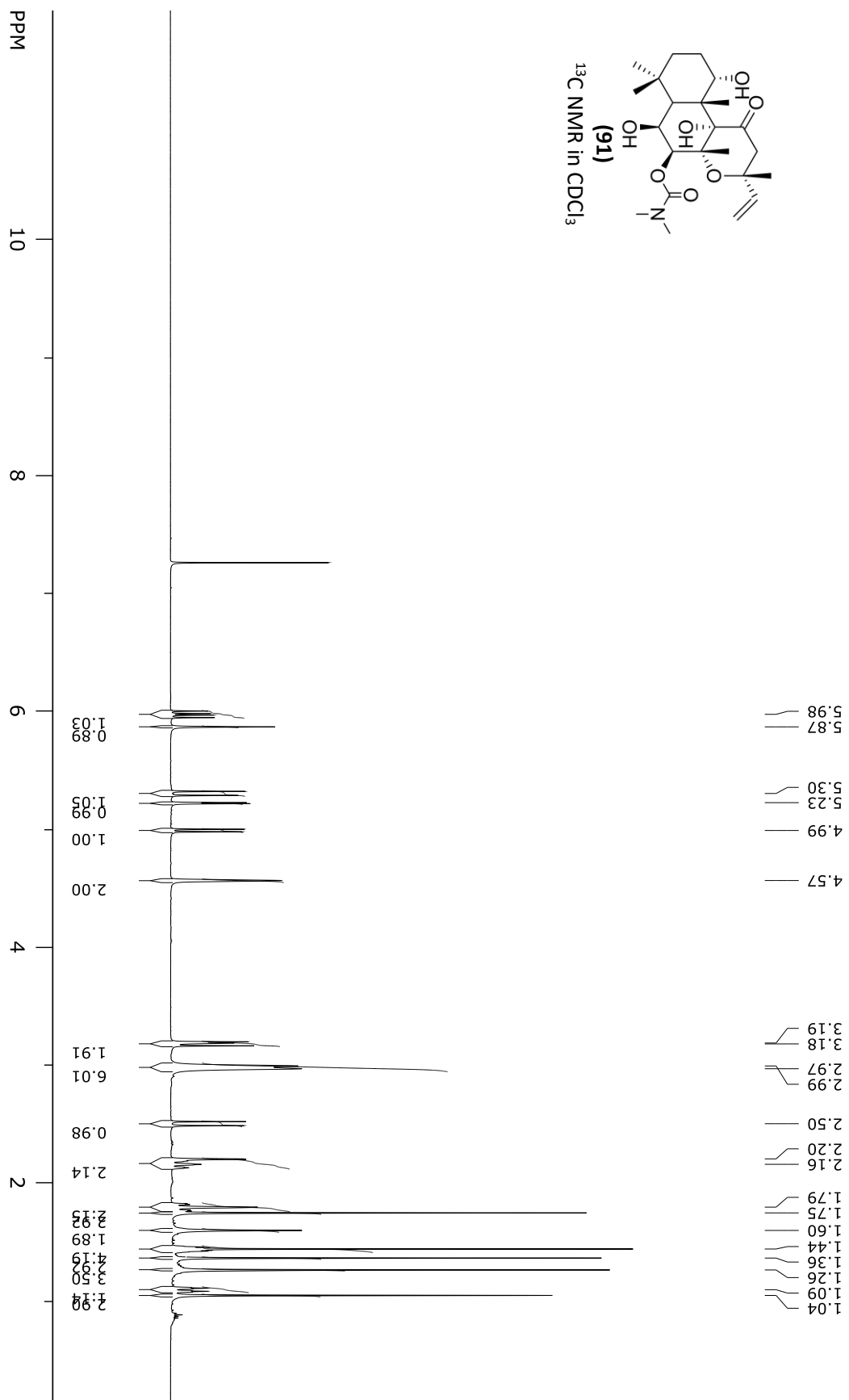


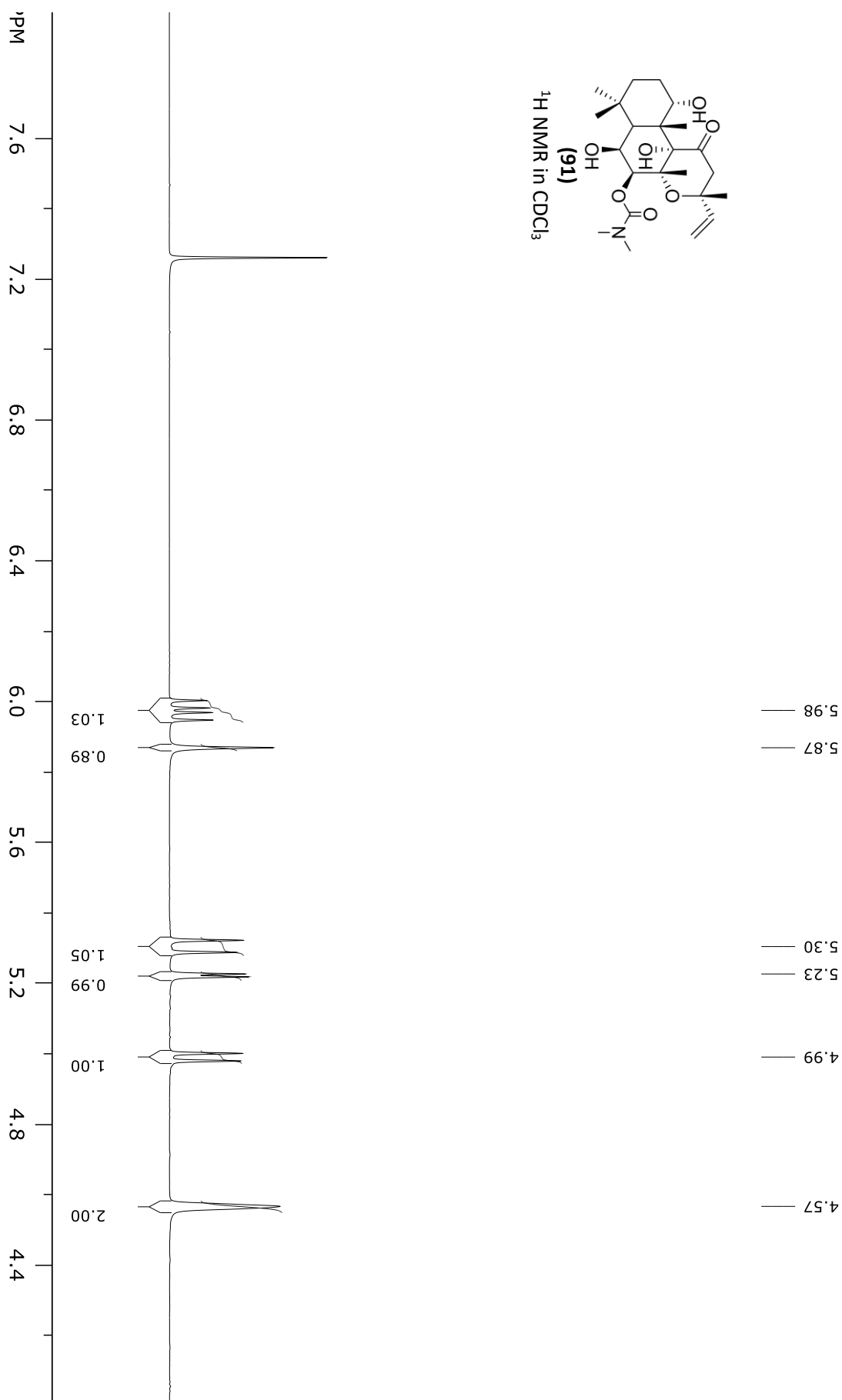
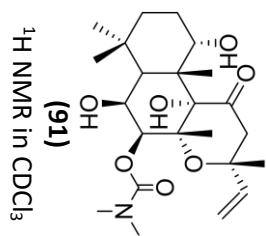


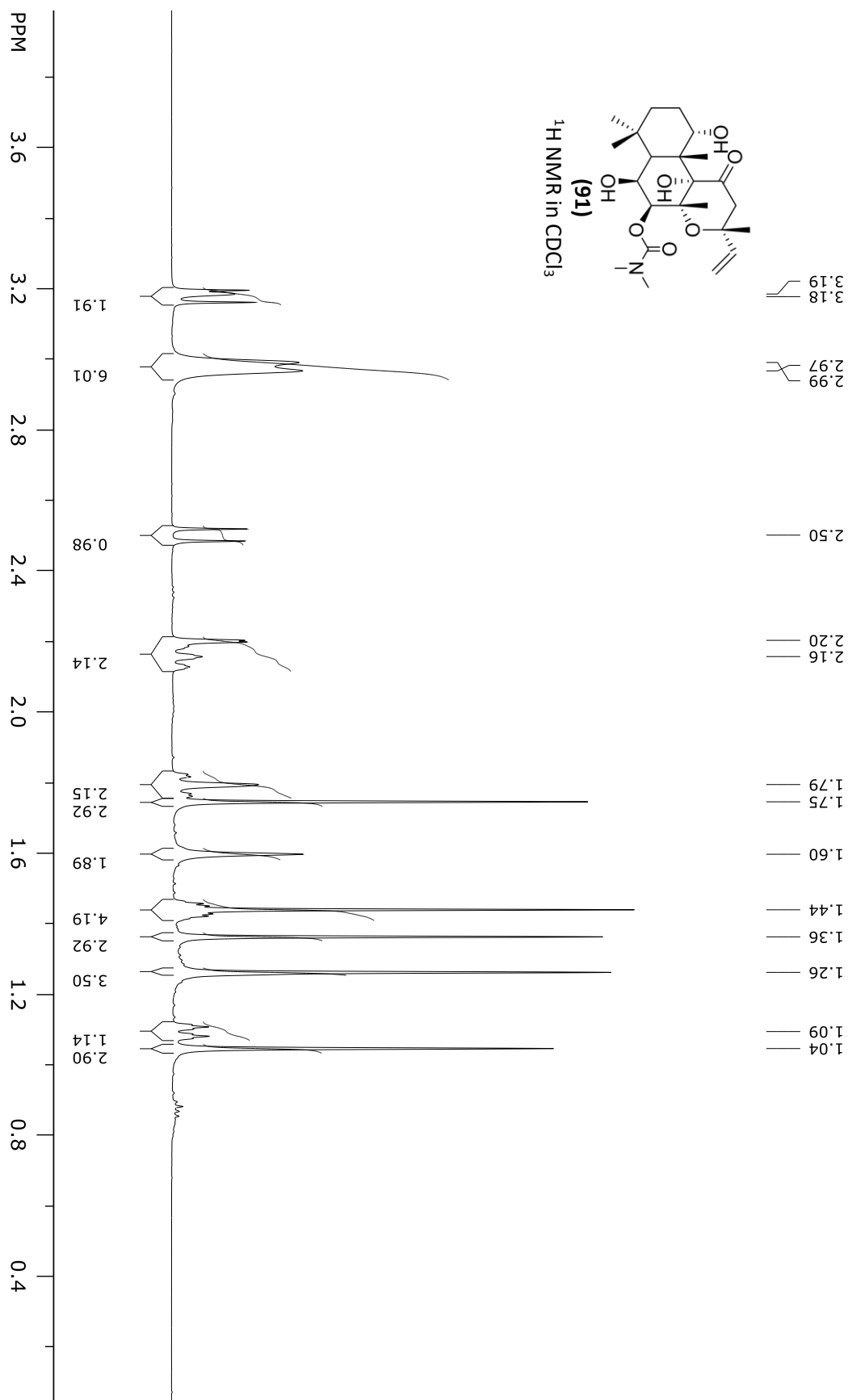


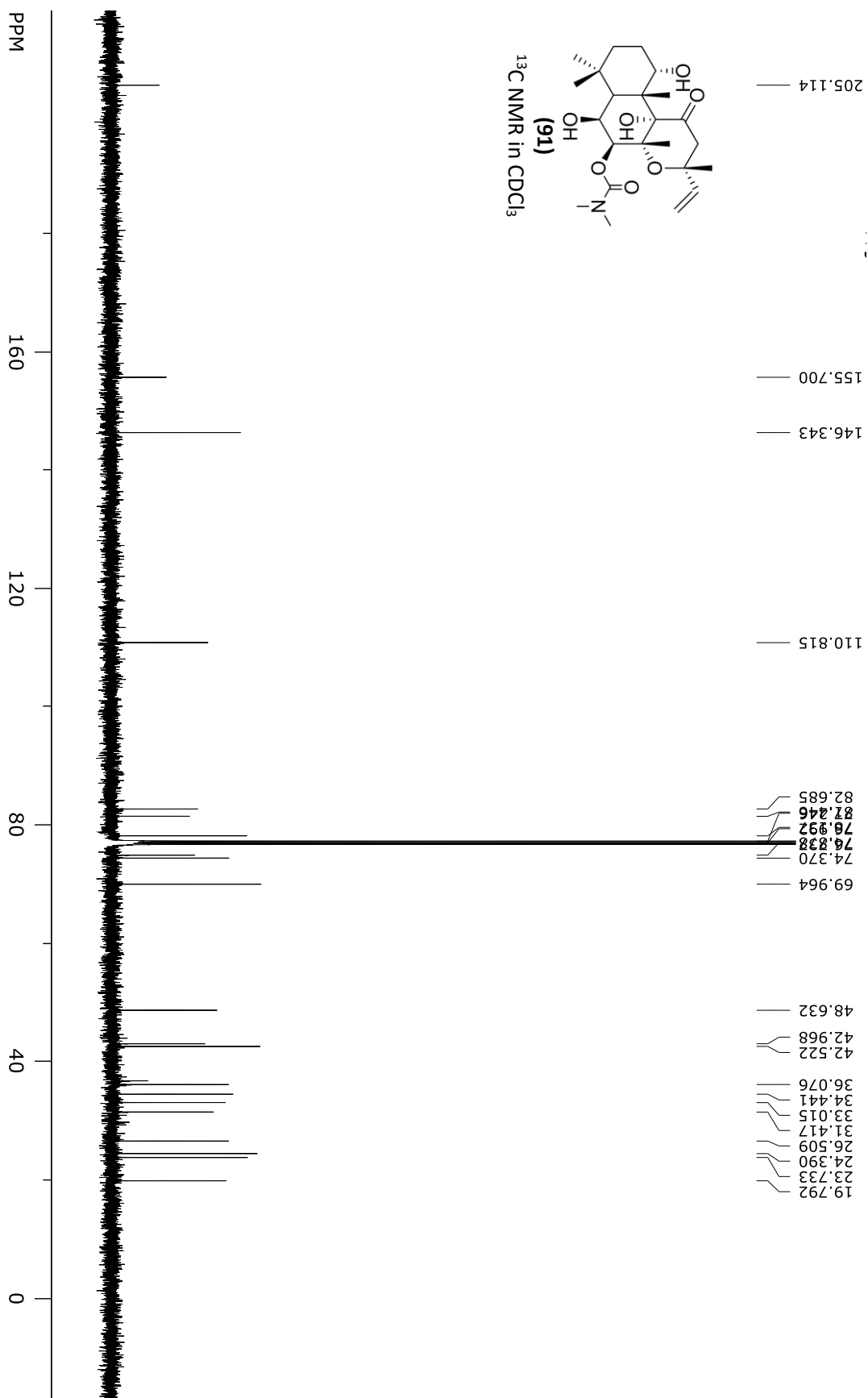


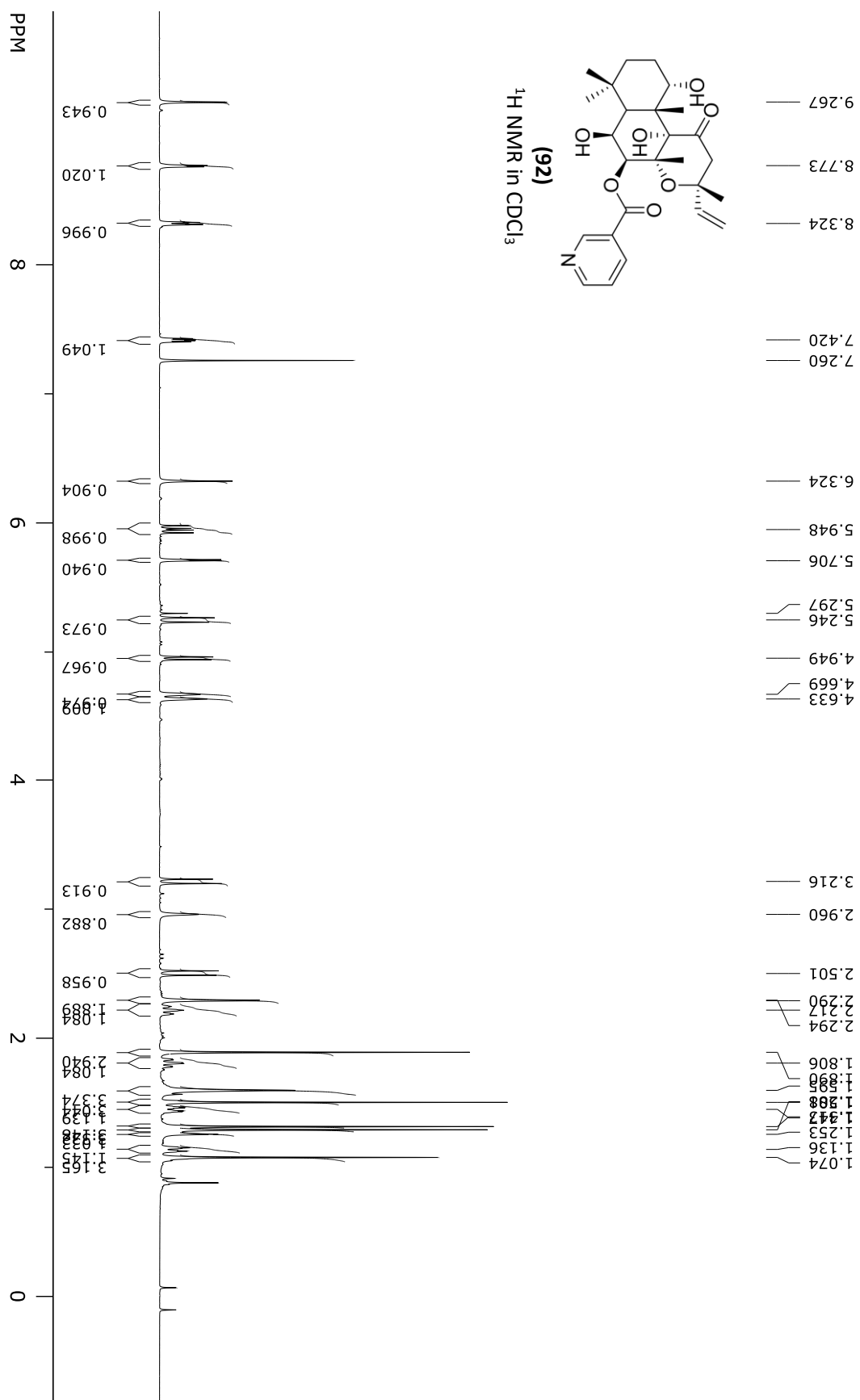


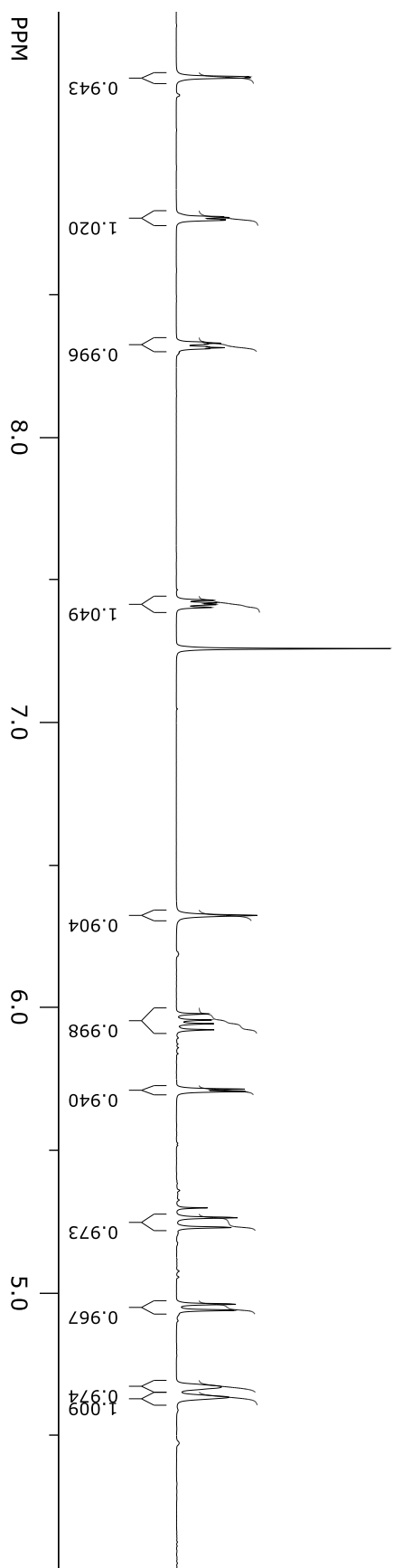
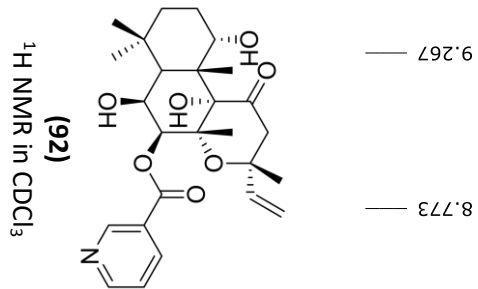


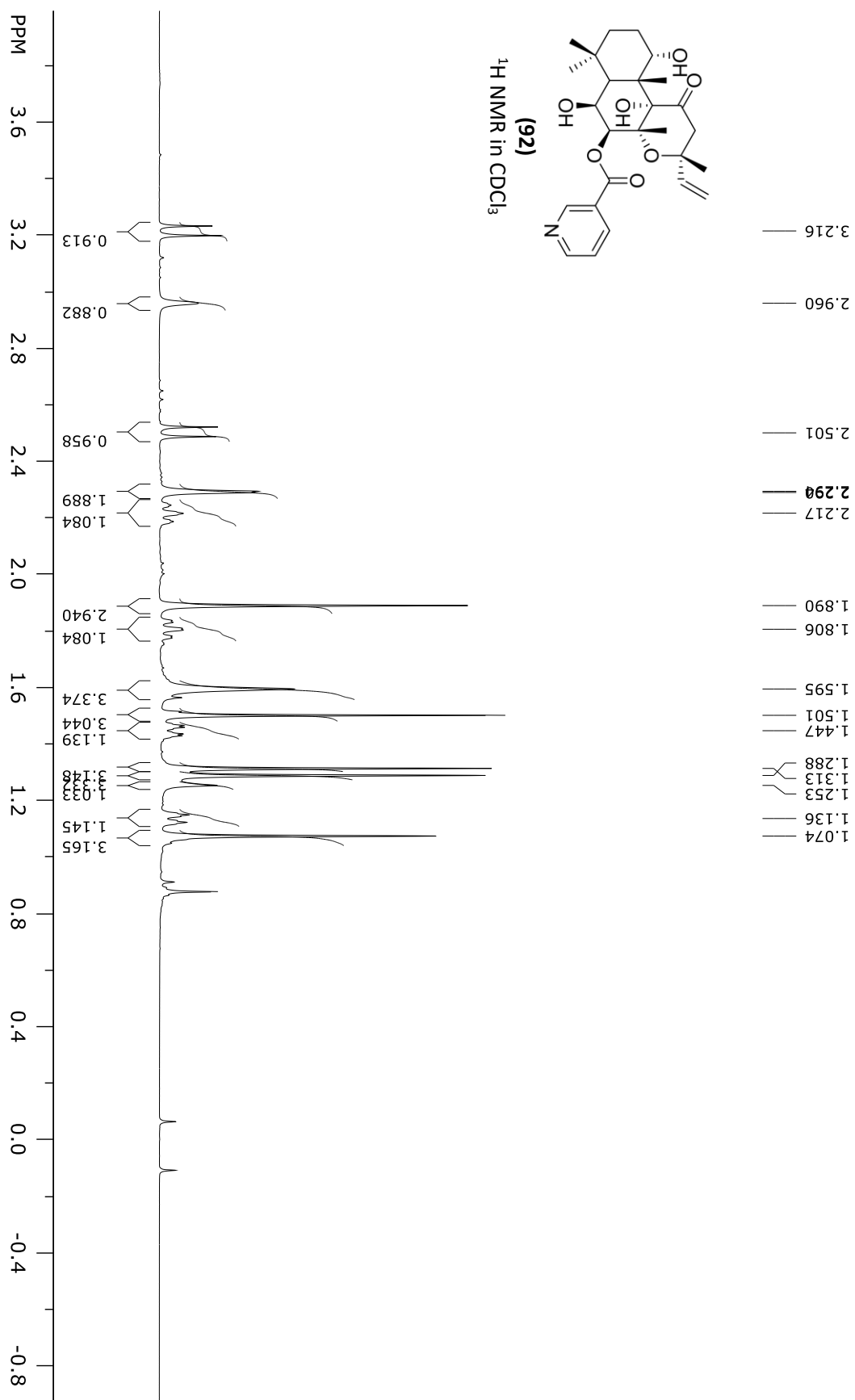


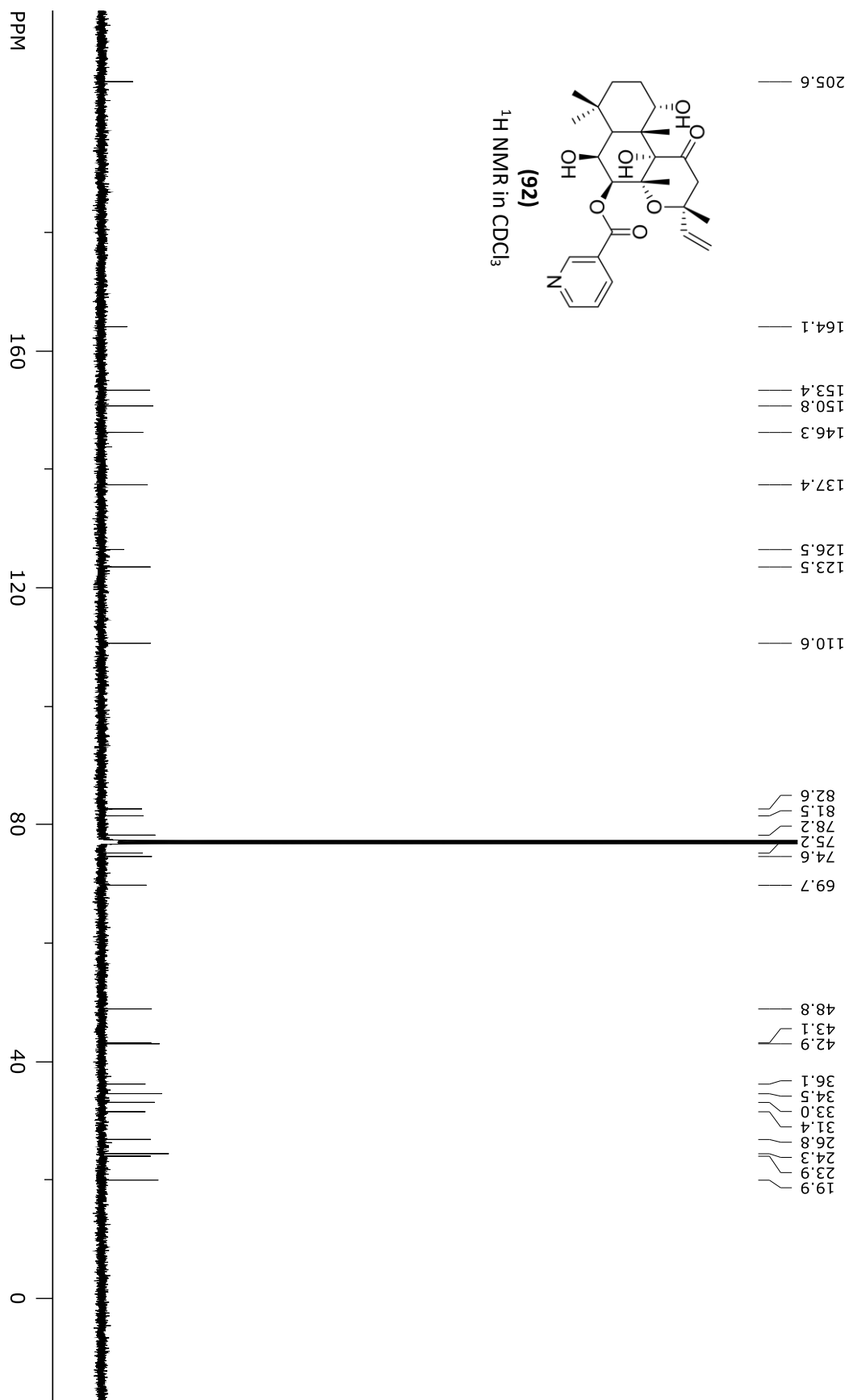












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