Design, Synthesis and Antitumor Properties of Glycosylated Antitumor Ether Lipid (GAEL) – Hybrids

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To my cute little niece, Bliss Temitope Adedeji, that came just at the nick of time.

ABSTRACT

The development of resistance to apoptotic pathways by cancer cells is one of the great impediments to the successful use of chemotherapeutic agents. The development of resistance may be attributed to the role of cancer stem cells in the progression of this disease. One approach to combat drug resistance involves the use of drug combinations that impact multiple targets simultaneously. Although this is believed to be better at controlling complex disease systems, it has often proven to be of limited benefits in terms of overall therapeutic outcomes in cancer treatment. Glycosylated antitumor ether lipids (GAELs) are an emerging class of novel anticancer molecules that is being investigated as potential anticancer drugs. Interest in this class of drug is based on their ability to kill cancer stem cells as well as their non-apoptotic mechanism of action. This provides new opportunities to manipulate cell death in a therapeutic context, especially the renewed ability to kill apoptosis-resistant cancer cells. We therefore hypothesized that hybrid molecules that combine apoptosis-dependent and apoptosis-independent mode of actions in a single molecule may lead to better therapeutic outcomes. We also posited that the amphiphilic nature of GAELs could be modulated and fine-tuned to give a more potent analog.

This dissertation describes the antitumor activities of different analogs of GAEL–chlorambucil hybrids and different triamino analogs. In all, eleven GAEL analogs were synthesized. Their activities, as well as that of reference compounds, were assessed against breast (JIMT1, MDA-MB-231, BT474), pancreas (MiaPaCa2) and prostate (DU145, PC3) cancer cell lines using the MTS assay. Our results reveal that the hybrid concept is a potential viable avenue to pursue as a therapeutic option especially when the other domain is carefully selected (Chapter 3). Moreover, the evidently more potent triamino analog not only corroborate the plausibility of a hybrid drug, it also revealed an important detail about the amphiphilic–cytotoxic relationships of GAELs (Chapter 4).

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Finally, all glory to the immortal, invisible and only wise God. Nothing compares to you.

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Ac_2O	acetic anhydride
AEL	antitumor ether lipid
AgOTf	silver trifluoromethanesulfonate (silver triflate)
$BF_3{\cdot}Et_2O$	boron triflouride diethylether
Boc	*tert*-butyl carbonyl
Boc_2O	di-*tert*-butyl dicarbonate (boc anhydride)
$CDCl_3$	deuterated chloroform
COSY	correlation spectroscopy
DCM	dichloromethane
DEPT	distortionless enhancement by polarization transfer
DIPEA	diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMF	*N, N*-dimethylformamide
DNA	deoxyribonucleic acid
ESI-MS	electrospray ionization mass spectrometry
Et_3N	triethylamine
GAEL	glycosylated antitumor ether lipid
GDG	glucosamine-derived glycerolipid
HSQC	heteronuclear single quantum coherence
IL	interleukin
J	NMR coupling constant (in hertz)

MALDI	matrix assisted laser desorption ionization
MeOD	deuterated methanol
MeOH	methanol
NAD	nicotinamide adenine dinucleotide
NaN_3	sodium azide
NaOH	sodium hydroxide
NaOMe	sodium methoxide
NIS	*N*-iodosuccinimide
NMR	nuclear magnetic resonance
p-TsCl	para toluenesulfonylchloride
PCC	pyridinium chlorochromate
PhSH	thiophenol
PMe_3	trimethylphosphine
ppm	parts per million
RT	room temperature
TBTU	*O*-(1H-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate
TFA	trifluoroacetic acid
WHO	world health organization
δ	chemical shift in parts per million

CHAPTER ONE: INTRODUCTION AND BACKGROUND

1 INTRODUCTION

The dynamics of human physiology and the interplay of innumerable complex processes involved in our day-to-day activities continue to provoke the drive towards understanding exactly how the human body works. These processes start from the point of embryonic fusion of zygotes, through various stages of organ differentiations, and till death. Basically, the human body is made up of bundle of cells, which are the basic and structural unit of life, and are constantly dividing via mitosis or meiosis to repopulate themselves. This cell division is as a result of growth and/or specialized cell differentiation that give rise to various tissues, organs, etc. The body therefore needs to carry out these tasks in a controlled manner such that it produces more cells when needed (to keep the body fully functional), and replaces damaged or dead cells with new ones. The complex feed-in and feedback mechanisms that co-ordinate these orderly processes require very high precision. Occasionally, things go wrong with these control mechanisms, where cells die when they should not and new ones form when not needed. These extra cells may sometimes form a mass within the tissues known as a tumor. Tumors can be classified based on type, nature and site of development or aggregation. Benign tumors are mostly non-proliferative and non-invasive, and may not require urgent clinical interventions except where they exert pressure on vital tissues or organs. On the other hand, some tumors may proliferate rapidly, are invasive and metastasize to distant sites. These are referred to as malignant tumors and are of immediate medical concern. The term ‘malignant tumor’ and ‘cancer’ are sometimes used interchangeably in clinical settings. It should be noted however that some cancers do not form tumors e.g. leukemia (cancer of the blood and bone marrow).

During cell division, a distorted and/or impaired control process may result in an uneven differentiation of a parent cell, thereby giving rise to entirely different daughter cells. These distortions may be amplified and transferred to subsequent generations, culminating into

permanently mutated cells with new genetic information. Mutations may occur entirely by chance as an error in DNA replication when cells are dividing, although many changes are needed in order to make a cell malignant. Mutated cells may self-destruct or they may be recognized as abnormal by the immune system and destroyed.[1] All of these mechanisms mean that it is not easy for a normal cell to become a cancer cell and most pre-cancerous cells die before they become cancerous. It also means that once a cell becomes cancerous, it is often difficult and nearly impossible to revert to a normal cell, as they now possess different genetic information. This leads to loss of growth coordination and confers the ability to evade death from the immune system on them.

Cancer can therefore be defined as a class of disease in which a group of cells exhibit uncontrollable growth of abnormal (mutated) cells, invade neighboring tissues or organs, and sometimes metastasize. It is a leading cause of death worldwide with the number of new cases, which stood at about 14 million in 2012, expected to rise by 70 % over the next two decades, if left unchecked.[2] These numbers are quite worrisome especially when reconciled with the amount of resources invested in the search of a holistic cancer treatment over the years. Although the exact cause of this disease remain largely unknown, various risk factors such as smoking, alcohol, obesity, genetics, physical inactivity, carcinogens, radiation, etc. have been identified to possibly trigger these cascade of events.[3]

1.1 Management of Cancer

Classical surgery, radiotherapy and chemotherapy are, without doubt, the leading efficient therapeutic options for the treatment of cancer. Before chemotherapeutics were developed in the 1940s, surgical removal of tumor tissue was the only available treatment, and is still being used for removal of localized cancers.[4] Radiation therapy is widely used to target cancer cells by directly damaging their DNA.[5] However, the ability of several cancer types to rapidly advance, reoccur or metastasize makes these two methods less desirable, thus giving chemotherapy an edge as the preferred choice of treatment. Notwithstanding the type of treatment employed, chemotherapy remains an integral part of cancer management plans. The use of chemotherapeutic drugs, whether alone or in combination with other modalities of cancer treatment, is a mainstay in the management of many forms of cancer, especially advanced and/or metastatic ones. They are classified into six major groups (Table 1-1), according to WHO (L01 class by ATC criteria).[6] These drugs kill cancer cells either by disrupting/destroying the DNA, inhibiting the synthesis of new DNA, targeting microtubules so that the cells stop dividing, fragmentation of the DNA strands or starving the cells of hormones needed for growth.[7] Other classes of anticancer agents include hormonal therapy (steroids, anti-estrogens, anti-androgens, luteinizing hormone-releasing hormone analogs, anti-aromatase agents) and immunotherapy (interferon, interleukin 2, vaccines).[8] More recently, antibody-based drugs (biological) such as trastuzumab (Herceptin) that target aberrant receptors have also been used to down-regulate growth signals and inhibit tumor growth.[9] Other antibodies often characterized as *'naked antibodies'* mediate anti-oncogenic activities by activation of the host's immune system, thus triggering the activation of signaling pathways that control tumor growth and results into apoptosis.[10]

	Class	Examples
1	**Platinum coordinating complex**	• Cisplatin • Carboplatin • Oxaliplatin
2	**Antimicrotubule agents**	• Vinca alkaloids (vinblastine, vinorelbine) • Taxanes (paclitaxel, docetaxel)
3	**Antimetabolites**	• Pyrimidine antimetabolites (5-fluorouracil, capecitabine, cytarabine) • Purine antimetabolites (mercaptopurine, fludarabine) • Dihydrofolate reductase inhibitors (methotrexate) • Thymidilate synthase inhibitors • Adenosine deaminase inhibitors • Ribonucleotide reductase inhibitors
4	**Antiumor antibiotics**	• Actinomycin D • Mitomycin C • Bleomycin • Anthracyclines (doxorubicin, daunorubicin) • Podophyllotoxins (etoposide, teniposide) • Camptothecins (irinotecan, topotecan)
5	**Alkylating agents**	• Cyclophosphamide • Mustard-based agents (chlorambucil, mephalan) • Nitrosoureas (carmustine) • Alkane sulfonates (busulfan)
6	Others include drugs that do not fall into any of these categories	

Table 1-1: Classes of chemotherapeutic agents

1.1.1 Shortcomings of current chemotherapeutic agents

The goal of cancer treatment is usually to get rid of cancer cells while preserving the integrity and functioning of normal ones. Most anticancer agents do not or are unable to discriminate between cancer cells and normal cells, resulting in severe side effects. Also, since many of these drugs interact with DNA, they are usually mutagenic and can lead to secondary malignancies.[11] These limitations bedeviling chemotherapeutic agents have threatened the continuous use of some drugs while the deployment of others have been severely restricted.[12,13] Furthermore, most anticancer drugs are clinically ineffective in some progressive tumors and highly advanced stages of carcinoma. In fact, some cancer types such as brain, pancreatic and ovarian cancers do not have any specific treatment *per se*. Although our inability to detect cancer at its early stages is believed to be responsible for a large proportion of death, it is by no means the sole reason for the mortality associated with cancer. The lack of efficacy of cancer-treating drugs is also a major factor in the high mortality rates. This is largely due to the acquisition of drug resistance that encompasses entire classes of drugs by the cancer cells. One major mechanism of resistance to anticancer agents is via the acquisition of an active efflux system that pumps out drugs as soon as they are transported into the cells, thus rendering them ineffective. This is achieved by an increased expression of multidrug resistant (MDR) proteins, *P*-glycoproteins, of the ATP-binding cassette transporter family by cancer cells.[14,15] Other mechanisms of drug resistance include: decreased uptake or influx of drugs, active DNA repair mechanisms, drug inactivation, alterations in drug targets and evasion of apoptosis.[15,16]

More significantly is the issue of relapse, which is a re-occurring phenomenon that is often associated with cancer management. Chemotherapeutic agents tend to shrink the size of tumors to a point where they may become undetectable, but over time, the cancer reappears more aggressively and survives treatment with the previous and other drugs. A leading

hypothesis, the cancer stem cell (CSC) hypothesis, that seeks to explains this phenomenon, posits that tumors are initiated by progenitor or stem-like cells that have the ability to self-renew and also generate differentiated drug-resistant cells.[17] Whether CSCs originate from normal stem cells that retain self-renewal properties but acquire epigenetic and genetic changes required for tumorigenicity or whether tumor stem cells are proliferative progenitors that acquire self-renewal capacity remain a critical question to be answered. The CSC theory has indeed generated lots of controversies,[17–20] with leading experts in this field agreeing to a consensus definition of CSC as a cell within a tumor that possesses the capacity to self-renew and generate the heterogeneous lineages of cancer cells that comprise the tumor.[21] It is believed that the rarity of CSCs and their ability to enter the cell cycle infrequently, make them intrinsically resistant to chemotherapeutic actions that kill cells of the bulk tumor.[22] Following drug treatment, the surviving CSCs are thought to repopulate the tumor with differentiated cells that are refractory to treatment.[23–25] Unfortunately, only few drugs have been reported to kill CSCs.[26] Strategies to improve therapeutic outcome will have to target CSCs in order to effectively address issues of tumor relapse and drug resistance.

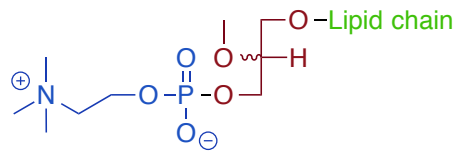
1.2 Antitumor Ether Lipids

Antitumor Ether Lipids (AELs), a class of drug that has been widely reported for their anticancer activities, is a broad term that describes a group of unnatural (synthetic) compounds developed to be long-lived analogs of lysophosphatidylcholine. The prototypic AELs were initially designed as a stable analog of the naturally occurring phospholipid, lysophosphatidylcholine, to be used for immunomodulatory activity studies in animals. AELs were however found to inhibit the biosynthesis of phosphatidylcholine, a major constituent of cell membrane, thus inducing apoptosis (programmed cell death).[27] The

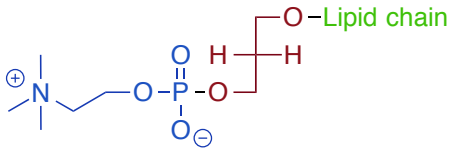
serendipitous discovery of their cytotoxic activity *in vitro* and *in vivo* opened up studies on their prospects as a new class of anticancer agent.[28,29] AELs are characterized by a distinct structural motif of ether bonds linked at *C*-1 and *C*-2 positions of a glycerol moiety and are generally classified into three groups: alkyllysophospholipids (ALPs), alkylphospholipids (APLs), and glycosylated antitumor ether lipids (GAELs) (**Figure 1-1**).

The ALPs are phospholipid-like compounds with a long alkyl chain at the *sn*-1 position, a short chain at the *sn*-2 position and a phosphobase at the *sn*-3 position of the glycerol. Interest in this class of drugs stems from their ability to deliver specific antitumor activity without the mutagenicity associated with conventional chemotherapeutic agents, since they do not interact with DNA.[28,29] Also, characteristics such as the ability to discriminate between normal and cancer cells, amphiphilicity, metabolic stability to hydrolases and acyl transferases, and cytotoxic activity independent of p53 status of cells, make them desirable as a potential class of anticancer drug.[30,31] Edelfosine (**Figure 1-1**), which represents the gold standard and prototype of this class of drug, has been extensively studied together with other analogs modified at the *sn*-2 position of the glycerol.[32] On the other hand, APLs, which represents the second class of AELs, do not have glycerol backbone. Instead, the alkyl group is directly esterified to the phosphobase as exemplified by hexadecylphosphocholine (miltefosine, **Figure 1-1**), an approved molecule currently in used for the topical treatment of skin metastasis in breast cancer.[33] Miltefosine and several other APL analogs have been shown to induce apoptosis via the perturbation of several processes involved in cell growth and survival.[33] The third class of AELs, the GAELs, differs from the other two groups in that the phosphobase is replaced with a sugar moiety at the *sn*-3 position of the glycerol backbone. Surprisingly, they were discovered to possess a distinct mode of action that is independent of apoptosis, as opposed to the other two groups that induce apoptosis.[34] This discovery gave renewed momentum to pursue the development of

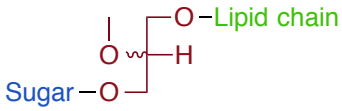
this group of drugs as possible alternative to existing apoptosis-inducing chemotherapeutic agents.



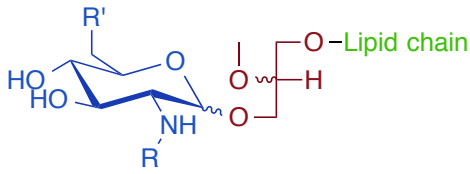
Alkyllysophospholipids (ALPs)



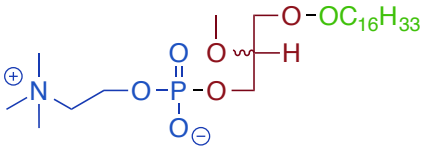
Alkylphospholipids (APLs)



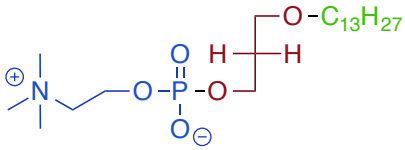
Glycosylated antitumor ether lipids (GAELs)



Glucosamine-derived glycerolipids (GDGs)



Edelfosine



Miltefosine

Figure 1-1: Generic structures of subclasses of antitumor ether lipids (AELs)

1.3 Glycosylated Antitumor Ether Lipids

Glycosylated antitumor ether lipid (GAEL) is a broad and vague subclass of AELs that encompasses a number of AEL compounds that have a sugar moiety. It includes phosphorus-GAELs (glycosyl-containing phospholipid ethers) such as glucosamine-PAF,[35] and non-phosphorus GAELs in which the sugar moiety replaces the phosphobase moiety (**Figure 1-2**). The non-phosphorus GAEL can further be grouped according to the types and nature of sugars substituted for the phosphocholine base. Their anticancer activities have been established and reported.[18] Despite these widely known cytostatic and cytotoxic

activities, development of GAELs as potential anticancer agents has seriously lagged behind the ALPs, perhaps, due to the mediocre cytotoxic activities exhibited by early analogs, relative to edelfosine.[38] Moreover, with the initial assumption that all AELs possess similar mechanism of actions, it was only logical to pursue the most promising of this category (edelfosine, ALPs). The discovery that GAELs mediate cytotoxicity via a novel non-apoptotic mechanism,[34] injected new enthusiasm towards developing clinically relevant analogs. The synthesis of a glucosamine-derived glycerolipid (GDG) analog, in which the sugar moiety bears an amino substituent at the *C*-2 position [1-*O*-hexadecyl-2-*O*-methyl-3-*O*-(2'-amino-2'-deoxy-β-D-glucopyranosyl)-*sn*-glycerol, **1**] (**Figure 1-2**), revolutionized the GAELs as it was found to display better cytotoxic activities compared to previous analogs as well as the much-celebrated edelfosine.[39] Mechanistic studies demonstrated that GAELs kill cells via an apoptosis-independent mechanism which may involve perturbation of the endocytic pathway and release of cathepsins to mediate cell death independent of apoptosis.[34,40,41] Despite the initial development of GAELs being staggered and non-systematic, the identification of glucosamine-derived non-phosphorus analog **1** and their recent demonstration of cytotoxicity against breast cancer stem cells,[42] have provided the needed impetus to unravel the pharmacophore responsible for their cytotoxicity.

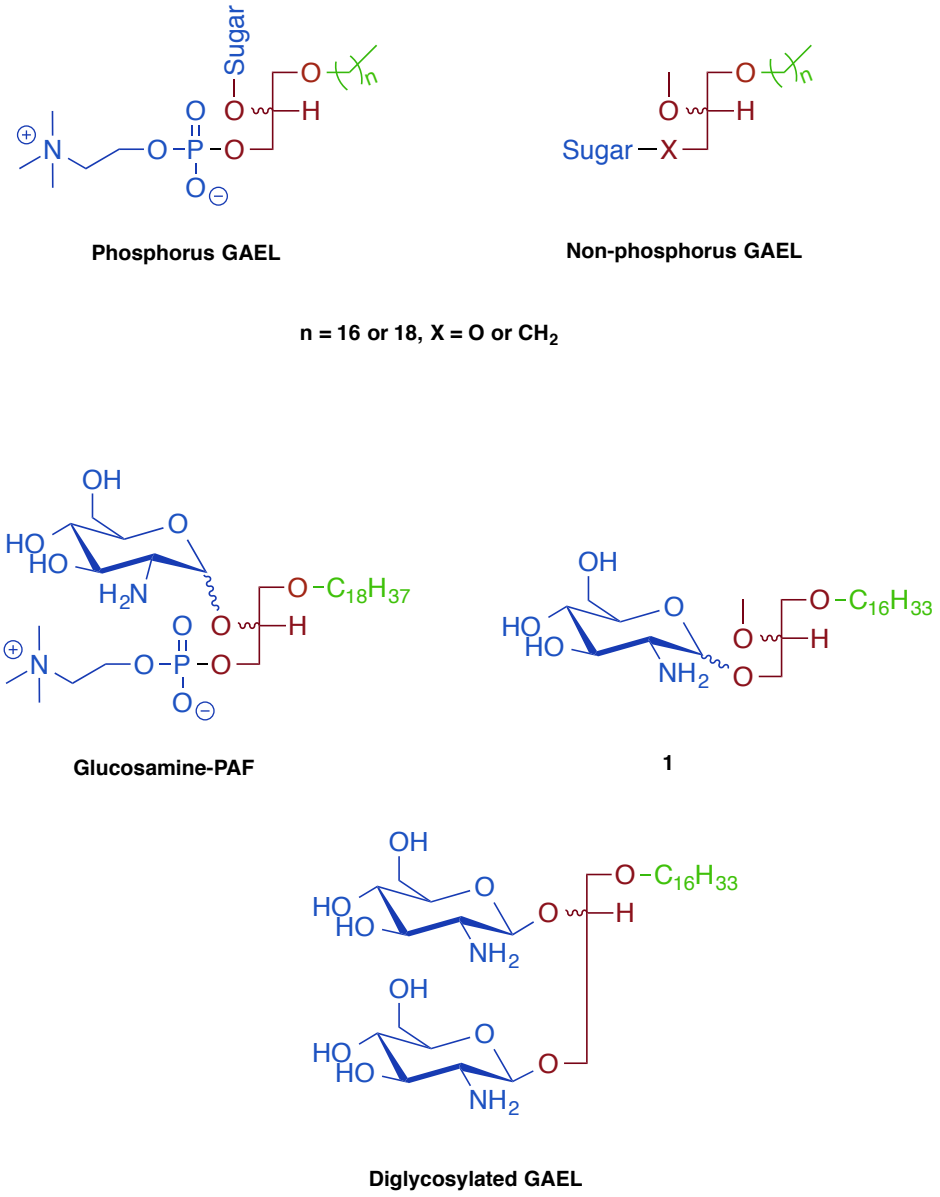


Figure 1-2: Types of GAELs and examples

1.3.1 Structure-Activity Relationships (SAR)

The synthesis of **1**,[39] which has become the prototype and most studied analog of the non-phosphorus GAELs, gave a good lead for SAR studies. Replacing various parts of the lead compound with other classical isosteres and substitutions with new moieties have been thoroughly investigated (**Figure 1-3**). For example, the role of the free NH_2 group at the *C*-2 position of glucose was investigated by *N*-methylation, conversion to an azido group as

well as a phthalimide group. In all instances, there were significant loss of activity relative to the reference compound **1**, with CC_{50} increasing by more than three fold.[39,43] This suggests that the cationic nature of the free amine might be crucial for activity. CC_{50} value is the concentration required to decrease cell viability by 50 % relative to the untreated control (set at 100 %). To further demonstrate how the nature of the cationic moiety at *C*-2 position might affect activity, the free amino group was benzylated and also guanidinylated. It was projected that the lipophilic effect of the benzyl group might enhance activity while the enhanced basicity of guanidine was expected to improve its antitumor activities. Contrary to these hypotheses, there was significant drop in activity, with the guanidinylated analogs not displaying any antitumor activity at the highest concentration tested (> 30 μM).[41,43] This again reinforces the indispensability of a free amino substituent for overall cytotoxicity. Furthermore, the nature of the glycosidic linkage of GAELs was examined. As opposed to ALPs that are desirably metabolically stable, GAELs, like other glycosidic drugs, may not be stable *in vivo*. This is due to the abundance of glycosidases in humans that readily cleave glycosidic bonds. Several approaches have often been used to address this challenge such as replacing the *O*-glycosidic bonds with metabolically stable *S*-, *N*- or *C*-glycosidic bonds. The biological evaluations of various *S*- and *N*-glycosidic analogs of **1** show very little to no promise, as there was significant (3- to 5-fold) loss of activity across all cell lines tested.[42,44] Gratifyingly, the *C*-analogs displayed cytotoxic activities comparable to the reference *O*-glycoside, but its synthetic approach is an arduous task and may not be economically viable on a large scale.[45,46] Nonetheless, they potentially represent a better choice for pre-clinical studies as they will not be susceptible to hydrolysis by glycosidases *in vivo*.

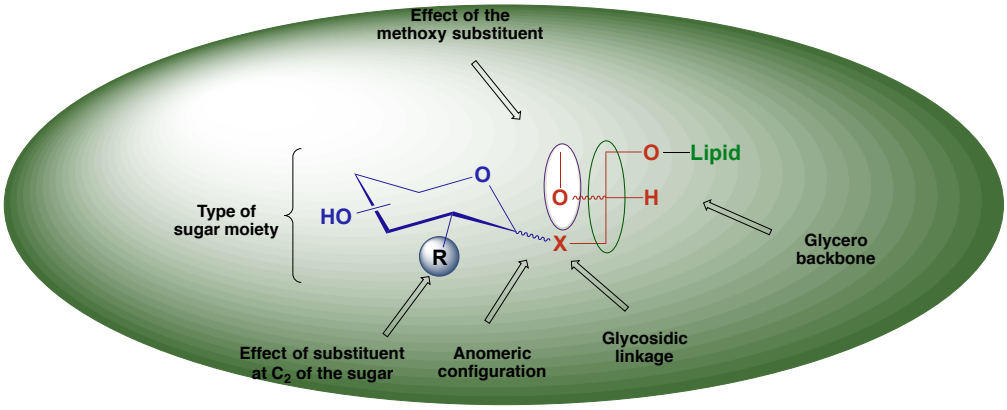


Figure 1-3: Schematic diagram for the structural activity studies of GAELs.[47]

In addition, the glycerol backbone and effect of various sugars as it affects overall cytotoxicity have also been explored. While the exact role and relevance of the methoxy group on *sn*-2 is not immediately obvious, it has been established that the glycerol backbone is important for activity.[43,44] Also, a variety of both furanoside- and pyranoside-based non-cationic GAELs have been studied alongside the early non-cationic disaccharides. The activities of these compounds were either lower or comparable to edelfosine. Only recently have studies revealed the potential utility of the disaccharide analogs with maltose and lactose as their sugar core as possible cytostatic agents. They were found to be potent inhibitors of cell migration and might be relevant in controlling cancer metastasis.[48,49] Other amino-sugar-derived GAELs such as mannosamine and galactosamine have also shown modest activity comparable to glucosamine-derived analogs. Worthy of mention is the fact that α-galactosamine-derived analogs displayed greater activity than α-GDG **1**, whereas its β-counterpart showed between 4- to 5-fold loss of activity.[42] Diglycosylated GAEL analog (**Figure 1-2**) has also been investigated to evaluate the effects of additional glucosamine moiety in the structure.[44] The hypothesis that an additional glucosamine moiety will enhance anticancer activities is based on the reported cytotoxicity of glucosamine against human epidermoid carcinoma cells in tissue

culture[50] and YD-8 human oral cancer cells.[51] This hypothesis was not valid as there was significant loss of activity, compared to **1**.[44]

1.3.2 Challenges and Limitations of GAELs

Some of the celebrated attractiveness of AELs include their metabolic stability to hydrolases and acyltransferases, coupled with their selectivity for cancer or transformed cells at concentrations that do not affect normal cells.[29,52–56] In lieu of this, the cytotoxicity of GAELs in normal and transformed cells were also investigated but no distinction or significant selectivity between the two was observed *in vitro*.[57,58] While *in vitro* studies may not be adequate to extrapolate *in vivo* activities and *vice-versa*, the lack of selectivity by GAELs might pose some concerns in the development of these molecules as potential anticancer drugs. Various factors such as selective absorption or accumulation in tumor versus normal tissues, distribution, extent of metabolism etc. are key determinants of how chemical entities behave in physiological models. The extent to which these factors will affect GAELs in animals is currently unknown. Moreover, only few currently used chemotherapeutic agents, if at all, show distinctions between normal and cancer cells, accounting for the side effects usually associated with their use. It is instructive to note that, in spite of the demonstrated selectivity of edelfosine for tumor cells relative to cancer cells, a major factor preventing its use in treating cancers is gastrointestinal toxicity,[33] presumably due to its effect on the normal epithelial of the gut. Thus, *in vitro* selectivity may not necessarily translate into *in vivo* selectivity.

The unlikelihood of glucose-derived GAELs being metabolically stable to glycosidases in humans also presents another challenge that needs to be resolved. Compound **1**, in fact, does not display any *in vivo* activity, perhaps, due to its metabolism by glycosidases in animals. This metabolic hydrolysis is highly improbable in cell lines used to characterize this compound and could account for the observed discrepancy *in vivo*. β-*O*-glycosidic

bonds of alkyl glycosides has been shown to be rapidly metabolized in the intestines and liver of mice,[59] suggesting a possible reason for the lack of *in vivo* activity of **1**. *C*-glycosides are however not susceptible to glycosidases and it is expected that *C*-glycosidic analogs of GAELs will display *in vivo* activity.

1.4 Justification for the Study

The astonishing statistics of cancer-related death, despite the huge resources dedicated to its research and the large number of approved anticancer drugs, sends a clear message that current drugs have not been as effective as desired. The limited benefits provided by current chemotherapeutic agents, which are mostly pro-apoptotic, is not that surprising given the nature and complexity of tumor biology.[60] The molecular pathways responsible for tumor growth and metastasis are highly adaptive and sometimes redundant, and they also vary between individual patients or between tumor cell sub-clones within the same patient. Some cells also harbor mutations in the apoptotic pathways that enable them to escape spontaneous or therapy-induced apoptosis thereby developing resistance to the very pathways activated by chemotherapeutic agents.[61–64] This implies that the anticipated mechanism of action of chemotherapeutic agents could be easily circumvented, thus rendering them ineffective. Moreover, it is unlikely that a single mono-functional 'targeted' drug will be effective for treating the most advanced carcinomas due to the highly sophisticated mechanism by which they adapt to drug actions.[65,66] In light of these, combined drugs that impact multiple targets simultaneously have been shown to be better at controlling complex disease systems. They are also less prone to drug resistance and are usually the typical standard care approach in cancer management.[67–69] A major drawback of combination therapy has to do with the different pharmacokinetics of the drugs involved. When two or more drugs are administered simultaneously, they are often transported and

absorbed with different efficiencies, resulting in different bioavailability and metabolic predisposition. This creates a problem where desired drugs do not get to the site of action at the same time, as when needed and/or for as long as needed. Other factors such as drug–drug interactions, the inconvenience of using many drugs, etc. also make this less desirable. To improve the efficiency of using a two-drug cocktail, a common approach involves the use of hybrid drugs.[70]

1.4.1 The Hybrid Concept

Hybrid drugs can be described as chemical entities with two or more structural domains that have different biological functions and dual activity. They are usually covalently-linked to form a single molecule with the intention of modulating, amplifying or exerting dual drug actions.[71] They may be classified based on the manner in which they are linked together i.e. the two entities can be linked directly or via a spacer/tether. Directly linked hybrid drugs are connected via a functional group on each of the participating molecules (mostly leading to formation of esters, carbamates, amides, etc.) while spacer-linked hybrids involves the use of a tether as a bridge between the two compounds. The tether in itself could possess or contribute significantly to the overall activity of the resulting hybrid molecule. It should be noted that both classes could be designed as either cleavable or non-cleavable.[72] A merged hybrid entity can also be obtained by overlapping the structural motifs or pharmacophores of two drugs in which the resulting hybrid product differ significantly in their structures, compared to the parent drugs from which they were designed. The resulting hybrid drug may retain the functional properties of either or both of the overlapped drugs e.g. azatoxin hybrid retains the activities of its parent drugs, ellipticine and etoposide (Figure 1-4).[73]

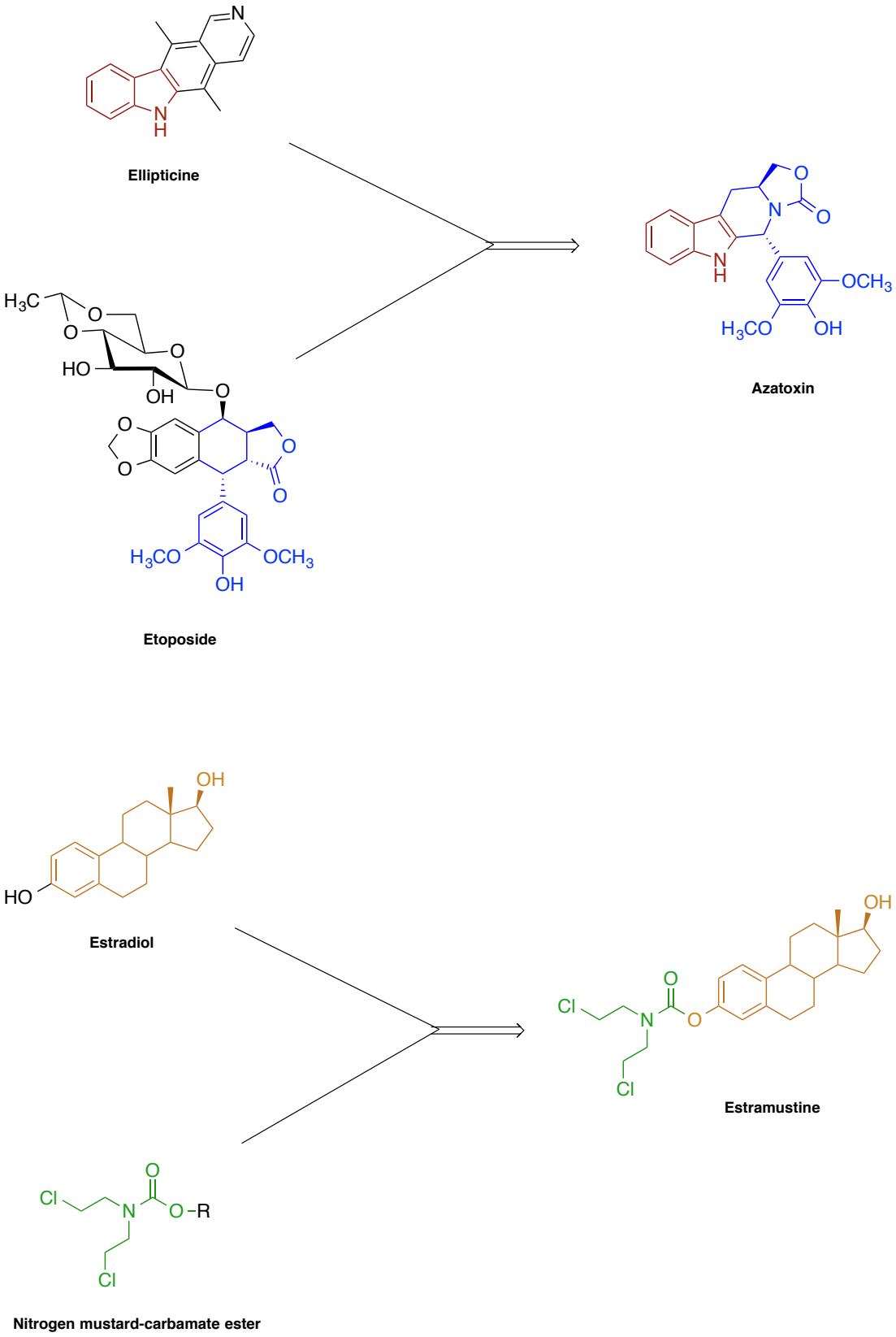


Figure 1-4: Examples of hybrid compounds

The anticancer hybrid concept was greatly influenced and espoused by the mechanism of action of the glycopeptide 'bleomycin', an efficient anticancer agent that was originally isolated from *Streptomyces verticillus*. This drug has three distinct structural domains: one for DNA binding, a second for metal binding and a third containing carbohydrate (**Figure 1-5**). After cell penetration facilitated by the carbohydrate domain, the bithiazole entity and its positively charged terminal chain binds to GC-rich sequences of DNA while its amine-rich domain strongly chelate redox-active metal ions such as iron. The BLM–Fe^{III} complex is easily reduced inside cells to BLM–Fe^{II}, which reacts with molecular oxygen, a second electron, and a proton to generate an "activated bleomycin".[71,74–78] Thus, bleomycin is an excellent example of the design of a hybrid molecule containing three structural entities with three different biological roles by a microorganism. Hybrid drugs may therefore possess enhanced efficacies compared to their individual parent entities.

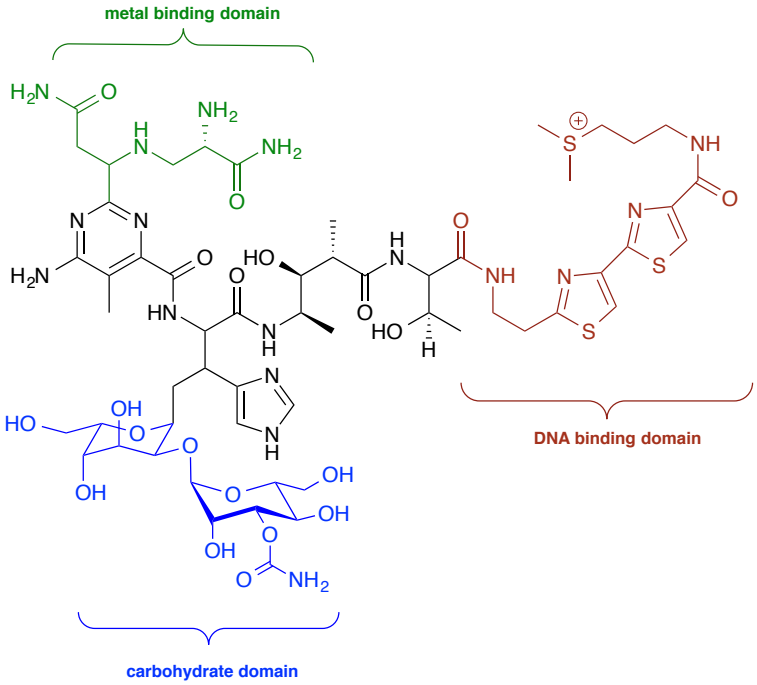


Figure 1-5: Structure of bleomycin (BLM) and its domains

1.4.1.1 Limitations

While some hybrid drugs have been successful, some combinations have resulted in outright loss of activity or may act via an entirely different mechanism of action.[72,79,80] More so, as an emerging area in drug development, the hybrid concept is beset with initial challenges that are not remarkably unexpected of any new field of endeavor. For instance, the physicochemical properties of most hybrid drugs, owing to their large molecular weight, are highly unpredictable and they tend to deviate from the generally acknowledged drug-like properties of medicinal agents. Also, they may be poorly soluble in water thereby making it difficult to develop satisfactory intravenous formulations. Moreover, because the development of most hybrid molecules involves complicated chemistry with many synthetic steps and maneuvering, a lot of resources and time is required for research and development. Regulatory requirements for hybrid molecules will also be more rigorous as each hybrid molecule will be expected to justify their benefits over conventional therapy as well as surmount the challenges associated with its development.[72,80]

1.4.2 Cancer Cell Death

1.4.2.1 Apoptotic pathways

Apoptosis (programmed cell death) differs distinctly from necrosis ('painful' cell death) although they could occur independently, sequentially as well as simultaneously.[81] Cells that die as a result of acute injury typically swell and burst, thereby spilling their contents all over the neighboring cells. This process, known as necrosis, often results into traumatic and potentially damaging inflammatory responses. By contrast, a cell that undergoes apoptosis dies neatly without damaging its neighbors.[82] The cell shrinks and condenses, the cytoskeleton decouples from plasma membrane and collapses (blebbing), the nuclear envelope disassembles and the DNA breaks up into fragments.[83–85] Most importantly, the cell surface is altered and displays properties that cause the dying cells to be phagocytosed,

either by a neighboring cell or a macrophage, before any leakage of its content occurs. This not only avoids the damaging consequences of necrosis, but also allows the organic components of the dead cell to be recycled by the cell that ingests it.[86,87] The intracellular machinery responsible for apoptosis depends on a family of proteases, known as caspases, which have cysteine at their active sites and cleave their target protein at specific aspartic acid.[88,89] Caspases are synthesized in the cells as inactive precursors, or *procaspases*, and are usually activated by cleavage at the aspartic acid by *initiator procaspases* either by extracellular or intracellular death signals. Once activated, caspases cleave and activate other *procaspases* resulting in an amplifying proteolytic cascade. These activated caspases then cleave other key proteins in the cell such as; nuclear lamins that causes irreversible nuclear lamina breakdown and the protein that anchors a DNA-degrading enzyme (DNAse), thus, freeing the DNAse to cut up the DNA in the cell nucleus.[90,91] While the early stages of apoptosis (pseudoapoptosis) has been demonstrated to be reversible,[92] it is mostly believed that the activation of intracellular cell death pathway is triggered in a complete, all-or-none fashion, such that when a cell reaches a critical 'point-of-no-return' (i.e. mitochondria permeabilization) along the path to destruction, it cannot turn back.[90] Indeed, most of the chemotherapeutic agents currently in use are pro-apoptotic in action i.e. they induce a caspase-dependent proteolytic cascade of cell death. Several studies have also demonstrated that ALPs, including edelfosine, kill cells by initiating events that ultimately result in cell death by caspase-dependent apoptosis.[27,31,93,94] Unfortunately, the development of resistance to these apoptotic pathways has been reported as one of the leading causes of chemotherapeutic failures in cancer management.[62–64] The resistance to the very pathways activated by these drugs can also be transferred across all classes of pro-apoptotic agents, leading to cross-resistance. A possible way out of this quagmire will be the development of

molecules whose mechanistic pathways do not exclusively rely on defined apoptotic pathways.

1.4.2.2 Non-apoptotic pathways

Non-apoptotic cell death, as the name implies, may serve as a back up to failed apoptosis or occur independent of apoptosis. The ability to engage non-apoptotic cell death provides new opportunities to manipulate cell death in a therapeutic context – for example, to enable the killing of apoptosis-resistant cancer cells. Although not as widely studied as apoptosis, several regulated non-apoptotic forms of cell death such as necroptosis, autophagic cell death, pyroptosis and paraptosis have also been described.[34,95] Despite the morphological resemblance between necroptosis and necrosis, necroptosis differs substantially in that it is a regulated active type of cell death that could be triggered by various stimuli and death-receptor ligands such as tumor necrosis factor (TNF) and Fas.[96–100] Autophagic cell death can be described as a lysosome-dependent process that degrades several cargoes, varying from molecules to organelles.[101] During this process, an isolation membrane that engulfs cytosolic cargo is formed in the cytoplasm. This creates an autophagosome, which then fuses with lysosomes, leading to the breakdown of the engulfed material and allowing macromolecules to be recycled.[102,103] Another form of lysosomal cell death where inhibition of acid sphingomyelinase (ASM) enhances membrane permeabilization and destabilization of cell lysosomes has also been reported.[104] Pyroptosis is a caspase-dependent form of programmed cell death that differs in many respects from apoptosis. It is an inflammatory type of cell death and unlike apoptosis, depends on the activation of caspase-1 or caspase-11 (caspase-5 in humans).[105–107] Caspase-I plays a key role in the processing of inactive IL-β and IL-18 into mature inflammatory cytokines and can also trigger cell death in some circumstances.[108] Conversely, paraptosis is a type of programmed cell death that is morphologically distinct from apoptosis and necrosis in that they are

characterized by cytoplasmic vacoulation, rounding of cells, and maintenance of membrane integrity. It is caspase-independent and is often associated with late loss of mitochondria membrane potential (swelling and clumping).[109,110]

The initial report on **1** that noted its ability to kill a drug-resistant ovarian cancer cell line, NIH OVCAR 3, which the other AEL subclasses could not kill,[39] hinted at the possibility that GAELs had a different mechanism of action than the ALPs. It was subsequently reported to kill cells via a non-apoptotic paraptosis-like cell death that requires an active endocytosis pathway.[34,41]

1.4.3 Hypothesis

In light of the non-apoptotic mechanism of cell death induced by GDG **1** relative to other clinically-used anticancer agents,[34] their reported cytotoxicity against cancer stem cells,[42] the development of resistance to clinically-used pro-apoptotic agents by cancer cells,[15,111] the challenges associated with combination cocktails,[70,72] and the potentials of non-apoptosis-inducing cytotoxic drugs in the fight against cancer; we conceived that a hybrid molecule comprising **1**, with an apoptosis-inducing anticancer molecule could present two separately functioning domains that may trigger two or more cytocidal mechanistic pathways to inhibit tumor growth more effectively than either compound alone.

We were therefore interested in investigating whether the development of this hybrid molecule with two distinct parts of apoptotic and non-apoptotic domains (Figure 1-6) could present a fortified or synergistic activity, as observed in bleomycin. Our concept was inspired by the synthesis of azatoxin hybrid, which was found to retain the activity of both parent molecules from which it was developed (Figure 1-4).[73]

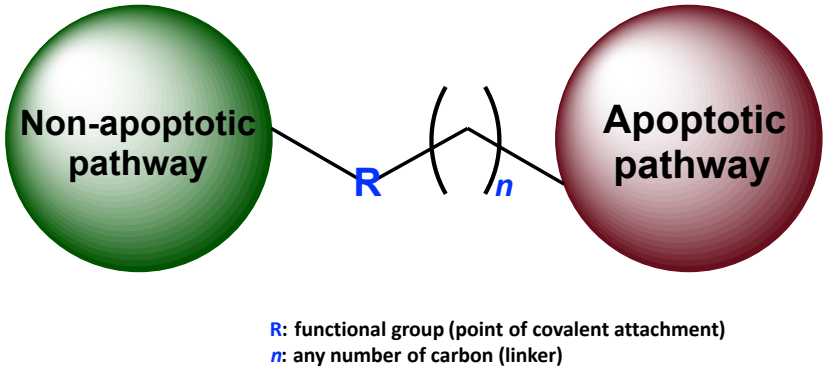


Figure 1-6: Generic hybrid approach

1.5 Measurement of Cytotoxicity (cell viability)

Analyzing the effects of chemical entities on cell growth inhibition and/or cell death *in vitro* has been an important component of oncology drug discovery. It involves the evaluation of potency and sensitivity of various cell lines to specific agents. The earliest, simplest and least expensive method of determining cell viability is by direct visual counting of dye-stained cells using a haemocytometer. However, this is an onerous task that is time consuming, subject to operators' errors and of limited value for large-scale viability testing. Newer techniques developed to evaluate cell number, cell proliferation, and/or cell viability, depending on the investigator's interest, involves the quantification of some aspect of cellular metabolism per well or determining the biomass as a proxy for the number of viable cells. Metabolic assays are nonetheless the preferred method to quantitate changes in cell growth although these assays may also not accurately reflect cellular proliferation rates due to a miscorrelation of metabolic activity and cell number.[112] Several proliferation assays have been developed based on the metabolic discrepancies of viable and non-viable cells, but three of the commonly used ones include: MTT/MTS assay, LDH (lactate dehydrogenase) assay and ATP-dependent bioluminescence.

1.5.1 MTT/MTS Assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium) assays are colorimetric assays that are based on the bioreduction of tetrazolium salts from pale yellow to a dark-blue (purple) formazan dye. The unique biochemical properties of tetrazolium salts that have led to their wide spread use is dependent on the positively charged quaternary tetrazole ring core containing four nitrogen atoms, surrounded by three aromatic groups that usually involve phenyl moieties. It is believed that the net positive charge on tetrazolium salts facilitates cellular uptake across the plasma membrane of viable cells via membrane potential. Following mild reduction, the tetrazole ring core is disrupted thereby transforming the weakly coloured tetrazolium salts structures into brightly coloured formazan products (Figure 1-7). This reduction process is mediated by the biochemical activities of the NAD(P)H-dependent oxidoreductases and dehydrogenases of metabolically active cells.[113]

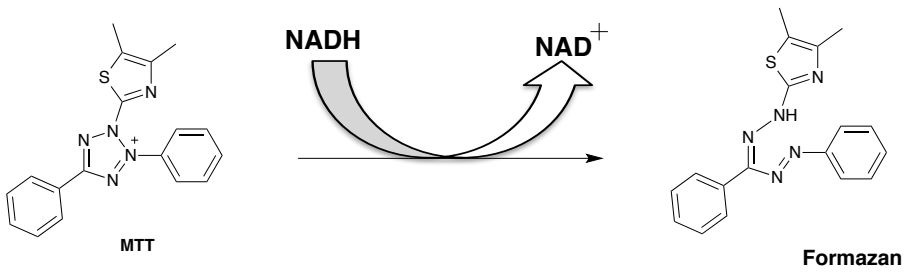


Figure 1-7: Metabolic reduction of MTT to formazan

Asides MTT forming an insoluble formazan and often used as end-point assay, it also differs from MTS in that it is positively charged and readily penetrates viable cells. On the contrary, negatively charged tetrazolium salts such as MTS, XTT [sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium] and WST-1 [sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium] do not

readily penetrate cells, hence, are typically used with an intermediate electron pair acceptor such as phenazine ethyl sulfate (PES) that can shuttle electrons between the cytoplasm and plasma membrane to facilitate the reduction of tetrazolium into the coloured formazan product. These negatively charged monotetrazolium salts also result in the formation of a soluble formazan and are thus used in real-time assay.

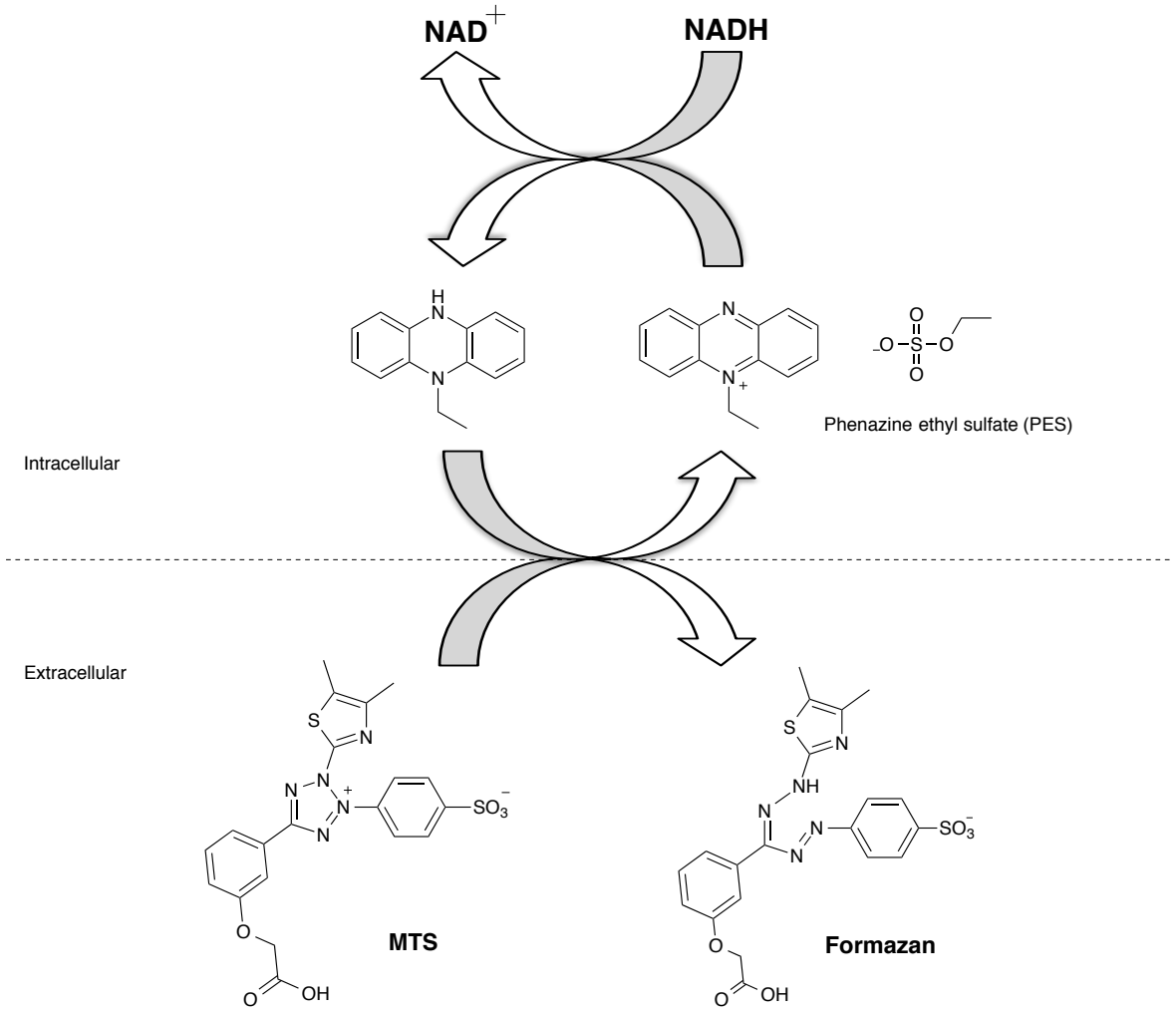


Figure 1-8: PES-assisted metabolic reduction of MTS to formazan

The intensity of the final product, which gives an indication of cell viability, is measured with a micro-plate reader that relates the attenuation of light to the properties of the material through which light is travelling (Beer-Lambert law).

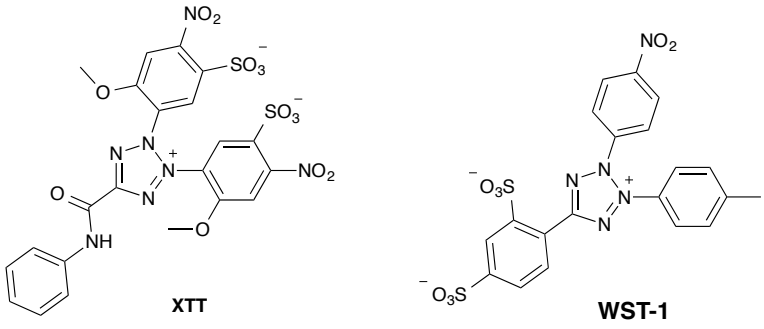


Figure 1-9: Structure of XTT and WST-1

The ease of use, ready application to high throughput micro-based assays, intense colouration of formazan, high sensitivity and reproducibility, and an integrated metabolic signal read by tetrazolium dyes under defined growth conditions make this method a moderately robust way of measuring cell viability.[113,114] However, factors such as the use of combined live/dead cells for extrapolation, timing of reading out results and the significant variability of formazan formation amongst different cells may limit the extent to which results could be interpreted.[115,116] In spite of its limitations, the MTT/MTS assay is still the most widely used method of cytotoxic evaluation.

1.5.2 LDH (lactate dehydrogenase)-based Assay

The LDH leakage assay is based on the measurement of lactate dehydrogenase activity in the extracellular medium. Firstly, LDH catalyzes the conversion of lactate to pyruvate via the reduction of NAD+ to NADH. Secondly, diaphorase uses NADH to reduce a tetrazolium salt, INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride] to a red formazan product. Therefore, the level of formazan formation is directly proportional to the amount of LDH released in the medium. The loss of intracellular LDH and its release into the culture medium during tissue damage is an indicator (biomarker) of irreversible cell death due to cell membrane damage. Characteristics such as reliability, speed and simplicity make this method of evaluation very desirable.[117] Since the LDH

assay solely depends on the integrity of cell membranes, it may provide satisfactory responses to cell membrane-damaging agents but could be misleading if the toxic agent only influences intracellular activities. Poor sensitivity of cell lines has also been reported to be associated with this method of assay.[118,119] Moreover, the LDH enzyme has a limited half-life in the medium and their activity decreases significantly after 24 hours, thus putting a time constraint on when reliable data could be collected.

It should be noted that while the MTT/MTS assays measures viable/live cells, the LDH measures non-viable/dead cells.

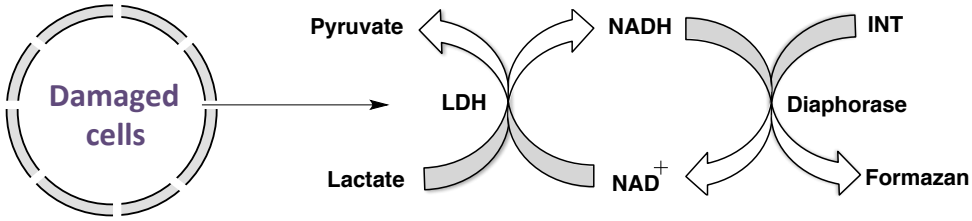


Figure 1-10: Metabolic reduction of lactate dehydrogenase (LDH)

1.5.3 ATP-dependent Bioluminescence

Chemoluminescence is the emission of light (luminescence) as one of the end result of a chemically exothermic reaction. It is the antithesis of a photochemical reaction in which light is used to drive an endothermic chemical reaction. This process could take place as well in a biological system and is known as bioluminescence. The principal chemical reaction in bioluminescence involves the light-emitting pigment luciferin and the enzyme luciferase. This enzyme catalyzes the oxidation of luciferin and requires cofactors such as calcium or magnesium ions, and also the energy-carrying molecule adenosine triphosphate (ATP) i.e. Luciferin (L) reacts with oxygen to create light:

$$L + O_2 + (ATP) \xrightarrow[Mg^{2+}]{\text{luciferase}} \text{oxy–L} + CO_2 + AMP + PP + Light$$

Carbon dioxide (CO_2), adenosine monophosphate (AMP) and phosphate groups (PP) are released as by-products

Since ATP is the primary energy currency of living systems, virtually all energy-requiring processes utilize the chemical energy stored in the phosphate bond of ATP. The extracellular signaling by ATP is a ubiquitous process in cell biology and physiology, and is formed exclusively in the mitochondria. Interruptions in/with the mitochondria activities will therefore affect ATP formation and this variation in ATP production ultimately affects the oxidation of luciferin by luciferase. The net effect of this commensurate with the glow of light emitted, which then gives an indication about the viability and/or non-viability of cells in the medium. Thus, if ATP becomes the limiting component in the luciferase (bioluminescence) reaction as a result of mitochondria-dependent cell death, the intensity of the emitted light is proportional to ATP concentration. The intensity of emitted light is measured using a luminometer that permits a direct quantitation of ATP.[120,121] Although this method of assay has been credited with the ability to distinguish between cytocidal (lethal) and cytostatic (growth inhibitory) drug effects,[122] its reliance on the concentration of luciferase, oxygen and luciferin, as well as the need for transfection, makes the limiting factor subjective thereby inadvertently resulting in prejudiced results.[123]

The suitability of the type of assay used in the evaluation of cytotoxicity is therefore often influenced by the predicted mechanism of cell death and care must be taken to prevent the likely possibilities of false-positive or false-negative results.

CHAPTER TWO

2 THESIS OBJECTIVE

We decided to initiate the GAEL-hybrid studies by covalently linking GDG **1** to chlorambucil and myristylamine analogs (cationic amphiphiles), based on the reasons discussed in Chapters 3.1 and 4.1 respectively.

For the GAEL-chlorambucil study, we were interested in:

a) Exploring the cytotoxic effects and/benefits of having two different modes of action in a single GAEL hybrid molecule

b) Investigating the contribution and/or role of chlorambucil to the overall activity, and

c) Investigating the significance of a cationic charge on the activity of the hybrid molecule against cancer cell lines.

Based on the preliminary results obtained from the GAEL-chlorambucil study, we sought to investigate the amphipathic nature of GAELs *viz-a-viz* how modulating amphiphilicity could influence their cytotoxicity. Thus, we set out to:

a) Explore the cytotoxicity of tricationic GAEL hybrids with an appropriately compensating hydrophobicity as exemplified by myristylamine

b) Investigate the cationic–hydrophobic relationship using different carbon chain lengths

c) Examine the exact contribution of a third cationic charge as well as the role of the glycerol backbone of the triamino analogs

To fully capture the roles of these additional moieties, we needed to synthesize some compounds that could serve as control to the hybrid molecules. This require us to:

- Synthesize structurally-diverse analogs of GAEL-chlorambucil hybrid and tricationic molecules
- Examine different points of covalent attachment between the parent molecules

The resulting molecules were then characterized using NMR spectroscopy and mass spectrometry, screened against a panel of human epithelial cancer cell lines and cytotoxicity assessed via MTS assay.

CHAPTER THREE: GAEL – CHLORAMBUCIL HYBRIDS

3 ABSTRACT

Glycosylated antitumor ether lipids (GAELs) kill cancer cells and cancer stem cells via a novel, apoptosis-independent mechanism. In contrast, chlorambucil, a drug in clinical use for the treatment of chronic lymphocytic leukemia reacts with nucleophiles within the major groove of DNA leading to apoptosis. We hypothesized that hybrid molecules which combine apoptosis-dependent and apoptosis-independent mode of actions in a single molecule may lead to greater cytotoxicity relative to the individual molecules. Here, we describe the antitumor activities of different analogs of chlorambucil-linked glucosamine-derived glycerolipid hybrids and investigate the role of the chlorambucil moiety and the effect of cationic charge on the hybrid molecule. Three hybrids and two control GAELs were synthesized and their activities against breast (JIMT1, MDA-MB-231, BT474), pancreas (MiaPaCa2) and prostate (DU145, PC3) cancer cell lines were determined using MTS assay. Hybrid **3** displayed the most potent activity on DU145 at CC_{50} of 6.0 μM while hybrid **4** displayed the best activity on JIMT1 at 7.5 μM. Hybrid **5** exhibited no activity at the highest concentration tested (CC_{50} > 20 μM), underscoring the significance of the cationic charge at C-2 position as previously reported. Although chlorambucil **(2)** itself showed very little activity against all the six cell lines (CC_{50} > 150 μM), GAELs **6** and **7** which lack the chlorambucil moiety were consistently less active than **3** and **4**, suggesting that the chlorambucil moiety contributes to the overall activity. The hybrids were however not as active as the parent GAEL **1** against MiaPaCa2 whereas **6** restored activity to levels comparable to **1**.

3.1 Introduction

In light of the novel non-apoptotic mechanism of cell death induced by GDG **1**, relative to other anticancer compounds,[34] and the potential of non-apoptosis-inducing cytotoxic drugs in the fight against cancer, we conceived that a hybrid molecule comprising **1**, with a pro-apoptotic inducing anticancer molecule could present two separately functioning domains that may trigger two or more cytocidal mechanistic pathways to inhibit tumor growth more effectively than either compound alone.

The alkylating agent chlorambucil **2,** which is used in the treatment of chronic lymphocytic leukemia (CLL) and management of other types of cancer,[124] was selected to form hybrid molecules with GDG **1**. The mechanism of action of alkylating agents involves the formation of adducts with DNA, RNA and proteins which ultimately leads to apoptotic cell death.[125] Furthermore, the ability to couple chlorambucil to other moieties without losing its inherent activity[126–128] made it an attractive molecule to explore the hybrid concept. In the studies described herein, we a) explored the cytotoxic effects of chlorambucil-linked GAEL hybrids b) investigated the contribution of chlorambucil to the overall activity and c) investigated the significance of a cationic charge on the activity of the hybrid molecule against cancer cell lines.

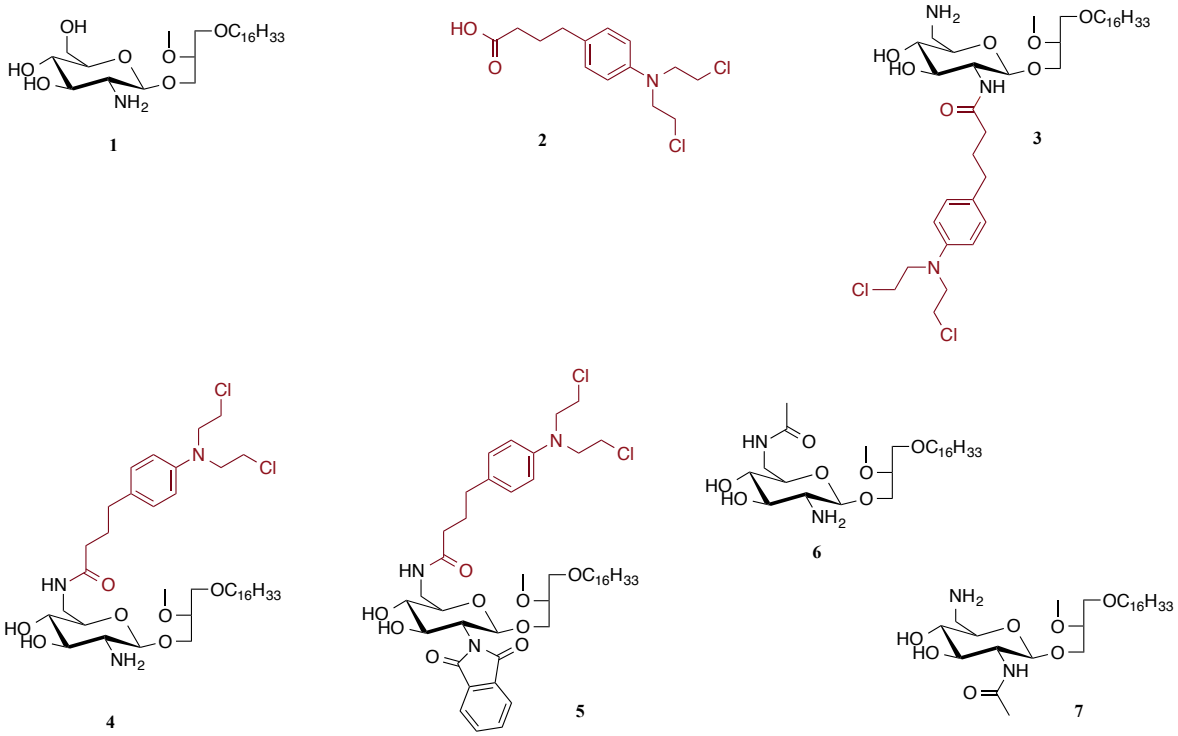


Figure 3-1: Structures of GAEL-chlorambucil hybrids and corresponding reference compounds used in this study

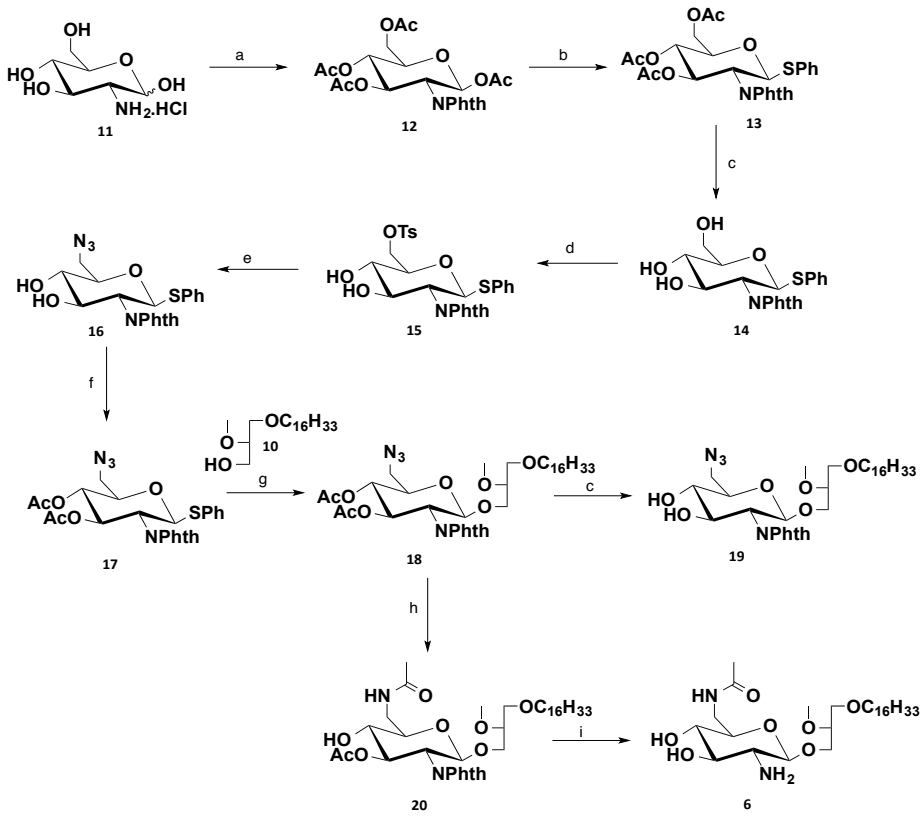
3.2 Results and Discussion

3.2.1 Chemistry

In order to preserve the pharmacophores responsible for the antitumor activities of the parent drugs, structurally diverse analogs of chlorambucil-linked GDG hybrids differing in the position of covalent attachment between C-2` and C-6` were synthesized. Compounds **3** and **4** were prepared to ensure the preservation of the cationic charge on the GDG analog at physiological pH as well as the alkylating portion of chlorambucil. Similarly, compound **5** was synthesized to evaluate and verify our initial report on the role of cationic charge on antitumor activities of GAEL,[43] while **6** and **7** were prepared to distinguish the exact role of chlorambucil (attached by an amide linkage) as opposed to an ordinary amide group.

The synthesis of compounds **3 – 7** commenced from a phthalimide and acetate-protected glucopyranoside **12** prepared from commercially available glucosamine hydrochloride **11** (Scheme 3-1), as previously described.[43] Conversion of anomeric acetate **12** into a thioglycoside donor **13** was accomplished by boron trifluoride diethyletherate-promoted thioglycosylation with thiophenol to afford β-thiophenyl glycoside **13** in 62 % yield. Deacetylation of **13** using sodium methoxide in methanol produced **14** in 81 % yield while the primary alcohol of **14** was tosylated to **15** with *p*-toluenesulfonylchloride and pyridine as the base, in 70 % yield. The tosyl-leaving group of **15** was then converted to azido-group with sodium azide under dry aprotic condition to afford **16** in 93 % yield and subsequently re-protected with acetate to give **17** in high yield. Thioglycoside **17** was then glycosylated with commercially available lipid alcohol **10** under *N*-iodosuccinimide (NIS)/silver triflate-promoted conditions to afford β-glycolipid anomer **18** in 70 % yield. Compound **18** was thereafter deacetylated with sodium methoxide in methanol to afford **19** in 80 % yield. Rather than preparing **6** from **18** in four steps, the azide on **18** was reduced with trimethylphosphine/water to afford **20** (where the acetate on C-4` migrates to C-6` *in situ*)

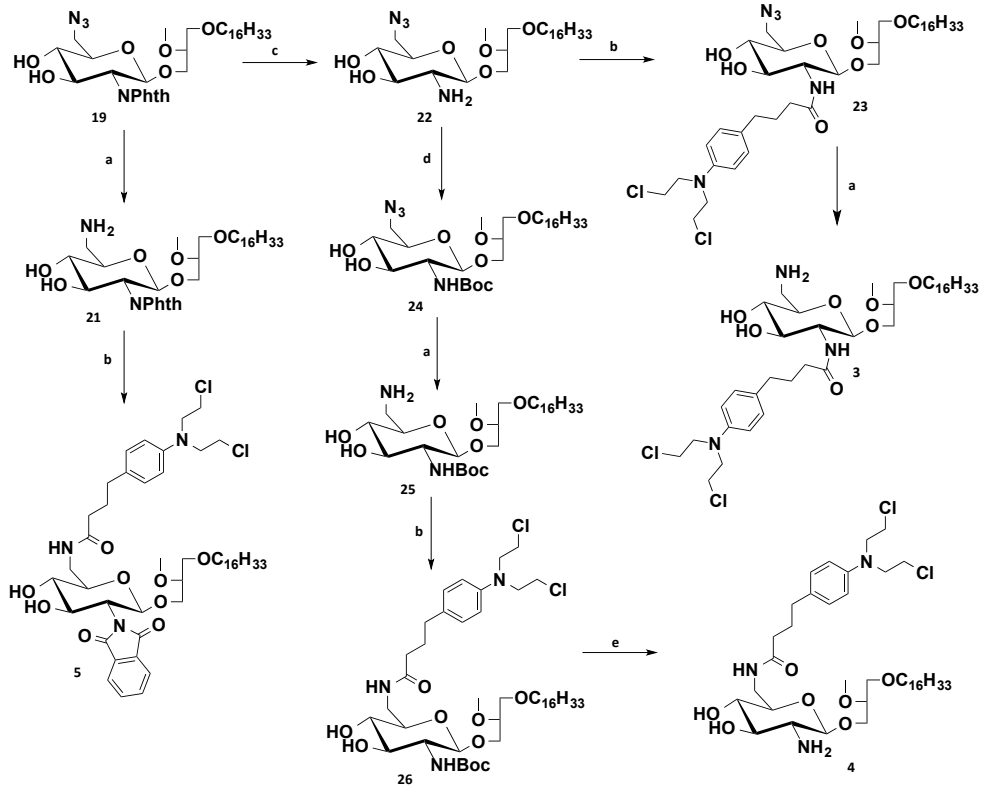
in a one-step reaction (72 % yield). The C – 3` acetate and phthalimide protecting groups in **20** were then removed using ethylenediamine in butanol to afford compound **6** in 65 % yield (Scheme 3-1).



Scheme 3-1: Synthesis of compound **6**.

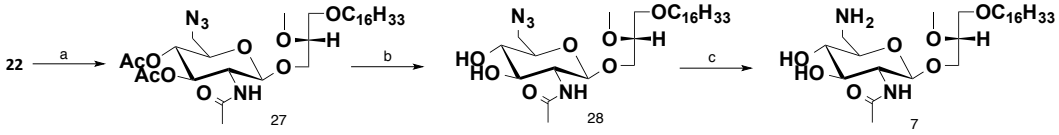
Reagents and conditions: (a) i) NaOH, H_2O, phthalic anhydride, RT, 16 h; ii) pyridine, Ac_2O, DMAP, RT, 16 h (65 %); (b) PhSH, $BF_3.Et_2O$, DCM, RT, overnight (62 %); (c) NaOMe, methanol, RT, 25 mins, DOWEX (76 – 81 %); (d) *p*-TsCl, DMAP, pyridine, RT, overnight (70 %); (e) NaN_3, DMF, 70 °C, overnight (93 %); (f) pyridine, Ac_2O, DMAP, RT, 16 h (87 %); (g) NIS, AgOTf, CH_2Cl_2, RT, 3 h (70 %); (h) PMe_3, THF, H_2O, RT, 1 h (72 %); (i) ethylenediamine, butanol, 90 °C, 3 h (65 %)

With compound **19** now handy, we could then begin exploring the routes towards the hybrid molecules. Compound **5** was prepared by catalytic hydrogenation of **19** to **21**, in 80 % yield, and subsequently coupled to a TBTU-activated chlorambucil **2**. The reaction was carried out in dry DMF using diisopropylethylamine as a base, to afford **5** in 92 % yield (Scheme 3-2). In a similar fashion, **3** was prepared by removing the phthalimide in **19** (using ethylenediamine) to give **22**, in 76 % yield, followed by coupling with an already activated chlorambucil **2** to give **23** and then catalytic hydrogenation to afford **3** in 80 % yield (Scheme 3-2). Unfortunately, **4** could not be prepared directly from **5** due to the certainty of knocking off the chlorine atoms under conditions (strong nucleophile and heat) sufficient enough to cleave phthalimido. Hence, we devised a new strategy of re-protecting the amine in **22** with di-*tert*-butyl dicarbonate (which could be cleaved under mild acidic condition) in methanol to give **24** in 93 % yield and then catalytic hydrogenation to give **25** in very good yield. **25** was thereafter coupled to an activated chlorambucil **2** to give **26**, which was then deprotected using trifluoroacetic acid in dichloromethane to afford **4** in 93 % yield (Scheme 3-2). Finally, compound **7** was prepared by acetylating **22** to give **27**, followed by deacetylation with sodium methoxide to give **28** and then catalytic hydrogenation to afford **7**, in good yields (Scheme 3-3).



Scheme 3-2: Synthesis of compounds **3 – 5**.

Reagents and conditions: (a) $Pd(OH)_2/C$, methanol, H_2, RT, 1 h (79 – 81 %); (b) chlorambucil, DIPEA, TBTU, DMF, RT, overnight (80 – 93 %); (c) ethylenediamine, butanol, 90 °C, 3 h (76 %); (d) Boc_2O, Et_3N, methanol, 50 °C (93 %); (e) TFA, CH_2Cl_2, RT, 1 h (90 %)



Scheme 3-3: Synthesis of compound **7**

Reagents and conditions*:* (a) pyridine, Ac_2O, DMAP, RT, 5 h (85 %); (b) NaOMe, methanol, RT, 30 mins, DOWEX (80 %); (c) $Pd(OH)_2/C$, methanol, RT, 1 h (76 %)

3.2.2 Cytotoxicity

The cytotoxic effects of compounds **2 – 7** against a panel of human epithelial cancer cell lines was assessed using the MTS assay.[129] The cell lines were derived from cancers of the breast (JIMT1, MDA-MB-231, BT474), pancreas (MiaPaCa2) and prostate (DU145, PC3). Exponentially growing cells were treated with test compounds and then incubated for 48 h. All compounds except compound **7** were tested up to 30 μM whereas compound **7** was tested up to a concentration of 15 μM because of limited availability. The results for **2 – 6** are displayed in Figure 3-2 and the CC_{50} values are displayed in Table 3-1 along with the values obtained for **1** from previous studies.[43]

The results showed that compound **2**, chlorambucil, exhibited very little activity against all the cell lines with CC_{50} greater than 150 μM, the highest concentration tested. A previous study also reported this high CC_{50} value of chlorambucil.[128] Compounds **3** and **4** proved to be the most active hybrids against all the cell lines with the exception of MiaPaCa2. The activities of **3** and **4** were quite similar against these cell lines, the only exception being with DU145 cells where **3** was almost twice as active as **4** with a CC_{50} of 6.0 μM for **3** versus 11.0 μM for **4**. JIMT1 cells were also sensitive to the compounds with CC_{50} of 7.5 and 8.5 μM. Both compounds also reduced the viability of the cells to around zero at a concentration of 20.0 μM. Thus, on the whole, it did not seem to matter whether the chlorambucil was linked at the C-2 or C-6 position of the glucose moiety.

As the activities displayed by the hybrid molecules **3** and **4** were somewhat similar to the activity obtained with **1**, the contribution of chlorambucil (**2**) to the overall activity was not obvious. To assess any contribution by chlorambucil, the activity of **3** and **4** were compared with the activity obtained with compounds **6** and **7**, which possessed the GDG-**1** and the amide linker but not the alkylating moiety of chlorambucil. The results showed that with

the exception of MiaPaCa2, **3** and **4** were significantly more active than **6** or **7**. These results suggest that the active group of **2** contributes to the overall activity of the GAEL-chlorambucil hybrid. This is readily apparent in the results obtained with DU145 cells where the presence of the chlorambucil moiety in **3** resulted in CC_{50} that was even lower than that observed with **1**. It is unclear why compound **4**, which differed from **3** in the positioning of the chlorambucil moiety, did not yield similar results as **3**. It is worth noting that estramustine, the only anticancer hybrid drug currently approved by FDA and is used for treating prostate cancer had a CC_{50} value of 40 – 60 μM against DU145 cells in *in vitro* studies[130] compared to 6 μM for compound **3.**

The presence of the amide moiety by itself appears to have a negative impact on the activity of GDG, as the activities of compounds **6** and **7** were lower than the activity reported for compound **1** with all the cell lines except for MiaPaCa2. It is also unclear why the compound **6** is more active than compound **7** especially since the differences between **3** and **4** were minimal for most of the cell lines.

In MiaPaCa2 cells, the chlorambucil molecule in the hybrid appears to have a negative effect on the overall activity. The CC_{50}'s of **3** and **4** were much higher than the activities observed with **1**. Furthermore, removal of the alkylating moiety either had no effect (compound **7**) or decreased the CC_{50} (compound **6**) when compared to the hybrid molecules **4** and **3** respectively. Indeed, the activity of compound **6** was similar to that of **1**.

Compound **5,** which is similar to **4** but without the C-2 NH_2 group, exhibited no activity at the highest concentration tested (CC_{50} > 20 μM). This result shows that even in the hybrid molecule, the presence of a cationic charge on the glucose moiety is essential for activity. This is in line with our previous report which also reinforced the importance of the NH_2 group at the C–2 position of the glucose.[43]

In summary, the results of the current study have revealed that in the GAEL-chlorambucil hybrid, both constituents of the molecules contribute to the overall activity, although the GAEL portion appears to be the dominant moiety. In addition, the effect of the chlorambucil moiety on the overall activity may be positive or negative depending on the cell type. Further development of the compounds may require studies to determine if the hybrid compounds functions as a unit or whether it is metabolized to yield the two constituents.

Cytotoxicity of compounds 1-7 on a panel of human epithelial cancer cell lines.
CC_{50} values (μM)

Compd	JIMT1	MDA-MB-231	BT474	MiaPaCa2	DU145	PC3
1*	9.0	7.1	8.0	9.0	10.0	13.5
2	>150	>150	>150	>150	>150	>150
3	8.5	8.5	12.5	16.0	6.0	12.0
4	7.5	10.5	12.5	20.0	11.0	11.5
5	>20	>20	>20	>20	>20	>20
6	11.0	13.5	13.5	10.0	15.0	14.5
7	>15	>15	>15	15.0	>15	15.0

Breast (JIMT1, MB-MDA-231, BT474), pancreas (MiaPaCa2) and prostate (DU145, PC3) cancer cell lines. The CC_{50} value is defined as the concentration required to decrease cell viability by 50% relative to the untreated control. Values were determined by MTS assay. The CC_{50} values were obtained by estimating the drug concentration at 50% viability on the *y-axis* of the plots for each cell line. (*Previously published).[43]

Table 3-1: Cytotoxicity of compounds **1 – 7** against human epithelial cancer cell lines

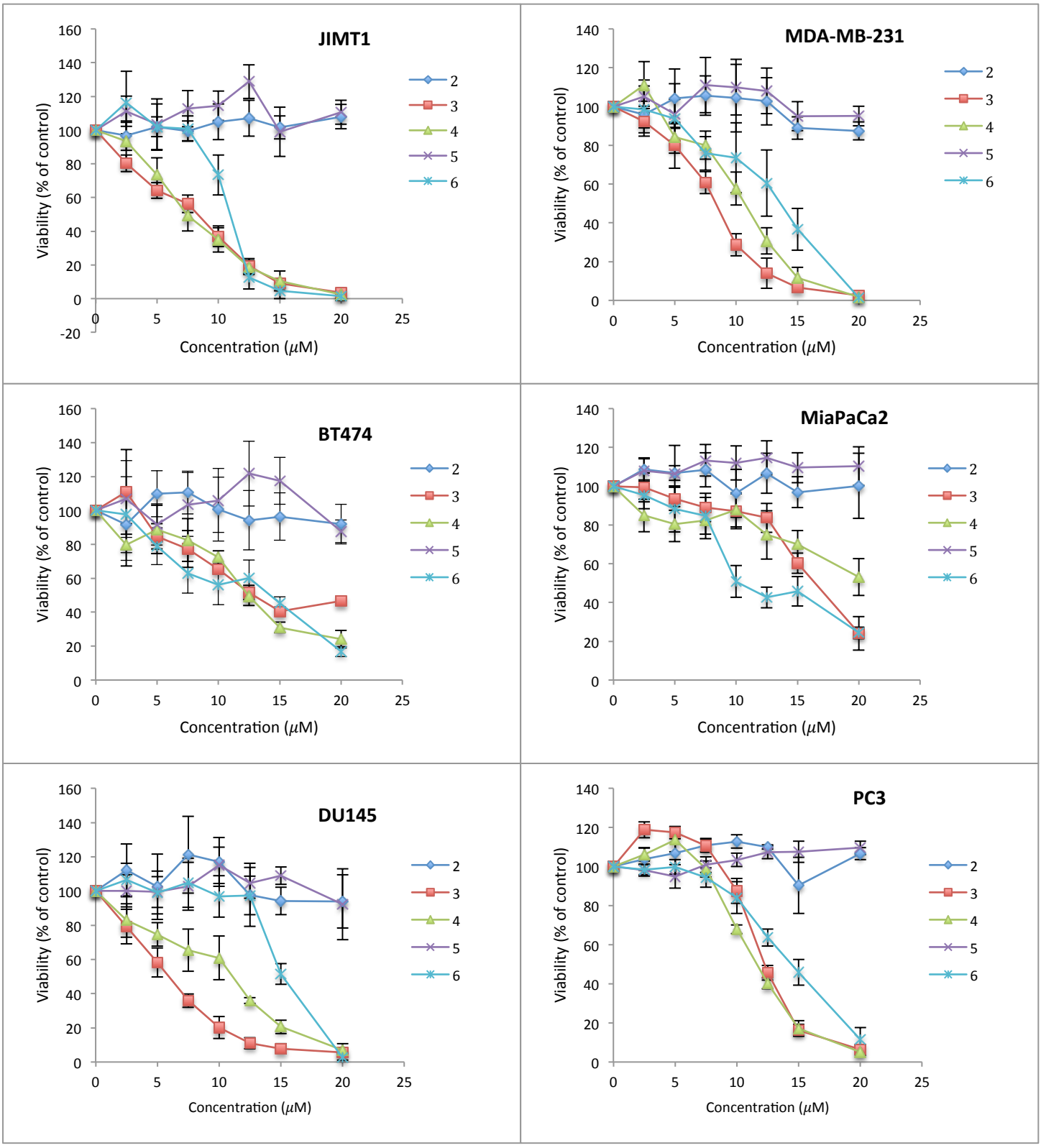


Figure 3-2**:** Effects of compounds **2** – **6** on the viability of epithelial cancer cells

Error bars indicate standard deviations from six representative experiments (n=6)

3.3 Conclusions

The hybrid analogs **3 – 7** were synthesized in good yields via the adaptation of several synthetic protocols. We were particularly interested in the benefits that could accrue from a chlorambucil-linked GDG hybrid and it was discovered that the hybrid compounds **3** and **4** were generally more active than reference compound **2** but comparable to **1**. The hybrids were similarly more active than **6** and **7** derivatives. Our results also demonstrated the capacity to fuse pro-apoptotic drugs with apoptosis-independent glucosamine-derived glycerolipids, with a prognosis of enhanced activity. The ability to retain antitumor activities in chlorambucil-linked GDG hybrid molecules suggests that future GDG-hybrids might hold better promises. This is due to the likely dominance of the GAEL domain of the hybrid while the exact role of the chlorambucil moiety is still unclear. Perhaps, chlorambucil might be acting as a new hydrophobe that fine-tunes the GDGs to mediate its antitumor activities. It will be worthwhile to explore the GAELs hybrid concept with classical cationic amphiphiles and other cationic amphiphilic drugs (CADs).

CHAPTER FOUR: TRICATIONIC GAELs

4 ABSTRACT

Glycosylated antitumor ether lipids (GAELs) are an emerging class of potential anticancer molecules that are being investigated as possible anticancer drugs. Interest in this class of drug is based on their non-apoptotic mechanism of action, as opposed to conventional anticancer agents that are mostly pro-apoptotic. On the other hand, myristylamine is a non-specific cytotoxic lipid that possesses inherent amphipathic properties. The membranolytic mode of action of cationic amphiphiles is believed to be responsible for their selectivity towards cancer cells and the possible reason for their synergism with conventional chemotherapeutic agents. We therefore postulated that a combination of GAELs and myristylamine as a hybrid drug, would present an amphipathic molecule with fine-tuned amphiphilicity. This approach is expected to result in triamino analogs that will be protonated at physiological pH, electrostatically attracted to cancer cells, as well as possess the requisite hydrophobicity to cross the cancer cell membrane. Here, we report the antitumor properties of different triamino analogs. We investigated the role of the glycerolipid backbone and examined the effect of the third cationic charge on the hybrid molecule. Four triamino GAEL analogs and two controls were synthesized and their cytotoxicity against breast (JIMT1, MDA-MB-231, BT474), pancreas (MiaPaCa2) and prostate (DU145, PC3) cancer cell lines were determined using MTS assay. Compound **32a** evidently displayed the best activity across all cell lines with CC_{50} values ranging from 1.5 to 4.0 μM, while **31a** that differed only in the point of covalent attachment displayed lower activity at CC_{50} values between 5.5 to 13.5 μM. The shorter chain length analogs **31b** and **32b** were not as active as the longer chain analogs. Interestingly, compound **33** was more active against MDA-MB-231 and BT474 than **31b** and **32b** while **34** was not active against any cell line at the highest concentration tested (> 20 μM). All of these suggest that amphiphilicity plays a significant role in the cytotoxicity of GAELs.

4.1 Introduction

Glycosylated antitumor ether lipids (GAELs), a subclass of antitumor ether lipids, kill cancer cells via a non-apoptotic mechanism of action.[34,41] Structure-activity studies have revealed the important role of the *C*-2 amino group in GAELs as it affects cytotoxicity.[39,43] Some cationic amphiphilic drugs (CADs) have also been shown to induce non-apoptotic lysosomal cell death,[104] perhaps, due to their amphipathic nature. In addition, several cationic amphiphiles have been proposed to have membranolytic mode of action, which may possibly explain their selectivity towards cancer cells.[131] It should be noted that most CADs are easily protonated at normal body pH, leaving them with a net positive charge. Meanwhile, cancer cell membranes typically carry a net negative charge due to elevated expression of anionic molecules such as phosphatidylserine,[132,133] *O*-glycosylated mucins,[134,135] sialilated gangliosides,[136] and heparan sulfates,[137] whereas, normal mammalian cell membranes are basically composed of zwitterionic phospholipids and sterols.[138] The electrostatic gradient generated between negatively charged cancer cell membranes and positively charged CADs may be responsible for their affinity towards cancer cells. Upon binding to cell membranes, hydrophobicity of molecules becomes very crucial to determine how well it permeates such membrane.[139–141] In addition, membrane fluidity is typically increased in cancer cells relative to their healthy counterparts,[142,143] which may facilitate cancer cell membrane destabilization by membrane-bound CADs. Consequently, classical cationic amphiphiles are often believed to possess intrinsic properties that could be selective for cancer cells as well as being more suited to cross the negatively charged hydrophobic membranes of cancer cells.

In another vein, some cationic antimicrobial peptides (CAPs) have been reported to show additive or synergistic effects in cancer therapy when combined with conventional chemotherapeutic agents.[144–146] Several models have been developed to explain the possible

mechanisms of interactions between CAPs and cell membranes.[147,148] A leading hypothesis for short CAPs is the 'in-plane-diffusion model' that proposes that in-plane insertion of CAPs disturbs the packing of membrane bilayer, leading to membrane thinning and formation of transient pores. This destabilization then allows CAPs to gain access to the cytoplasmic membrane, which is subsequently disrupted by hydrophobic interactions between amphipathic CAPs and the membrane lipid bilayer.[139,149,150] This, perhaps, could be responsible for facilitating the activities of other anticancer agents.

We have therefore been probing the effects of additional cationic groups on GAELs, being a cationic glycolipid, with a view to modulating its amphiphilic nature. Initial studies done with diglycosylated compounds resulted in significant loss of activity.[44] The diglycosylated GDG analogs were synthesized to impart two amino functionalities into one molecule. However, unpublished data has lately revealed that GAELs with two amino substituents on a single sugar molecule, **35** and **36** (Figure 4-1), exhibit better cytotoxic activities compared to the reference GDG **1**. These amino groups that can be easily protonated at normal physiological pH could interact with the highly negative mitochondria trans-membrane potential of eukaryotic cells,[151] and could be potentially trapped within the cytosol of the cell. The fact that analogs with two amino groups on a single sugar molecule display better activities than analogs with two amino groups on separate sugars might suggest an important amphipathic property of the active analogs. Therefore, we were curious about what the effect of having three amino groups with an appropriately compensating hydrophobicity will be. Our previous study that demonstrated the ability to fuse other domains with GDGs without significantly altering cytotoxicity (Chapter 3) gave insights on possible modifications that could be made on GAELs. We decided to investigate the tricationic (triamino) concept by imparting a third amino functionality to **35** using myristylamine analogs. Myristylamine **37** (Figure 4-1) is a cationic lipid that has been

investigated as a non-specific cytotoxic lipid. It exhibited little activity (high CC_{50} values) against epithelial cancer cells, and as such, not as active as GDGs.[43] The amphipathic properties of myristylamine, and the compensating hydrophobicity it may impart on the triamino molecule made it an attractive starting point. We envisaged that myristylamine analogs will not only impart amphiphilicity on GAELs but may also amplify overall cytotoxicity due to the presence of an additional amino group. In the studies described herein, we: a) explored the cytotoxic effects of various triamino analogs at different positions of covalent attachment b) investigated the contribution of a third cationic charge and c) investigated the role of the glycerol backbone of the triamino analogs against cancer cell lines.

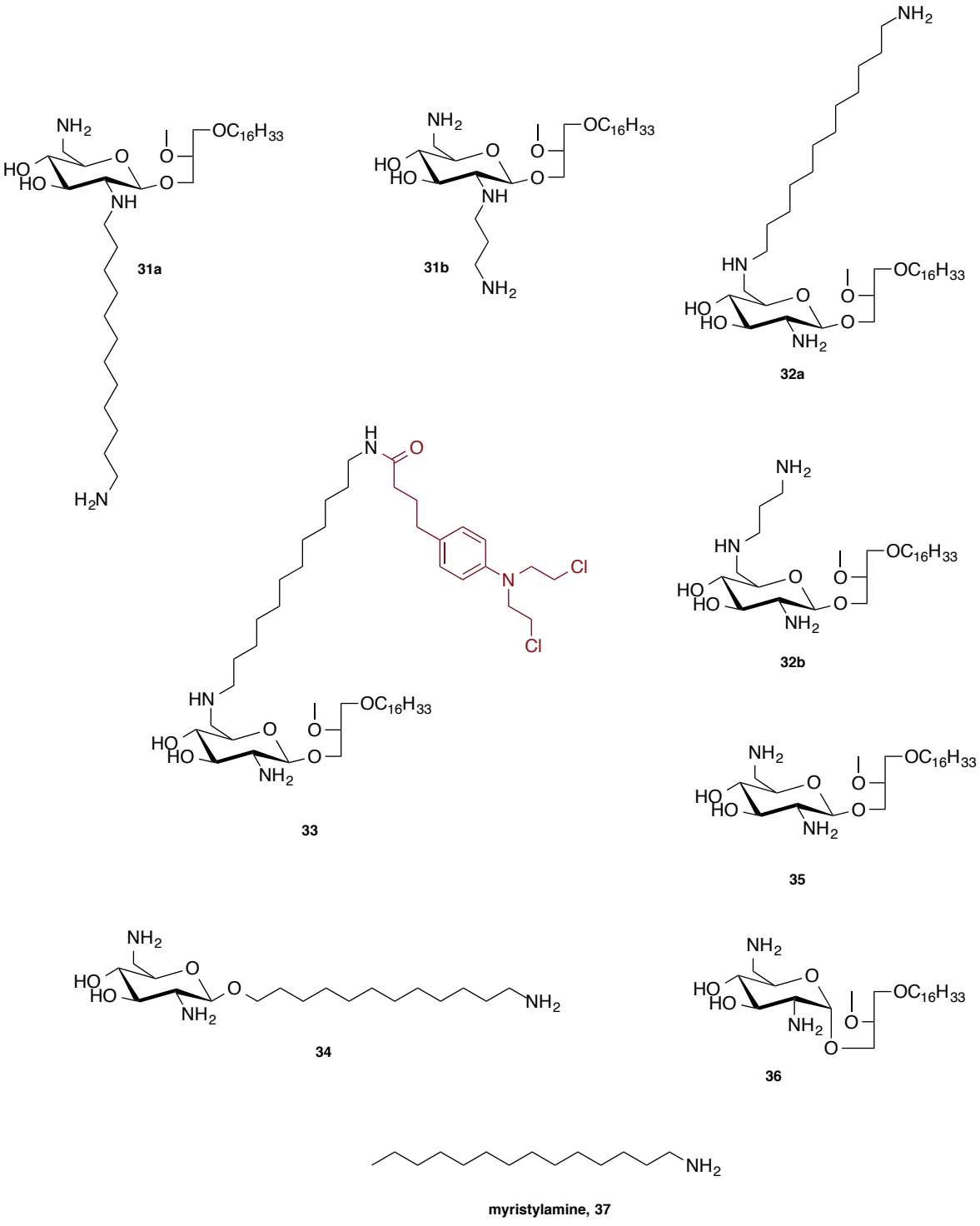


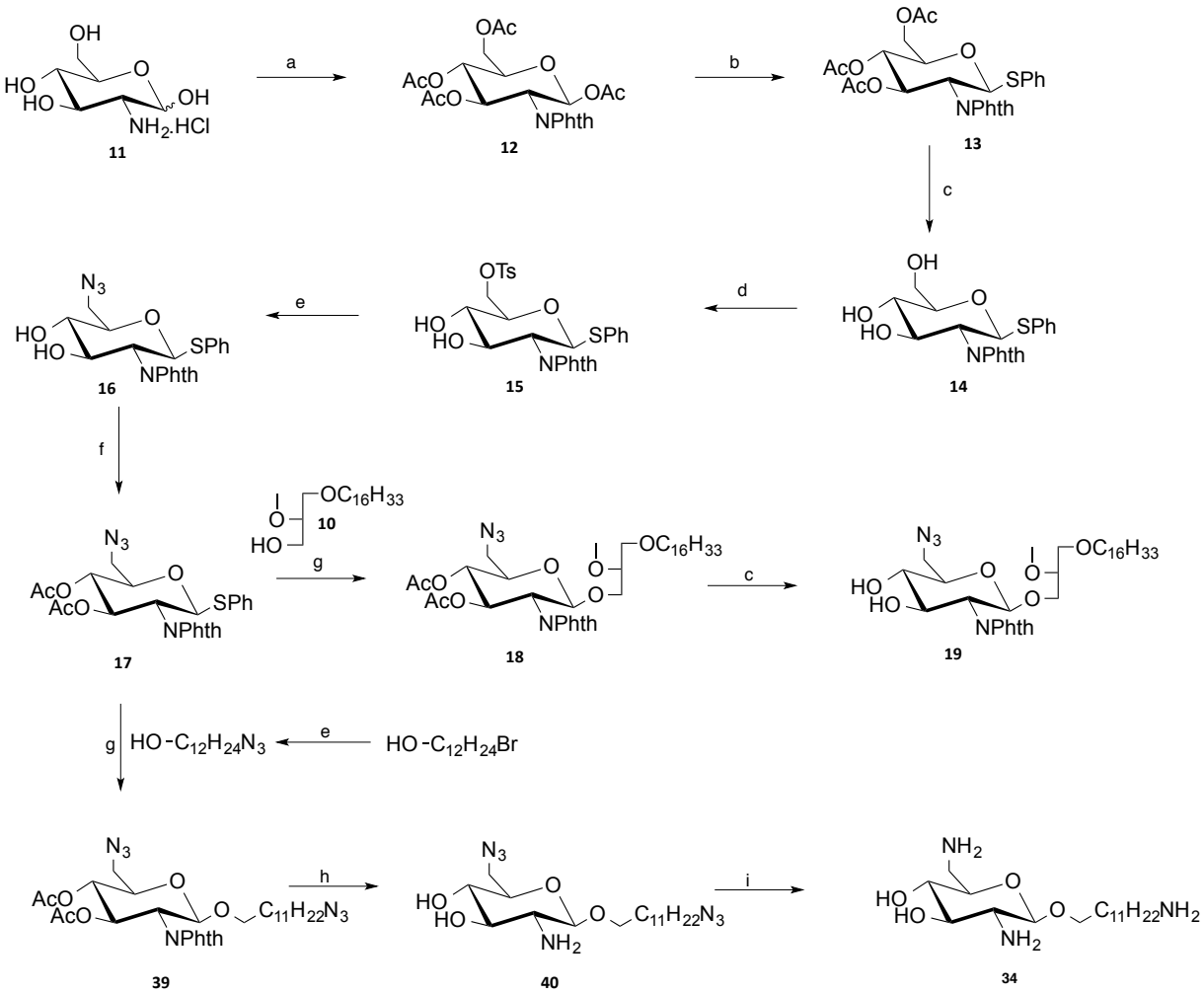
Figure 4-1: Structures of polycationic GAELs and myristylamine

4.2 Results and Discussion

4.2.1 Chemistry

The design of the tricationic hybrids is such that all the functional groups in the dicationic analog would be preserved. Hence, we decided to alkylate either of the amino groups of the dicationic analog, **35** (Figure 4-1). This is to ensure the preservation of their respective charges, which is the ultimate goal of the study. Compounds **31a** and **32a** were thus prepared via reductive amination (Scheme 4-2). The different points of covalent attachment (*C*-2 and *C*-6) were meant to probe the influence of the position of the free amine of GAELs. The amphiphilic nature of myristylamine domain was also investigated by keeping the terminal amino substituent constant while varying the length of the carbon chain to give **31b** and **32b**, which were prepared similarly as **31a** and **32a**. Also as a proof of concept study of the third cationic charge (amino group), we decided to couple chlorambucil with the terminal myristylamine amino group. This converts the free amine into an amide bond that cannot be protonated at physiological pH. It should be noted that the carbon chain length was kept constant and the original dicationic charges (diamino) of the parent compound were maintained. Compound **33** was thus synthesized (Scheme 4-2) using chlorambucil moiety **2,** a molecule that has been earlier reported to be less cytotoxic to epithelial cancer cells (Chapter 3).

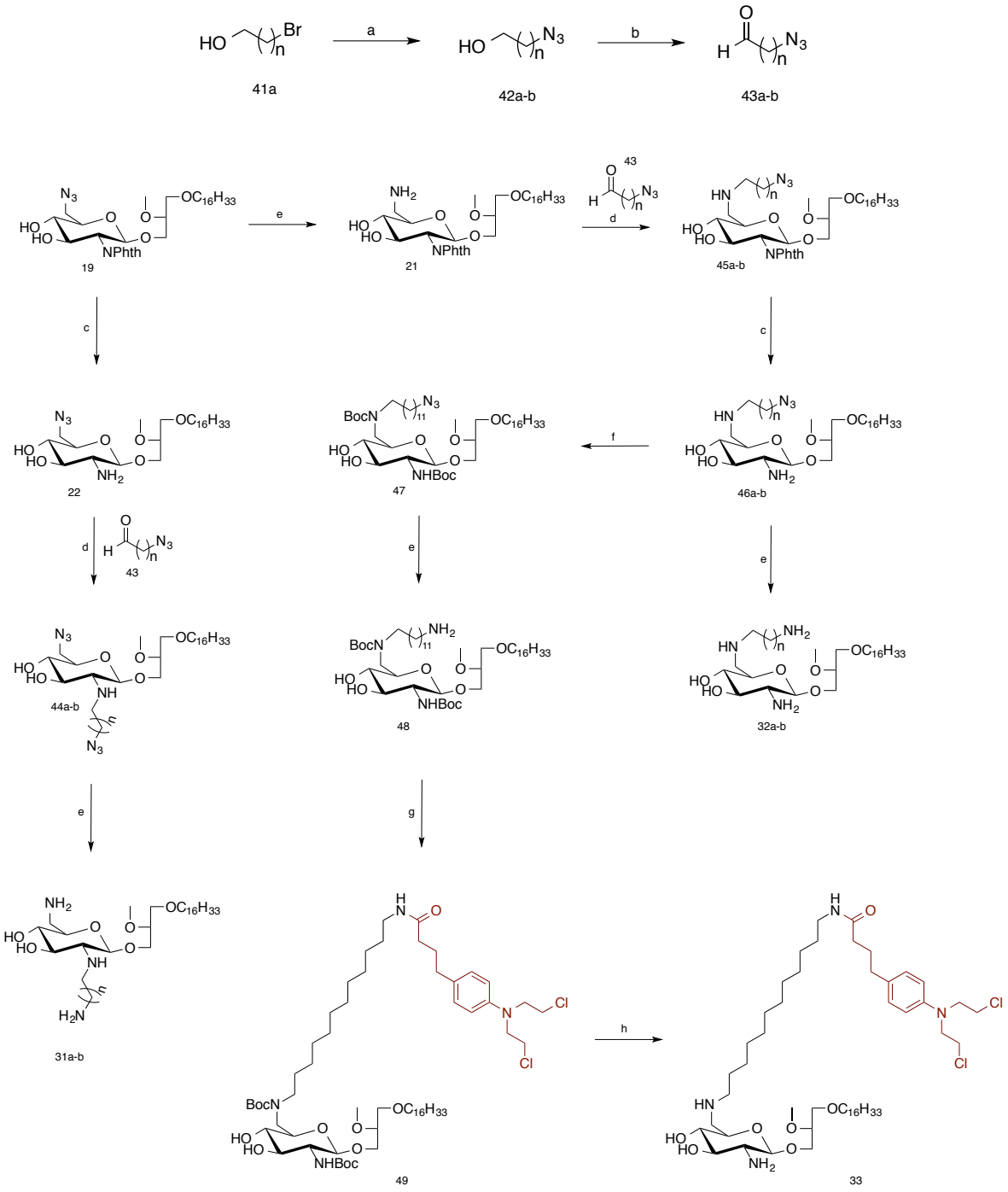
To put all these into context, we also explored the tricationic concept holistically without the glycerol backbone of GAELs. We wanted to examine whether cytotoxicity will be based solely on the triamino group or on the entire molecule as whole. This was informed by the fact that glycerol backbone and lipid moiety is considered essential for cytotoxicity of GAELs.[43,44] Compound **34** was therefore synthesized to validate the generalization of this assumption.



Scheme 4-1: Synthesis of compound **34**

Reagents and conditions: (a) i) NaOH, H_2O, phthalic anhydride, RT, 16 h; ii) pyridine, Ac_2O, DMAP, RT, 16 h; (b) PhSH, $BF_3.Et_2O$, DCM, RT, overnight; (c) NaOMe, methanol, RT, 25 mins, DOWEX; (d) *p*-TsCl, DMAP, pyridine, RT, overnight; (e) NaN_3, DMF, 70 °C, overnight (88 %); (f) pyridine, Ac_2O, DMAP, RT, 16 h; (g) NIS, AgOTf, DCM, RT, 3 h (66 %); (h) ethylenediamine, butanol, 90 °C, 3 h (67 %); (i) $Pd(OH)_2/C$, H_2, methanol, 2 h (80 %)

The synthesis of compounds **31 – 34** commenced from a commercially available glucosamine hydrochloride **11**, with compounds **12 – 22** prepared as previously described in Chapter 3 (Scheme 3-1). Compounds **43a-b** were prepared by S_N2 displacement of the bromine in **41** with sodium azide (anhydrous condition) to give **42a-b** and a successive oxidation of the primary alcohol to aldehyde by pyridinium chlorochromate (PCC) to afford **43a-b** in good yield (Scheme 4-2). This was immediately reacted with **22** via reductive amination (under dry conditions) to afford **44a-b**. It should be noted that an imine intermediate was formed before subsequent reduction with sodium borohydride to prevent double alkylation of the amine. A milder reducing agent such as sodium triacetoxyborohydride [$NaBH(OAc)_3$] may also lead to the formation of tertiary amine (double alkylation) as it could preferentially reduce an imine over an aldehyde, resulting in a second reaction of the secondary amine with the aldehyde.[152,153] Compounds **31a-b** were finally synthesized by the catalytic hydrogenation of **44a-b**. In the same token, **45a-b** were prepared in similar manner from **21** and deprotected by ethylenediamine/butanol (1:1, v/v) to give **46a-b**. **32a-b** were finally synthesized by the catalytic hydrogenation of **46a-b**. However, compound **33** could not be prepared directly from **45a** due to an almost certain nucleophilic displacement of the chlorine atoms of **33** under conditions sufficient enough to cleave phthalimido (strong nucleophile and heat). Compound **47** was thus prepared by re-protecting the primary and secondary amines of **46a** with di-*tert*-butyl dicarbonate (which could be cleaved under mild acidic condition) in methanol and then catalytically hydrogenated to give **48**. This was thereafter coupled to an activated chlorambucil **2** to give **49** in good yield. Compound **49** was finally deprotected with trifluoroacetic acid (95 % v/v) to yield **33** (Scheme 4-2).



Scheme 4-2: Synthesis of compounds **31 – 33** (**a**: n = 11, **b**: n = 2)

Reagents and conditions: (a) NaN_3, DMF, 70 °C, 3 h (94 %) (b) PCC, DCM, 2 h (c) ethylenediamine, butanol, 90 °C, 3 h (70 – 75 %); (d) i) DCM, 0 °C to RT, overnight; ii) $NaBH_4$, acetic acid, 2 h (64 – 73 %); (e) $Pd(OH)_2/C$, methanol, H_2, 2 h (69 – 80 %); (f) Boc_2O, methanol, 50 °C, 4 h (90 %); (g) chlorambucil, DIPEA, TBTU, DMF, RT, overnight (73 %); (h) TFA, DCM, RT, 1h (85 %)

4.2.2 Cytotoxicity

The cytotoxic effects of compounds **31 – 34** against a panel of human epithelial cell lines were assessed using the MTS assay.[129] The cell lines were derived from cancers of the breast (JIMT1, MDA-MB-231, BT474), pancreas (MiaPaCa2) and prostate (DU145, PC3). Exponentially growing cells were treated with test compounds and then incubated for 48 h. All compounds were tested up to 20 μM while **32a** was re-tested at a much lower concentration to generate a more reliable calibration curve for extrapolation. The results for compounds **31 – 34** are displayed in Figure 4-3 and the CC_{50} values are displayed in Table 4-1 along with the values obtained for **1** and **37** from previous studies.[43]

The results revealed that triamino hybrid analogs **31a** and **32a** retained cytotoxic activities as previously demonstrated in GAEL-chlorambucil hybrids. Compound **32a** displayed the most potent activity of all the six triamino analogs tested across all cell lines. It is very encouraging that **32a** displayed CC_{50} values that range from 1.5 μM – 4.0 μM, a consistent 3- to 5-fold increase in activity across all cell lines when compared to **1**, and an amazing 7-fold increase in activity against PC3. It also reduced cell viability to almost zero across all cell lines at a concentration of about 6.0 μM. **32a** is evidently the most active GAEL analog known to date.

Surprisingly, the activity of **31a** was only slightly better than **1** with a barely 2-fold increase against DU145 and PC3. Its CC_{50} values range from 5.5 μM to 8.5 μM and it reduced cell viability to almost zero at a concentration of about 10 μM, except for BT474 cells. The reason for this low activity against BT474 cells is currently unknown, although BT474 human breast carcinomas are characterized by the over expression of human epidermal growth receptor 2 (HER2) and estrogen receptor (ER) but show resistance to ER antagonists such as tamoxifen.[154] Since the difference between compounds **31a** and **32a** is merely the position of myristylamine, it suggests that the point of covalent attachment

might indeed play a significant role in the cytotoxicity of the final molecules. Our results revealed that covalent attachment of myristylamine analog at *C*-6 position of the sugar (**32a**) affords a more active compound than attachment at *C*-2 position (**31a**). The exact implication of these points of attachment is not immediately clear but substitution at *C*-6 position of glucose is known to preserve the exact pharmacophore of the lead GDG compound **1**.

Compounds **31b** and **32b** displayed poor activity against all cell lines with CC_{50} values ranging from 12.5 μM to > 20 μM. The activities of these compounds were less than the reference monoamino analog **1**. The low activity is not surprising as carbon chain length is expected to play a significant role in modulating the amphiphilicity of the final compounds. Longer carbon chain length as presented by **31a** and **32a** is expected to impart a compensating hydrophobicity to the terminal amino group while a shorter carbon chain length with terminal amino group is expected to make the final molecule less hydrophobic. Hydrophobicity plays a vital role in the movement of amphiphilic molecules across cell membranes. While the positively charged groups on the compounds (protonated amino groups) are expected to be electrostatically attracted to the abundantly expressed anionic charges on cancer cell membranes, its hydrophobic nature will determine its uptake, as cell membranes are richly lipophilic. Compounds **31a** and **32a** with longer chain (12 carbons) probably transverse the cell membrane more easily than **31b** and **32b** with shorter chain (3 carbons), thus explaining the significant difference in activity. Interestingly, **32b** that was substituted at *C*-6 position of the sugar also display a slightly better activity than **31b** that was substituted at *C*-2 position, except for MDA-MB-231 cells. This phenomenon seems to be in agreement with the earlier explanation provided on their longer chain counterparts **31a** and **32a**, except that the differences in activity were not as pronounced. In what appears like a pattern, **31b** was also not very active against BT474 similar to **31a**.

Substitution at the *C*-2 position perhaps decreases sensitivity of BT474 cells to GAELs. Mechanistic studies might provide insight on this observation.

Compound **33** was also not as active as **31a** and **32a** against any cell line (CC_{50} values of 10.5 μM to > 20 μM). This is not surprising as compound **33** was synthesized to neutralize the cationic effect of the terminal amino group through the formation of an amide bond that removes the possibility of protonation, while retaining the long carbon chain length. Chlorambucil, a clinical anticancer drug, was attached to the terminal amino group of the triamino molecule as it displays low activity (CC_{50} > 150 μM) against epithelial cells (chapter 3). It is therefore not expected to contribute any significance to cytotoxicity, as it is believed to merely impart hydrophobicity on previous GAEL-chlorambucil hybrids (chapter 3). This could probably explain the significant loss of activity of **33,** as the overall lipophilic-charge ratio is extremely high.

A proof of concept study with compound **34** supports this argument of cationic-hydrophobic balance. Compound **34**, which lacks the glycerol backbone and the long lipid tail of GAELs, was undeniably far less active than all the analogs tested against all cell lines. This result leaves us with two conclusions: 1) glycerol backbone and long lipid tail is essential for the activity of all GAEL analogs, as previously reported.[43] 2) cationic charges presented by amino groups alone are not enough for activity, but a balance of cationic charges and an adequately compensating hydrophobicity.

In summary, the results of the current study have revealed that triamino compounds with longer carbon chain length are more potent than those with shorter chain. Also, diamino compound **35** substituted with myristylamine at *C*-6 position was more active than the one substituted at *C*-2 position. Further study will be to investigate the exact role of myristylamine, since it has been shown to be less cytotoxic to the cancer cell lines used.

Cytotoxicity of compounds 31-34 on a panel of human epithelial cancer cell lines. CC_{50} values (μM)						
Compd	**JIMT1**	**MDA-MB-231**	**BT474**	**MiaPaCa2**	**DU145**	**PC3**
1*	9.0	7.1	8.0	9.0	10.0	13.5
31a	8.0	5.5	13.5	8.5	7.5	8.5
31b	12.5	17.5	>20	14.5	18.5	14.5
32a	3.4	1.5	1.6	4.0	3.8	2.0
32b	13.5	13.5	>20	18.5	16.5	12.5
33	16.0	10.5	13.0	>20	>20	>20
34	>20	>20	>20	>20	>20	>20
37*	27.0	NT	NT	18.0	16.5	24.0

Breast (JIMT1, MB-MDA-231, BT474), pancreas (MiaPaCa2) and prostate (DU145, PC3) cancer cell lines. The CC_{50} value is defined as the concentration required to decrease cell viability by 50% relative to the untreated control. Values were determined by MTS assay. The CC_{50} values were obtained by estimating the drug concentration at 50% viability on the *y-axis* of the plots for each cell line. NT: Not Tested. (*Previously published).[43]

Table 4-1: Cytotoxicity of compounds **31 – 34** against human epithelial cancer cell lines

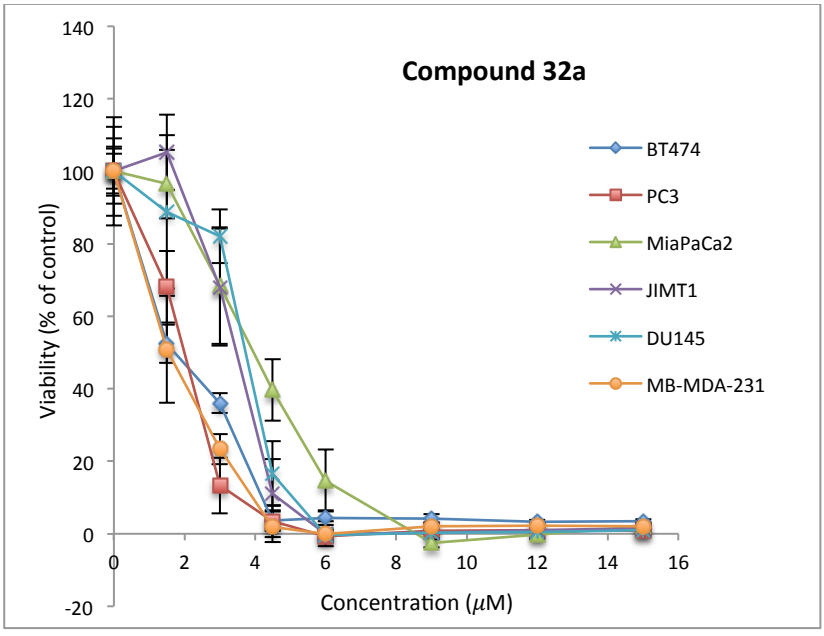


Figure 4-2: Effect of compound **32a** on the viability of epithelial cancer cells at lower concentrations

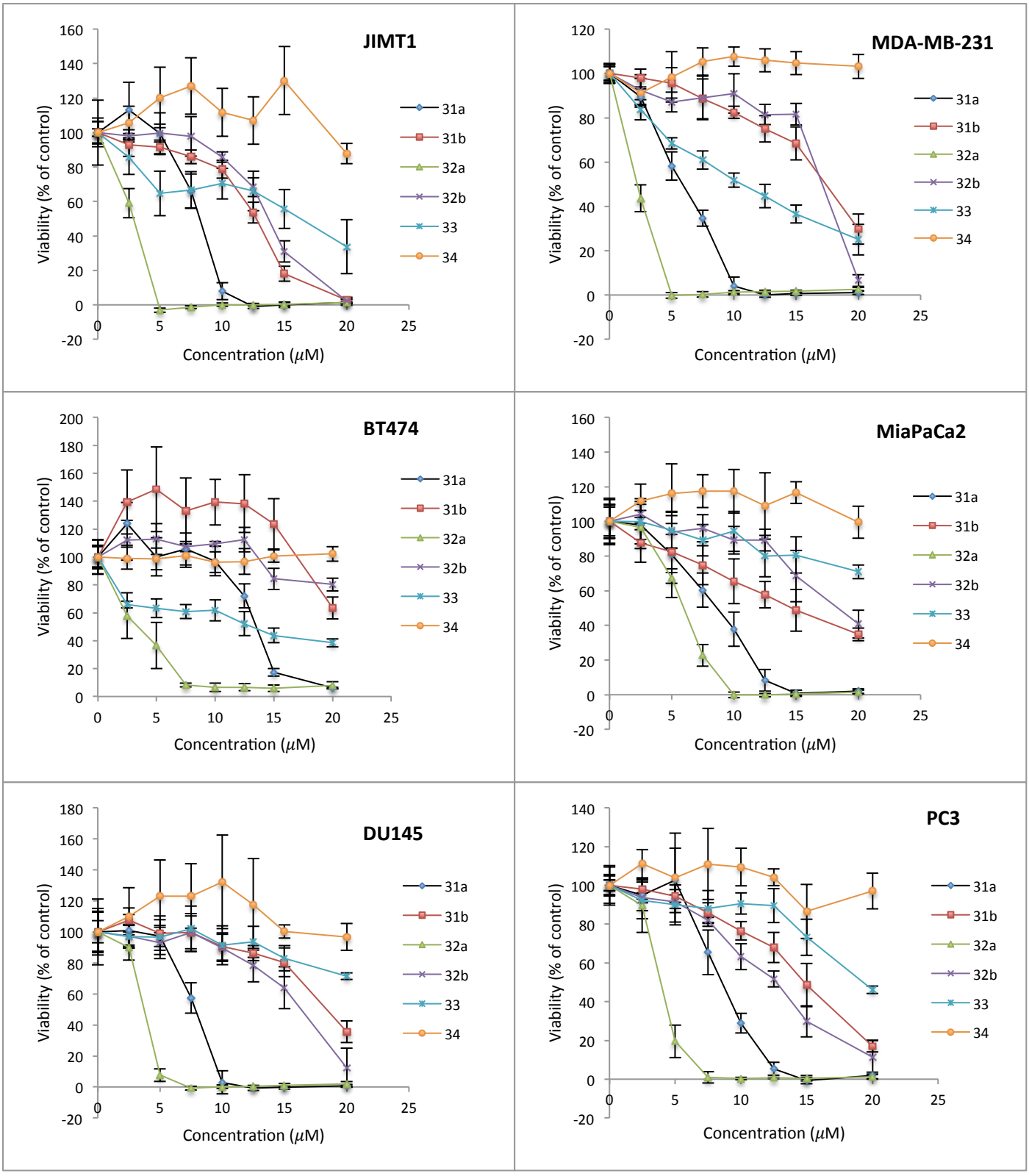


Figure 4-3: Effects of compounds **31** – **34** on the viability of epithelial cancer cells

Error bars indicate standard deviations from six representative experiments (n=6)

4.3 Conclusions

The triamino and reference compounds **31 – 34** were synthesized in good yields via the adaptation of several synthetic procedures. Our results have shown that triamino compounds with a compensating hydrophobicity are more active than the mono- and diamino analogs. We have been able to also demonstrate that amphiphilicity is key to modulating the activities of GAELs. Lastly, the observation that triamino analogs that were covalently attached at *C*-6 position of the sugar displayed better activities than those attached at the *C*-2 position will effectively guide future modifications on this class of drugs.

We believe that the synthesis of the triamino analog **32a**, which is the most potent GAEL known to date, has opened a new chapter in the development of clinically relevant GAELs.

CHAPTER FIVE: CONCLUSIONS AND OUTLOOK

5 CONCLUSIONS AND OUTLOOK

5.1 Conclusions

In spite of the challenges associated with the development of hybrid drugs (chapter 1.4.1.1), it remains one of the few practicable and viable approaches to confront the menace of drug resistance, not just to cancer, but also microorganisms. Hybrid anticancer agents are expected to possess enhanced efficacies compared to the parent agents or combination.[70] Notwithstanding the fact that only one hybrid drug (estramustine) has been approved for the treatment of prostate cancer,[130] it seems only a matter of time before many more hybrid drugs succeed in clinical trials and ultimately make it into the clinic.

CSCs have generally been implicated in drug resistance and metastases, the two major problems that have bedeviled a cure for cancer.[23–25,155,156] With the recent reports of GAEL analogs as one of the very few agents capable of killing cancer stem cells (CSCs),[42] perhaps due to their apoptosis-independent mechanism of action, combining GAELs with other agents that can shrink the bulk tumor mass could be a promising avenue to develop novel therapeutics. The potentials of GDGs to overcome these twin-problem in cancer treatment, especially with the more potent triamino analog, may present an alternative regimen in cancer management if successful. Thus triamino GDG analogs should serve as the future lead for optimizing GAEL molecules.

5.2 Outlook

All synthesized and tested compounds in this project were β-glucosamine analogs. Our previous report,[42] and some unpublished data indicate that α-analogs of GAELs are more potent than β-analogs. If this phenomenon is universal, it is expected that α-triamino analogs will be more active than the β-analogs. In the same token, galactose analogs (especially α-galactosamine), which have been reported to be more active than the glucose analogs, may provide some additional benefits in the triamino concept.

The ability to fuse other domains on the lead GDG **1** molecule in a hybrid fashion, as demonstrated in chapters 3 and 4, gives a spark of hope for future development of GAELs as hybrid drugs. This might indeed become very relevant in the optimization of therapeutic efficacies, where additional domains on GAELs may be necessary to properly 'guide' their movement in the body, shield them from enzymatic activities and/or for targeted delivery to specific sites of action to minimize side effects.

Future work will involve carrying out hemolytic studies on this class of drugs, especially the triamino compounds, to determine their level of toxicity or otherwise to red blood cells. Tolerability studies in mice should also be performed to evaluate toxicity and determine its therapeutic index. In addition, the expanding knowledge on CSCs offers the possibility of generating novel targets that could discriminate between normal and cancer stem cells. This will improve therapeutic efficacy and could even make cancer curative while obviating systemic toxicity.

The future of GAELs holds a lot of promise and it will be interesting to see how all these parameters play out in the quest to finally develop clinically relevant drugs.

CHAPTER SIX: EXPERIMENTAL SECTION

6 EXPERIMENTAL PROCEDURES

6.1 Chemistry

General: With the exception of 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol **(10)** that was purchased from Chem-Implex Inc. (Wood Dale, IL, USA), all chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on Bruker AMX-300 and AMX-500 spectrometers (Germany), and chemical shifts, δ, reported in parts per million (ppm). ^{1}H and ^{13}C of compounds were assigned based on Proton, COSY, Carbon-13, DEPT-135 and HSQC experiments. Thin-layer chromatography (TLC) was carried out on aluminum-backed silica gel 60 F_{254} GF plates (Merck KGaA, Germany), and visualized under ultraviolet light and/or charring with 10 % H_2SO_4 in methanol. Compounds were purified by flash chromatography on silica gel 60 (230-400 ASTM mesh) or reverse-phase C18 silica gel (Silicyle, USA). Electrospray ionization mass spectrometry (ESI-MS) on a Varian 500-MS IT spectrometer (USA) was obtained for all samples while matrix assisted laser desorption ionization (MALDI) coupled with time of flight (ToF) mass analyzer was used to characterize the molecular weight of final compounds.

1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-*N*-phthalimido-glucopyranoside (12): D-Glucosamine hydrochloride **11** (2.00 g, 9.28 mmol) and NaOH (0.80 g, 20 mmol) were dissolved in water (20.0 ml) and stirred for 30 mins at RT. Phthalic anhydride (1.40 g, 9.45 mmol) was added to the mixture and stirred overnight at RT. The mixture was concentrated in *vacuo* and the resulting residue dissolved in pyridine (10.0 ml) with the addition of acetic anhydride (12.0 ml) and catalytic amount of dimethylaminopyridine (DMAP). This was stirred overnight at RT and concentrated to dryness. The resultant dark brown solid was dissolved in CH_2Cl_2 (20.0 ml) and washed with 10 % HCl (20.0 ml ×2), saturated $NaHCO_3$ (20.0 ml ×3), H_2O (20.0 ml ×1) and saturated brine (20.0 ml ×1) successively. The organic

layer was dried over anhydrous Na_2SO_4, concentrated under low pressure and purified by flash chromatography (hexanes/ethyl acetate, 1:1, v/v) to yield **12** (2.95 g, 65 %). NMR data were consistent with literature.[157]

Phenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-*N*-phthalimido-1-thio-glucopyranoside (13): A solution of **12** (2.50 g, 5.24 mmol) in CH_2Cl_2 (30.0 ml) was treated with thiophenol (0.6 ml, 10.8 mmol) and $BF_3.Et_2O$ (2.0 ml, 8.89 mmol) and stirred overnight at RT. The reaction mixture was washed with ice-cold water (×2), saturated $NaHCO_3$ (×3) and saturated aq. NaCl successively. It was then dried over anhydrous Na_2SO_4 and concentrated in *vacuo* to give an oily residue. The resulting residue was purified by flash chromatography (hexanes/ethyl acetate, 2:1, v/v) to give **13** (1.72 g, 62.2 %). NMR data were consistent with earlier reports.[158]

Phenyl 2-deoxy-2-*N*-phthalimido-1-thio-glucopyranoside (14): Compound **13** (1.5 g, 2.84 mmol) was dissolved in a solution of sodium methoxide (0.08 g, 1.48 mmol) in methanol (30.0 ml) and stirred for 25 mins at RT. The reaction was monitored with TLC and quenched with a catalytic amount of DOWEX ion exchange resin. The resulting mixture was filtered by suction, concentrated in *vacuo* and purified by flash chromatography (100 % ethyl acetate) to afford **14** (0.92 g, 80.7 %). ^{1}H NMR (300 MHz, MeOD): δ = 7.96 – 7.81 (m, 4H), 7.47 – 7.19 (m, 5H), 5.60 (d, *J* = 10.2 Hz, 1H, H-1), 4.28 (dd, *J* = 10.2, 7.9 Hz, 1H), 4.19 – 4.04 (m, 1H), 4.02 – 3.89 (m, 1H), 3.80 – 3.69 (m, 1H), 3.57 – 3.39 (m, 2H). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{20}H_{19}NO_8S_2Na^+$: 578.09, found: 578.1.

Phenyl 6-*O*-*p*-toluenesulfonyl-2-deoxy-2-*N*-phthalimido-1-thio-glucopyranoside (15): A solution of **14** (0.92 g, 2.29 mmol) in dry pyridine (15.0 ml) was treated with *p*-toluenesulfonylchloride (0.88 g, 4.64 mmol), DMAP (0.07 g, 0.57 mmol) and stirred overnight at RT. The mixture was concentrated in *vacuo*, re-dissolved in saturated $NaHCO_3$

and the resulting aqueous layer extracted with ethyl acetate (×3). The combined organic layer was dried over anhydrous Na_2SO_4, concentrated in *vacuo* and purified by flash chromatography (hexanes/ethyl acetate, 1:2, v/v) to give **15** (0.89 g, 70 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.89 – 7.07 (m, 13H), 5.49 (d, *J* = 10.3Hz, 1H, H-1), 4.40 – 4.21 (m, 2H), 3.79 – 3.67 (m, 2H), 3.62 – 3.48 (m, 2H), 2.40 (s, 3H). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{27}H_{26}NO_6SNa^+$: 424.08, found: 424.1.

Phenyl 6-deoxy-6-azido-2-deoxy-2-*N*-phthalimido-1-thio-glucopyranoside (16): A solution of **15** (0.80 g, 1.44 mmol) in dry DMF (10.0 ml) was treated with NaN_3 (0.94 g, 14.4 mmol) and stirred overnight at 70 °C under nitrogen gas. Resulting solution was concentrated in *vacuo*, re-dissolved in saturated brine and extracted with ethyl acetate (×3). The combined organic layer was dried over anhydrous Na_2SO_4, concentrated in *vacuo* and purified by flash chromatography (hexanes/ethyl acetate 1:2, v/v) to afford **16** (0.57 g, 93 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.86 – 7.65 (m, 4H), 7.47 – 7.18 (m, 5H), 5.53 (d, *J* = 10.4 Hz, 1H, H-1), 4.24 (dd, *J* = 10.4, 8.5 Hz, 1H, H-2), 4.16 – 4.04 (m, 2H), 3.61 – 3.35 (m, 3H). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{20}H_{18}N_4O_5SNa^+$: 449.09, found: 449.1.

Phenyl 3,4-di-*O*-acetoxy-6-deoxy-6-azido-2-deoxy-2-*N*-phthalimido-1-thio-glucopyranoside (17): Compound **16** (0.55 g, 1.30 mmol) was dissolved in pyridine (10.0 ml) and acetic anhydride (8.0 ml) was added. A catalytic amount of DMAP was added and the reaction was stirred overnight at RT. Methanol was added to quench excess acetic anhydride, concentrated in *vacuo* and purified by flash chromatography (hexanes/ethyl acetate, 1:1, v/v) to afford **17** (0.58 g, 87 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.94 – 7.74 (m, 4H), 7.36 – 7.24 (m, 5H), 5.78 (dd, *J* = 8.5, 9.8 Hz, 1H, H-3), 5.08 (d, *J* = 9.5 Hz, 1H, H-1), 4.36 (dd, *J* = 9.8, 10.4 Hz, 1H, H-4), 4.14 (dd, *J* = 9.5, 8.5 Hz, 1H, H-2), 3.90 – 3.80 (m, 1H), 3.43 – 3.33 (m, 2H), 2.06 (s, 3H, COC***H*****$_3$**), 2.01 (s, 3H, CO***CH*****$_3$**). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{24}H_{22}N_4O_7SNa^+$: 533.11, found: 533.1.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(6`-azido-6`-deoxy-3`,4`-di-*O*-acetoxy-2`-deoxy-2`-*N*-phthalimido-glucopyranoside)-*sn*-glycerol (18): A solution of **17** (0.58 g, 1.14 mmol), **10** (0.45 g, 1.36 mmol) and *N*-iodosuccinimide (NIS) (0.32 g, 1.40 mmol) in dry CH_2Cl_2 (20.0 ml) was treated with silver triflate, AgOTf (0.04 g, 0.14 mmol). After stirring for 3 h at RT under nitrogen gas, the insoluble NIS was filtered using celite, The filtrate was then washed with $Na_2S_2O_3$ (×2), $NaHCO_3$ (×3), H_2O (×1) and saturated brine (×1) successively. The organic layer was dried over anhydrous Na_2SO_4, concentrated in *vacuo* and purified by flash chromatography (hexanes/ethyl acetate, 3:2, v/v) to afford 18 (0.72 g, 70 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.90 – 7.70 (m, 4H), 5.85 (dd, *J* = 10.8, 8.9 Hz, 1H, H-3), 5.39 (d, *J* = 8.4 Hz, 1H, H-1), 5.06 (dd, *J* = 9.5 Hz, 1H, H-4), 4.33 (dd, *J* = 10.8, 8.4 Hz, 1H, H-2), 3.96 – 3.82 (m, 1H), 3.62 (dd, *J* = 10.7, 5.0 Hz, 1H), 3.57 – 3.40 (m, 4H), 3.37 – 3.05 (m, 7H), 2.04 (s, 3H, -COC***H₃***), 1.87 (s, 3H, -COC***H₃***), 1.39 (m, 2H), 1.26 (s, 26H), 0.88 (t, *J* = 6.9 Hz, 3H, CH_2C***H₃***). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{38}H_{58}N_4O_{10}Na^+$: 753.41, found: 753.4.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(6`-azido-6`-deoxy-2`-deoxy-2`-*N*-phthalimido-glucopyranoside)-*sn*-glycerol (19): Compound **18** (0.62 g, 0.85 mmol) was dissolved in a solution of sodium methoxide (0.05 g, 0.93 mmol) in methanol (20.0 ml) and stirred for 25 mins at RT. The reaction was monitored with TLC and quenched with a catalytic amount of DOWEX ion exchange resin. The resulting mixture was filtered by suction, concentrated in *vacuo* and purified by flash chromatography (hexanes/ethyl acetate, 1:1, v/v) to afford **19** (0.44 g, 80 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.95 – 7.68 (m, 4H), 5.35 (d, *J* = 8.5 Hz, 1H, H-1), 4.24 (dd, *J* = 10.4, 8.5 Hz, 1H) 4.16 – 4.04 (m, 3H), 3.70 – 3.51 (m, 3H), 3.50 – 3.37 (m, 9H), 1.45 – 1.35 (m, 2H), 1.35 – 1.15 (m, 26H), 0.89 (t, *J* = 6.8 Hz, 3H, -CH_2C***H₃***). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{34}H_{54}N_4O_8Na^+$: 699.82, found: 699.8.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(6`-acetamido-6`-deoxy-3`-*O*-acetoxy-2`-deoxy-2`-*N*-phthalimido-glucopyranoside)-*sn*-glycerol (20):** A solution of 18 (0.1 g, 0.14 mmol) in THF (6.0 ml) was treated with trimethyphosphine (1.2 ml) and water (1.0 ml). The reaction was stirred for 3 h at RT to give 20 (where the 4` acetate group migrates to the 6` position *in situ*). The mixture was concentrated in *vacuo* and purified by flash chromatography (hexanes/ethyl acetate, 1:1, v/v) to yield 20 (0.07 g, 71.8 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.90 – 7.68 (m, 4H), 5.74 (dd, *J* = 10.7, 9.0 Hz, 1H, H-3), 5.35 (d, *J* = 8.5 Hz, 1H, H-1), 4.47 (d, *J* = 4.2 Hz, 1H), 4.17 (dd, *J* = 10.7, 8.5 Hz, 1H, H-2), 4.12 – 4.04 (m, 1H), 3.86 (dd, *J* = 10.8, 4.7 Hz, 1H), 3.60 – 3.51 (m, 2H), 3.45 (m, 1H), 3.34 – 3.24 (m, 2H), 3.24 – 3.10 (m, 6H), 2.11 (s, 3H, -COC*H₃***), 1.92 (s, 3H, -COC***H₃***), 1.46 – 1.34 (m, 2H), 1.34 – 1.14 (m, 26H), 0.87 (t, *J* = 6.9 Hz, 3H, CH_2C***H₃***). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{38}H_{60}N_2O_{10}Na^+$: 727.41, found: 727.4.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(6`-acetamido-6`-deoxy-2`-amino-2`-deoxy-glucopyranoside)-*sn*-glycerol (6): A solution of **20** (0.06 g, 0.085 mmol) in butanol (2.0 ml) was treated with ethylenediamine (2.0 ml) and stirred for 3 h at 90 °C. The mixture was concentrated under high *vacuo* and purified by flash chromatography (dichloromethane/methanol, 7:1, v/v) and reverse-phase C18 silica gel to give **6** (0.0294 g, 65 %). $[\alpha]_D^{25}$ = -4.6° (*c* = 0.1, MeOH); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.23 (d, *J* = 7.9 Hz, 1H, H-1), 3.96 – 3.82 (m, 2H), 3.69 – 3.49 (m, 3H), 3.56 – 3.42 (m, 7H), 3.33 – 3.12 (m, 3H), 2.71 (dd, *J* = 9.7, 7.9 Hz, 1H, H-2), 2.05 (s, 3H, -COC***H₃***), 1.63 – 1.55 (m, 2H), 1.29 – 1.23 (m, 26H), 0.89 (t, *J* = 6.9

Hz, 3H, CH_2C***H₃***); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.5 (C-amide), 104.8 (C-1), 79.2, 75.2, 75.0, 71.9, 70.4, 70.1, 69.7, 58.0, 57.0, 40.1, 31.92, 29.6, 29.5, 29.4, 26.1, 23.0, 22.7, 14.1 ppm. HRMS: *m/z* $[M + Na]^+$ calc'd for $C_{28}H_{56}N_2O_7Na^+$: 555.3985, found: 555.391.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(6`-amino-6`-deoxy-2`-deoxy-2`-*N*-phthalimido-glucopyranoside)-*sn*-glycerol (21): A solution of **19** (0.05 g, 0.077 mmol) in methanol (5.0 ml) at RT was treated with catalytic amount of $Pd(OH)_2/C$ (10 % wt.) and stirred under H_2 gas atmosphere for 1h. The resulting mixture was filtered, concentrated in *vacuo* and purified by flash chromatography (dichloromethane/methanol, 6:1, v/v) to yield **21** (0.0382 g, 80 %). ^{1}H NMR (500MHz, MeOD): δ = 7.95 – 7.68 (m, 4H), 5.25 (d, *J* = 8.5 Hz, 1H, H-1), 4.30 (dd, *J* = 10.4, 9.5 Hz, 1H) 4.04 – 3.83 (m, 3H), 3.59 – 3.29 (m, 4H), 3.28 – 3.09 (m, 8H), 1.45 – 1.35 (m, 2H), 1.35 – 1.15 (m, 26H), 0.90 (t, *J* = 6.9 Hz, 3H, $-CH_2C\boldsymbol{H_3}$). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{34}H_{56}N_2O_8Na^+$: 643.39, found: 644.4.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-{6`-*N*-(4-[bis(2-chlorethyl)amino]benzenebutanoyl)-6`-deoxy-2`-deoxy-2`-*N*-phthalimido-glucopyranoside}-*sn*-glycerol (5): A solution of **2** (0.018 g, 0.058 mmol) in dry DMF (5.0 ml) was pre-activated with TBTU (0.0186 g, 0.058 mmol) and diisopropylethylamine (0.3 ml) and stirred for 20 mins at RT under nitrogen gas. Compound **21** (0.03 g, 0.048 mmol) was subsequently added and stirred overnight at RT under nitrogen gas. The resulting mixture was concentrated in *vacuo* and purified by flash chromatography (100 % ethyl acetate) to yield **5** (0.04 g, 92 %). $[\alpha]_D^{25}$ = -3.9° (*c* = 0.1, MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 7.90 – 7.76 (m, 4H), 7.07 (d, *J* = 8.4 Hz, 2H), 6.69 (d, *J* = 8.6 Hz, 2H), 5.12 (d, *J* = 8.4 Hz, 1H, H-1), 4.30 (dd, *J* = 10.7, 8.7 Hz, 1H, H-3), 3.98 (dd, *J* = 10.7, 8.6 Hz, 1H, H-2), 3.78 (dd, *J* = 10.9, 4.3 Hz, 1H), 3.71 (m, 4H), 3.71 – 3.60 (m, 8H), 3.52 (dd, *J* = 11.0, 4.6 Hz, 1H), 3.50 – 3.43 (m, 2H), 3.30 – 3.18 (m, 2H), 3.19 – 3.03 (m, 2H), 3.11 (s, 2H), 2.54 (t, *J* = 7.5 Hz, 2H), 2.26 (t, *J* = 7.4Hz, 2H), 1.95 – 1.80 (m, 2H), 1.37 – 1.33 (m, 2H), 1.28 (m, 26H), 0.89 (t, *J* = 6.9 Hz, 3H, $-CH_2C\boldsymbol{H_3}$); ^{13}C NMR (126 MHz, MeOD): δ = 175.4 (C-amide), 144.6, 134.0, 130.3, 129.3, 112.2, 112.1, 98.9 (C-1), 78.7, 74.7, 72.4, 71.1, 70.6, 69.3, 67.9, 57.2, 56.4, 53.2, 53.1,

40.4, 40.3, 33.8, 33.6, 32.8, 31.7, 29.4, 29.3, 29.2, 29.1, 29.0, 27.7, 26.7, 25.7, 22.3, 13.1 ppm. ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{48}H_{73}Cl_2N_3O_9Na^+$: 928.4662, found: 928.449.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(6`-azido-6`-deoxy-2`-amino-2`-deoxy-glucopyranoside)-*sn*-glycerol (22): A solution of **19** (0.06 g, 0.093 mmol) in butanol (2.0 ml) was treated with ethylenediamine (2.0 ml) and stirred for 3 h at 90°C. The mixture was concentrated under high *vacuo* and purified by flash chromatography (dichloromethane/methanol, 4:1, v/v) to give **22** (0.036 g, 75.5 %). ^{1}H NMR (300 MHz, MeOD): δ = 4.26 (d, *J* = 8.0 Hz, 1H, H-1), 3.91 (dd, *J* = 9.1, 4.5 Hz, 1H), 3.68 (dd, *J* = 10.6, 4.5 Hz, 1H), 3.61 – 3.51 (m, 3H), 3.51 – 3.37 (m, 8H), 3.30 – 3.21 (m, 2H), 2.60 (dd, *J* = 8.0, 3.8 Hz, 1H, H-2), 1.60 1.55 (m, 2H), 1.30 – 1.27 (m, 26H), 0.89 (t, *J* = 6.7 Hz, 3H, -$CH_2C\boldsymbol{H_3}$). ESI-MS: *m/z* $[M + H]^+$ calc'd for $C_{26}H_{53}N_4O_6^+$: 516.39, found: 516.4.

Compound (23): A solution of **2** (0.0131 g, 0.043 mmol) in dry DMF (4.0 ml) was pre-activated with TBTU (0.0138 g, 0.043 mmol) and diisopropylethylamine (0.2 ml) by stirring for 20 mins at RT under nitrogen gas. Compound **22** (0.02 g, 0.039 mmol) was subsequently added and stirred overnight at RT under nitrogen gas. The resulting mixture was concentrated in *vacuo* and purified by flash chromatography (100 % ethyl acetate) to yield **23** (0.0282 g, 90.1 %). ^{1}H NMR (300 MHz, MeOD): δ = 7.02 (d, *J* = 8.0 Hz, 2H), 6.62 (d, *J* = 8.1 Hz, 2H), 4.40 (d, *J* = 8.4 Hz, 1H, H-1), 3.80 (dd, *J* = 10.4, 4.4 Hz, 1H), 3.71 – 3.40 (m, 8H), 3.45 – 3.15 (m, 13H), 2.49 (t, *J* = 7.6 Hz, 2H), 2.17 (t, *J* = 7.6 Hz, 2H), 1.89 – 1.77 (m, 2H), 1.43 (t, *J* = 7.6 Hz, 2H), 1.31 – 1.21 (m, 2H), 1.29 – 1.23 (m, 26H), 0.88 (t, *J* = 6.9 Hz, 3H, $CH_2C\boldsymbol{H_3}$). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{40}H_{69}Cl_2N_5O_7Na^+$: 824.45, found: 824.5.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-{6`-amino-2`-*N*-(4-[bis(2-chlorethyl)amino] benzenebutanoyl)-2`,6`-dideoxy-glucopyranoside}-*sn*-glycerol (3): A solution of **23** (0.028 g, 0.035 mmol) in methanol (3.0 ml) at RT was treated with catalytic amount of $Pd(OH)_2/C$ (10 % wt.) and stirred under H_2 gas atmosphere for 1 h. The resulting mixture was filtered, concentrated in *vacuo* and purified by flash chromatography (dichloromethane/methanol, 6:1, v/v) to yield **3** (0.0218 g, 80.2 %). $[\alpha]_D^{25}$ = 12.6° (*c* = 0.1, MeOH); ^{1}H NMR (300 MHz, MeOD): δ = 7.01 (d, *J* = 8.0 Hz, 2H), 6.60 (d, *J* = 8.1 Hz, 2H), 4.40 (d, *J* = 8.4 Hz, 1H, H-1), 3.80 (dd, *J* = 10.4, 4.4 Hz, 1H), 3.71 – 3.40 (m, 8H), 3.45 – 3.15 (m, 13H), 2.49 (t, *J* = 7.6 Hz, 2H), 2.17 (t, *J* = 7.6 Hz, 2H), 1.89 – 1.77 (m, 2H), 1.43 (t, *J* = 6.7 Hz, 2H), 1.31 – 1.21 (m, 2H), 1.29 –1.23 (m, 26H), 0.88 (t, *J* = 6.9 Hz, 3H, $CH_2C\boldsymbol{H_3}$); ^{13}C NMR (75 MHz, MeOD): δ = 176.4 (C-amide), 146.0, 132.0 130.7, 113.6, 103.1 (C-1), 80.6, 76.6, 75.8, 73.8, 72.7, 71.5, 69.8, 58.4, 57.2, 54.7, 43.4, 41.8, 37.0, 35.3, 33.1, 30.9, 30.8, 30.7, 30.6, 30.5, 29.2, 27.3, 23.8, 14.6 ppm. ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{40}H_{71}Cl_2N_3O_7Na^+$: 798.4567, found: 798.456.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(6`-azido-6`-deoxy-2`-deoxy-2`-*N*-Boc-glucopyranoside)-*sn*-glycerol (24): A solution of **22** (0.0368 g, 0.0712 mmol) in methanol (3.0 ml) was treated with Boc_2O (0.023 g, 0.107 mmol) and Et_3N (0.1 ml). The resulting solution was then stirred overnight at 50 °C. The mixture was concentrated in *vacuo* and purified by flash chromatography (hexanes/ethyl acetate, 2:1, v/v) to afford **24** (0.0408 g, 93 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.41 (d, *J* = 8.2 Hz, 1H, H-1), 3.98 (dd, *J* = 11.3, 3.7 Hz, 1H), 3.70 (dd, *J* = 11.3, 4.1 Hz, 1H), 3.61 – 3.52 (m, 4H), 3.52 – 3.39 (m, 9H), 3.34 – 3.27 (m, 1H), 1.60 – 1.52 (m, 2H), 1.45 (s, 9H, $-COOC(C\boldsymbol{H_3})_3$), 1.37 – 1.18 (m, 26H), 0.88 (t, *J* = 6.9 Hz, 3H, $-CH_2C\boldsymbol{H_3}$). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{31}H_{60}N_4O_8Na^+$: 639.43, found: 639.5.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(6`-amino-6`-deoxy-2`-deoxy-2`-*N*-Boc-glucopyranoside)-*sn*-glycerol (25): A solution of **24** (0.037 g, 0.06 mmol) in methanol (5.0 ml) at RT was treated with catalytic amount of $Pd(OH)_2/C$ (10 % wt.) and stirred under H_2 gas atmosphere for 1h. The resulting mixture was filtered, concentrated in *vacuo* and purified by flash chromatography (dichloromethane/methanol, 6:1, v/v) to yield **25** (0.0285 g, 80.4 %). ^{1}H NMR (300 MHz, MeOD): δ = 4.43 (d, *J* = 8.2 Hz, 1H, H-1), 3.98 (dd, *J* = 11.2, 3.7 Hz, 1H), 3.70 (dd, *J* = 11.2, 4.1 Hz, 1H), 3.61 – 3.52 (m, 4H), 3.52 – 3.39 (m, 9H), 3.34 – 3.24 (m, 1H), 1.60 – 1.52 (m, 2H), 1.45 (s, 9H, -COOC(C***H*₃**)$_3$), 1.37 – 1.18 (m, 26H), 0.88 (t, *J* = 6.9 Hz, 3H, -CH_2C***H*₃**). ESI-MS: *m/z* [M + Na]$^+$ calc'd for $C_{31}H_{62}N_2O_8Na^+$: 613.44, found: 613.5.

Compound (26): A solution of **2** (0.0162 g, 0.0534 mmol) in dry DMF (4.0 ml) was pre-activated with TBTU (0.017 g, 0.0534 mmol) and diisopropylethylamine (0.2 ml) and stirred for 20 mins at RT under nitrogen gas. Compound **25** (0.028 g, 0.049 mmol) was subsequently added and stirred overnight at RT under nitrogen gas. The resulting mixture was concentrated in *vacuo* and purified by flash chromatography (100 % ethyl acetate) to yield **26** (0.0347 g, 80.7 %). ^{1}H NMR (300 MHz, MeOD): δ = 7.10 (d, *J* = 8.4 Hz, 2H), 6.71 (d, *J* = 8.6 Hz, 2H), 4.33 (d, *J* = 8.0 Hz, 1H, H-1), 3.88 (dd, *J* = 10.3, 4.4 Hz, 1H), 3.81 – 3.59 (m, 9H), 3.62 – 3.34 (m, 13H), 3.17 (dd, *J* = 9.5, 8.3 Hz, 1H), 2.57 (t, *J* = 7.6 Hz, 2H), 2.26 (t, *J* = 7.5 Hz, 2H), 1.97 – 1.85 (m, 2H), 1.62 – 1.51 (m, 2H), 1.47 (s, 9H, -COOC(C***H*₃**)$_3$), 1.42 1.26 (m, 26H), 0.87 (t, *J* = 6.9 Hz, 3H, CH_2C***H*₃**). ESI-MS: *m/z* [M + Na]$^+$ calc'd for $C_{45}H_{79}Cl_2N_3O_9Na^+$: 898.51, found: 898.5

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-{2`-amino-6`-*N*-(4-[bis(2-chlorethyl)amino] benzenebutanoyl)-2`,6`-dideoxy-glucopyranoside}-*sn*-glycerol (4): A solution of **26** (0.033 g, 0.038 mmol) in CH_2Cl_2 (3.0 ml) was treated with trifluoroacetic acid (1.0 ml) and stirred for 1 h at RT. Mixture was concentrated under low *vacuo* and purified by flash

chromatography (dichloromethane/methanol, 6:1, v/v) to give **4** (0.0247 g, 93 %). $[\alpha]_D^{25}$ = 8.9° (*c* = 0.1, MeOH); ^{1}H NMR (300 MHz, MeOD): δ = 7.09 (d, *J* = 8.4 Hz, 2H), 6.71 (d, *J* = 8.6 Hz, 2H), 4.58 (d, *J* = 8.3 Hz, 1H, H-1), 3.96 (dd, *J* = 10.7, 3.8 Hz, 1H), 3.81 – 3.59 (m, 10H), 3.63 – 3.51 (m, 4H), 3.50 – 3.43 (m, 6H), 3.43 – 3.39 (m, 1H), 3.25 – 3.17 (m, 1H), 2.94 – 2.81 (m, 1H), 2.56 (t, *J* = 7.6 Hz, 2H), 2.26 (t, *J* = 7.5 Hz, 2H), 1.99 – 1.82 (m, 2H), 1.63 – 1.52 (m, 2H), 1.42 – 1.26 (m, 26H), 0.87 (t, *J* = 6.9 Hz, 3H, $CH_2C\boldsymbol{H_3}$); ^{13}C NMR (75 MHz, MeOD): δ = 176.7 (C-amide), 146.1, 131.8, 130.6, 113.6, 100.5 (C-1), 80.4, 76.4, 73.5, 72.8, 71.2, 69.9, 58.3, 57.7, 54.6, 41.8, 41.3, 36.5, 35.3, 33.1, 30.8, 30.7, 30.7, 30.5, 29.2, 27.3, 23.8, 14.5 ppm. ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{40}H_{71}Cl_2N_3O_7Na^+$: 798.4567, found: 798.465.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2`-acetamido-2`-deoxy-6`-azido-6`-deoxy-3`,4`-di-*O*-acetoxy-glucopyranoside)-*sn*-glycerol (27): A solution of **22** (0.30 g, 0.58 mmol) in pyridine (10.0 ml) with acetic anhydride (8.0 ml) and a catalytic amount of DMAP was stirred at RT for 5 h. Methanol was subsequently added to quench excess acetic anhydride, concentrated in *vacuo* and purified by flash chromatography (100 % ethyl acetate) to afford **27** (0.32 g, 85 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.86 (d, *J* = 8.6 Hz, 1H), 5.23 (dd, *J* = 10.6, 9.2 Hz, 1H, H-3), 4.93 (t, *J* = 9.6 Hz, 1H, H-4), 4.71 (d, *J* = 8.4 Hz, 1H, H-1), 4.09 (dd, *J* = 7.1 Hz, 1H), 3.93 – 3.82 (m, 2H), 3.72 – 3.63 (m, 2H), 3.48 – 3.34 (m, 7H), 3.17 (dd, *J* = 13.4, 2.6 Hz, 1H), 2.01 (s, 6H, 2[-COC$\boldsymbol{H_3}$]), 1.91 (s, 3H, -COC$\boldsymbol{H_3}$), 1.57 – 1.48 (m, 2H), 1.33 – 1.19 (m, 26H), 0.85 (t, *J* = 6.9 Hz, 3H, $CH_2C\boldsymbol{H_3}$). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{32}H_{58}N_4O_9Na^+$: 665.41, found: 665.4

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2`-acetamido-2`-deoxy-6`-azido-6`-deoxy-glucopyranoside)-*sn*-glycerol (28): Compound **27** (0.25 g, 0.39 mmol) was dissolved in a solution of sodium methoxide (0.05 g, 0.93 mmol) in methanol (20.0 ml) and stirred at RT for 30 mins. The reaction was quenched with a catalytic amount of DOWEX ion exchange

resin, filtered, concentrated in *vacuo* and purified by flash chromatography (dichloromethane/methanol, 7:1, v/v) to give **28** (0.17 g, 79.6 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.44 (d, *J* = 8.1 Hz, 1H, H-1), 4.06 (dd, *J* = 11.8, 2.6 Hz, 1H), 3.76 (dd, *J* = 11.8, 3.8 Hz, 1H), 3.61 (dd, *J* = 9.6, 5.2 Hz, 1H), 3.57 – 3.41 (m, 13H), 2.04 (s, 3H, -COC***H₃***), 1.60 – 1.52 (m, 2H), 1.35 – 1.22 (m, 26H), 0.88 (t, *J* = 6.9 Hz, 3H, CH_2C***H₃***). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{28}H_{54}N_4O_7Na^+$: 581.39, found: 581.5

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2`-acetamido-2`-deoxy-6`-amino-6`-deoxy-glucopyranoside)-*sn*-glycerol (7): A solution of **28** (0.10 g, 0.18 mmol) in methanol (7.0 ml) was treated with a catalytic amount of $Pd(OH)_2/C$ (10 % wt.) and stirred under H_2 gas atmosphere for 1 h at RT. The resulting mixture was filtered, concentrated in *vacuo* and purified by flash chromatography (dichloromethane/methanol, 3:1, v/v) to afford **7** (0.07 g, 76 %). $[\alpha]_D^{25}$ = -3.9° (*c* = 0.1, MeOH); ^{1}H NMR (300 MHz, MeOD): δ = 4.43 (d, *J* = 8.1 Hz, 1H, H-1), 3.88 (dd, *J* = 7.6, 8.0 Hz, 1H, H-3), 3.66 – 3.34 (m, 12H), 3.32 – 3.13 (m, 1H), 3.04 (dd, *J* = 13.5, 3.0 Hz, 1H), 2.83 (dd, *J* = 13.4, 6.3 Hz, 1H), 1.99 (s, 3H, -COC***H₃***), 1.60 – 1.50 (m, 2H), 1.35 –1.22 (s, 26H), 0.87 (t, *J* = 6.9 Hz, 3H, CH_2C***H₃***). ^{13}C NMR (75 MHz, MeOD): δ = 174.2 (C-amide), 102.8 (C-1), 80.7, 79.1, 78.8, 78.6, 78.2, 77.4, 76.6, 73.3, 71.3, 69.9, 59.2, 58.1, 43.9, 33.3, 31.0, 30.9, 30.8, 30.7, 30.6, 27.4, 24.0, 15.3 ppm. ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{28}H_{56}N_2O_7Na^+$: 555.3985, found: 555.401.

1-(12-azidododecyl)-6-azido-6-deoxy-3,4-di-*O*-acetoxy-2-deoxy-2-*N*-phthalimido glucopyranoside (39): A solution of **17** (0.15 g, 0.294 mmol), **42a-b** (0.08 g, 0.353 mmol) and *N*-iodosuccinimide (NIS) (0.10g, 0.44 mmol) in dry CH_2Cl_2 (15.0 ml) was treated with silver triflate, AgOTf (0.011 g, 0.044 mmol) and stirred for 3 h at RT under nitrogen gas. The insoluble NIS was filtered using celite and the filtrate washed with $Na_2S_2O_3$ (×2), $NaHCO_3$ (×3), H_2O (×1) and saturated brine (×1) successively. The organic layer was dried over anhydrous Na_2SO_4, concentrated in *vacuo* and purified by flash chromatography

(hexanes/ ethyl acetate, 3:1, v/v) to give **39** (0.12 g, 66 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.90 – 7.65 (m, 4H), 5.77 (dd, *J* = 10.8, 9.0 Hz, 1H, H-3), 5.37 (d, *J* = 8.5 Hz, 1H, H-1), 5.02 (dd, *J* = 10.1, 9.0 Hz, 1H, H-4), 4.29 (dd, *J* = 10.8, 8.5 Hz, 1H, H-2), 4.15 – 3.96 (m, 2H), 3.90 - 3.77 (m, 2H), 3.50 – 3.38 (m, 1H), 3.26 – 3.15 (m, 2H), 2.01 (s, 6H), 1.63 – 1.50 (m, 4H), 1.48 – 1.28 (m, 16H). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{30}H_{41}N_7O_8Na^+$: 650.80, found: 650.8

1-(12-azidododecyl)-6-azido-6-deoxy-2-amino-2-deoxy glucopyranoside (40): A solution of **39** (0.11 g, 0.18 mmol) in butanol (4.0 ml) was treated with ethylenediamine (4.0 ml) and stirred for 3 h at 90 °C. The mixture was concentrated under high *vacuo* and purified by flash chromatography (dichloromethane/ methanol, 7:1, v/v) to afford **40** (0.049 g, 67 %). ^{1}H NMR (300 MHz, MeOD): δ = 4.28 (d, *J* = 7.9 Hz, 1H, H-1), 3.96 – 3.85 (m, 1H), 3.61 – 3.50 (m, 1H), 3.50 – 3.40 (m, 3H), 3.36 -3.20 (m, 4H), 2.70 – 2.55 (m, 1H), 1.73 – 1.52 (m, 4H), 1.48 – 1.28 (m, 16H). ESI-MS: *m/z* $[M + H]^+$ calc'd for $C_{18}H_{35}N_7O_4H^+$: 413.28, found: 413.3

1-(12-aminododecyl)-6-amino-6-deoxy-2-amino-2-deoxy glucopyranoside (34): A solution of **40** (0.045 g, 0.11mmol) in methanol (5.0 ml) was treated with catalytic amount of $Pd(OH)_2/C$ (10 % wt.) and stirred under H_2 gas atmosphere for 2 h. The resulting mixture was filtered, concentrated in *vacuo* and purified by reverse-phase C18 silica gel to give **34** (0.032 g, 80 %). $[\alpha]_D^{25}$ = 36.6° (*c* = 0.1, MeOH); ^{1}H NMR (300 MHz, MeOD): δ = 4.28 (d, *J* = 7.9 Hz, 1H, H-1), 3.96 – 3.85 (m, 1H), 3.61 – 3.50 (m, 1H), 3.50 – 3.40 (m, 3H), 3.36 -3.20 (m, 4H), 2.70 – 2.55 (m, 1H), 1.73 – 1.52 (m, 4H), 1.48 – 1.28 (m, 16H); ^{13}C NMR (75 MHz, MeOD) δ = 104.35 (C-1), 77.39, 77.22, 72.79, 70.81, 58.35, 52.83, 52.50, 30.76, 30.70, 30.66, 30.65, 30.50, 30.30, 29.95, 27.86, 27.18. HRMS: *m/z* $[M + Na]^+$ calc'd for $C_{18}H_{39}N_3O_4Na^+$: 400.2578, found: 400.259

Compound 43a-b: A solution of **41** in dry DMF was treated with NaN_3 at 90°C for 3 h. The mixture was then concentrated under *vacuo*, worked up with H_20 (×2) and brine (×1) successively and re-concentrated to give **42a-b** in excellent yield. **42a-b** was subsequently dissolved in dry DCM, treated with PCC and stirred at RT for 2 h. The reaction was monitored with TLC using $KMnO_4$ stain. The resulting mixture was filtered through a pad of silica and concentrated under low *vacuo* to give **43a-b**. The resulting compound was used immediately without further purification.

Compound 44a: A solution of **22** (0.20 g, 0.39 mmol) in dry DCM was treated with **43a** (0.088 g, 0.39 mmol) and stirred overnight at 0 °C to RT. Two drops of acetic acid and sodium borohydride, $NaBH_4$ (0.045 g, 1.161 mmol) in methanol were then added to the mixture and stirred further for 2 h at RT. The resulting mixture was concentrated in *vacuo,* extracted with ethyl acetate and purified by flash chromatography (dichloromethane/ methanol, 7:1, v/v) to afford **44a** (0.20 g, 69 %). ^{1}H NMR (300 MHz, MeOD): δ = 4.60 (d, *J* = 8.1 Hz, 1H, H-1), 3.98 (dd, *J* = 8.9, 4.2 Hz, 1H), 3.74 (dd, *J* = 10.5, 3.8 Hz, 1H), 3.64 – 3.38 (m, 11H), 3.36 – 3.24 (m, 4H), 3.18 – 3.04 (m, 1H), 3.02 – 2.90 (m, 1H), 2.69 (dd, *J* = 10.2, 8.1 Hz, 1H, H-2), 1.72 – 1.50 (m, 6H), 1.48 – 1.28 (m, 42H), 0.88 (t, *J* = 6.9 Hz, 3H). ESM-MS: *m/z* $[M + H]^+$ calc'd for $C_{38}H_{75}N_7O_6H^+$: 726.58, found: 726.7

Compound 44b: A solution of **22** (0.15 g, 0.29 mmol) in dry DCM was treated with **43b** (0.03 g, 0.30 mmol) and stirred overnight at 0 °C to RT. Two drops of acetic acid and sodium borohydride, $NaBH_4$ (0.041 g, 0.91 mmol) in methanol were then added to the mixture and stirred further for 2 h at RT. The resulting mixture was concentrated in *vacuo,* extracted with ethyl acetate and purified by flash chromatography (dichloromethane/ methanol, 7:1, v/v) to afford **44b** (0.11 g, 64 %). ^{1}H NMR (300 MHz, MeOD): δ = 4.43 (d, *J* = 8.1 Hz, 1H, H-1), 3.99 (dd, *J* = 10.6, 4.2 Hz, 1H), 3.89 (dd, *J* = 11.9, 2.0 Hz, 1H), 3.77 – 3.65 (m, 2H), 3.65 – 3.23 (m, 13H), 3.22 – 3.06 (m, 1H), 3.02 – 2.89 (m, 1H), 2.55 (dd, *J*

= 10.4, 8.1 Hz, 1H, H-2) 1.90 – 1.77 (m, 2H), 1.65 – 1.52 (m, 2H), 1.45 – 1.23 (m, 26H), 0.91 (t, *J* = 6.9 Hz, 3H). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{29}H_{57}N_7O_6Na^+$: 622.43, found: 622.5

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-[2`-*N*-(12-aminododecyl)-2`,6`-diamino-2`,6`-dideoxy-glucopyranoside]-*sn*-glycerol (31a): A solution of **44a** (0.20 g, 0.28 mmol) in methanol (5.0 ml) was treated with a catalytic amount of $Pd(OH)_2/C$ (10 % wt.) and stirred under H_2 gas atmosphere for 1 h. The resulting solution was filtered, concentrated in *vacuo* and purified by reverse-phase C18 silica gel to give **31a** (0.14 g, 76 %). $[\alpha]_D^{25}$ = -5.2° (*c* = 0.1, MeOH); ^{1}H NMR (300 MHz, MeOD): δ = 4.60 (d, *J* = 8.1 Hz, 1H, H-1), 3.98 (dd, *J* = 8.9, 4.2 Hz, 1H), 3.74 (dd, *J* = 10.5, 3.8 Hz, 1H), 3.64 – 3.38 (m, 11H), 3.36 – 3.24 (m, 4H), 3.18 – 3.04 (m, 1H), 3.02 – 2.90 (m, 1H), 2.69 (dd, J = 10.2, 8.1 Hz, 1H, H-2), 1.72 – 1.50 (m, 6H), 1.48 – 1.28 (m, 42H), 0.88 (t, *J* = 6.9 Hz, 3H); 13C NMR (75 MHz, MeOD) δ = 102.71 (C-1), 80.40, 77.38, 74.55, 72.88, 72.76, 71.54, 69.35, 63.97, 58.21, 52.67, 52.52, 33.16, 30.90, 30.86, 30.76, 30.71, 30.57, 30.38, 30.02, 29.26, 28.10, 27.93, 27.33, 23.83, 14.60. HRMS: m/z $[M + K]^+$ calc'd for $C_{38}H_{79}N_3O_6K^+$: 712.5606, found: 712.559

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-[2`-*N*-(3-aminopropyl)-2`,6`-diamino-2`,6`-dideoxy-glucopyranoside]-*sn*-glycerol (31b): A solution of **44b** (0.11 g, 0.18 mmol) in methanol (5.0 ml) was treated with a catalytic amount of $Pd(OH)_2/C$ (10 % wt.) and stirred under H_2 gas atmosphere for 1 h. The resulting solution was filtered, concentrated in *vacuo* and purified by reverse-phase C18 silica gel to give **31b** (0.072 g, 72 %). $[\alpha]_D^{25}$ = -3.6° (*c* = 0.1, MeOH); 1H NMR (300 MHz, MeOD): δ = 4.43 (d, *J* = 8.1 Hz, 1H, H-1), 3.99 (dd, *J* = 10.6, 4.2 Hz, 1H), 3.89 (dd, *J* = 11.9, 2.0 Hz, 1H), 3.77 – 3.65 (m, 2H), 3.65 – 3.23 (m, 13H), 3.22 – 3.06 (m, 1H), 3.02 – 2.89 (m, 1H), 2.55 (dd, *J* = 10.4, 8.1 Hz, 1H, H-2) 1.90 – 1.77 (m, 2H), 1.65 – 1.52 (m, 2H), 1.45 – 1.23 (m, 26H), 0.91 (t, *J* = 6.9 Hz, 3H); 13C NMR (75 MHz, MeOD): δ = 104.10 (C-1), 80.58, 78.18, 75.72, 72.70, 72.00, 71.52, 69.65,

64.36, 62.66, 58.18, 33.10, 30.80, 30.77, 30.62, 30.49, 29.35, 27.26, 23.76, 14.47. HRMS: m/z $[M + Na]^+$ calc'd for $C_{29}H_{61}N_3O_6Na^+$: 570.4458, found: 570.447

Compound 45a: A solution of **21** (0.45 g, 0.73 mmol) in dry DCM was treated with **43a** (0.17 g, 0.73 mmol) and stirred overnight at 0 °C to RT. Two drops of acetic acid and sodium borohydride, $NaBH_4$ (0.099 g, 2.19 mmol) in methanol were then added to the mixture and stirred further for 2 h at RT. The resulting mixture was concentrated in *vacuo,* extracted with ethyl acetate and purified by flash chromatography (dichloromethane/ methanol, 10:1, v/v) to afford **45a** (0.46 g, 73 %). ^{1}H NMR (500 MHz, MeOD): δ = 7.92 – 7.78 (m, 4H), 5.21 (d, *J* = 8.5 Hz, 1H, H-1), 4.32 (dd, *J* = 10.8, 8.6 Hz, 1H, H-3), 4.00 (dd, *J* = 10.8, 8.5 Hz, 1H, H-2), 3.84 – 3.72 (m, 1H)**,** 3.63 – 3.53 (m, 1H), 3.50 – 3.38 (m, 3H), 3.30 – 3.23 (m, 5H), 3.23 – 3.09 (m, 6H), 3.07 – 3.01 (m, 2H), 1.87 – 1.80 (m, 2H), 1.73 – 1.66 (m, 2H), 1.50 – 1.17 (m, 44H), 0.89 (t, *J* = 6.9 Hz, 3H). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{46}H_{79}N_5O_6Na^+$: 853.09, found: 853.1

Compound 45b: A solution of **21** (0.21 g, 0.34 mmol) in dry DCM was treated with **43b** (0.035 g, 0.34 mmol) and stirred overnight at 0 °C to RT. Two drops of acetic acid and sodium borohydride, $NaBH_4$ (0.005 g, 0.10 mmol) in methanol were then added to the mixture and stirred further for 2 h at RT. The resulting mixture was concentrated in *vacuo*, extracted with ethyl acetate and purified by flash chromatography (dichloromethane/ methanol, 10:1, v/v) to afford **45b** (0.17 g, 70 %). ^{1}H NMR (500 MHz, MeOD): δ = 7.92 – 7.78 (m, 4H), 5.17 (d, *J* = 8.0 Hz, 1H, H-1), 4.33 (dd, *J* = 10.5, 4.9 Hz, 1H), 3.90 (dd, *J* = 10.5, 3.9 Hz, 1H), 3.61 – 3.42 (m, 7H), 3.38 (t, *J* = 6.7 Hz, 4H), 3.26 (dd, *J* = 10.0, 8.7 Hz, 1H), 3.15 – 3.09 (m, 1H), 2.94 (dd, *J* = 14.2, 2.4 Hz, 1H), 2.68 – 2.55 (m, 5H), 1.83 – 1.68 (m, 3H), 1.61 – 1.51 (m, 2H), 1.39 – 1.27 (m, 26H), 0.89 (t, *J* = 6.8 Hz, 3H). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{37}H_{61}N_5O_8Na^+$: 726.44, found: 726.5

Compound 46a: A solution of **45a** (0.45 g, 0.53 mmol) in butanol (6.0 ml) was treated with ethylenediamine (6.0 ml) and stirred at 90 °C for 3 h. The mixture was concentrated under high *vacuo* and purified by flash chromatography (dichloromethane/ methanol, 3:1, v/v) to give **46a** (0.26 g, 68%). ^{1}H NMR (500 MHz, MeOD): δ = 4.35 (d, *J* = 8.0 Hz, 1H, H-1), 3.93 (dd, *J* = 10.6, 5.0 Hz, 1H), 3.75 (dd, 1H), 3.63 – 3.47 (m, 4H), 3.48 – 3.43 (m, 4H), 3.48 – 3.24 (m, 7H), 3.23 – 3.11 (m, 1H), 3.06 (dd, *J* = 13.1, 9.0 Hz, 1H), 3.01 – 2.91 (m, 1H), 2.63 (dd, *J* = 10.1, 8.0 Hz, 1H), 1.73 – 1.63 (m, 1H), 1.63 – 1.51 (m, 4H), 1.42 – 1.26 (m, 42H), 0.89 (t, *J* = 6.8 Hz, 3H). ESI-MS: *m/z* $[M + H]^+$ calc'd for $C_{38}H_{77}N_5O_6H^+$: 700.59, found: 700.5

Compound 46b: A solution of **45b** (0.17 g, 0.24 mmol) in butanol (4.0 ml) was treated with ethylenediamine (4.0 ml) and stirred at 90 °C for 3 h. The mixture was concentrated under high *vacuo* and purified by flash chromatography (dichloromethane/ methanol, 3:1, v/v) to give **46b** (0.089 g, 65%). 1H NMR (500 MHz, MeOD): δ = 4.23 (d, *J* = 8.0 Hz, 1H, H-1), 3.90 (dd, *J* = 10.5, 4.9 Hz, 1H), 3.66 (dd, *J* = 10.5, 3.9 Hz, 1H), 3.61 – 3.42 (m, 7H), 3.38 (t, J = 6.7 Hz, 4H), 3.26 (dd, *J* = 10.0, 8.7 Hz, 1H), 3.15 – 3.09 (m, 1H), 2.94 (dd, *J* = 14.2, 2.4 Hz, 1H), 2.68 – 2.55 (m, 5H), 1.83 – 1.68 (m, 3H), 1.61 – 1.51 (m, 2H), 1.39 – 1.27 (m, 26H), 0.89 (t, *J* = 6.8 Hz, 3H). ESI-MS: *m/z* $[M + Na]^+$ calc'd for $C_{29}H_{59}N_5O_6Na^+$: 596.44, found: 596.5

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-[6`-*N*-(12-aminododecyl)-2`,6`-diamino-2`,6`-dideoxy-glucopyranoside]-*sn*-glycerol (32a): A solution of **46a** (0.07 g, 0.096 mmol) in methanol (4.0 ml) was treated with a catalytic amount of $Pd(OH)_2$/C (10 % wt.) and stirred under H_2 gas atmosphere for 1 h. The resulting solution was filtered, concentrated in *vacuo* and purified by reverse-phase C18 silica gel to give **32a** (0.048g, 72 %). $[\alpha]_D^{25}$ = -5.9° (*c* = 0.1, MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 4.35 (d, *J* = 7.1 Hz, 1H, H-1), 3.93 (dd, *J* = 10.5, 6.7 Hz, 1H), 3.75 – 3.64 (m, 1H), 3.63 – 3.47 (m, 4H), 3.47 – 3.42 (m, 4H), 3.41 – 3.23 (m,

7H), 3.23 – 3.11 (m, 2H), 3.06 (dd, J = 13.1, 9.0 Hz, 1H), 3.01 – 2.91 (m, 1H), 2.65 (dd, *J* = 9.6, 7.1 Hz, 1H, H-2), 1.73 – 1.63 (m, 1H), 1.63 – 1.51 (m, 4H), 1.42 – 1.26 (m, 41H), 0.89 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (126 MHz, MeOD): δ = 103.11 (C-1), 78.97, 75.98, 73.75, 72.95, 71.21, 69.81, 68.27, 56.79, 56.67, 55.66, 54.70, 53.39, 40.24, 38.62, 35.53, 31.68, 30.23, 29.80, 29.39, 29.36, 29.33, 29.25, 29.22, 29.08, 29.06, 27.26, 26.53, 26.38, 25.88, 22.34, 13.07. HRMS: m/z $[M + Na]^+$ calc'd for $C_{38}H_{79}N_3O_6Na^+$: 696.5867, found: 696.588

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-[6`-*N*-(3-aminopropyl)-2`,6`-diamino-2`,6`-dideoxy-glucopyranoside]-*sn*-glycerol (32b): A solution of **46b** (0.082 g, 0.14 mmol) in methanol (4.0 ml) was treated with a catalytic amount of $Pd(OH)_2$/C (10 % wt.) and stirred under H_2 gas atmosphere for 1 h. The resulting solution was filtered, concentrated in *vacuo* and purified by reverse-phase C18 silica gel to give **32b** (0.054g, 69 %). $[\alpha]_D^{25}$ = -2.8° (*c* = 0.1, MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 4.23 (d, *J* = 8.0 Hz, 1H, H-1), 3.90 (dd, *J* = 10.5, 4.9 Hz, 1H), 3.66 (dd, *J* = 10.5, 3.9 Hz, 1H), 3.61 – 3.42 (m, 7H), 3.38 (t, *J* = 6.7 Hz, 4H), 3.26 (dd, *J* = 10.0, 8.7 Hz, 1H), 3.15 – 3.09 (m, 1H), 2.94 (dd, *J* = 14.2, 2.4 Hz, 1H), 2.68 – 2.55 (m, 5H), 1.83 – 1.68 (m, 3H), 1.61 – 1.51 (m, 2H), 1.39 – 1.27 (m, 26H), 0.89 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (126 MHz, MeOD): δ = 102.52, 78.97, 75.66, 74.45, 72.63, 71.24, 69.83, 68.35, 56.70, 54.70, 51.44, 49.10, 31.66, 29.37, 29.34, 29.26, 29.19, 29.06, 26.26, 25.83, 22.32, 13.04. HRMS: *m/z* $[M + K]^+$ calc'd for $C_{29}H_{61}N_3O_6K^+$: 586.4197, found: 586.420

Compound 47: A solution of **46a** (0.18 g, 0.26 mmol) in methanol (7.0 ml) was treated with boc_2O (0.23 g, 1.03 mmol) and Et_3N (0.2 ml). The reaction was stirred at 50 °C for 5 h. The mixture was then concentrated in *vacuo* and purified by flash chromatography (hexanes/ethyl acetate, 1:1, v/v) to give 47 (0.21 g, 90 %). ^{1}H NMR (500 MHz, MeOD): δ = 4.28 (d, *J* = 8.1 Hz, 1H, H-1), 3.81 (dd, *J* = 10.5, 4.6 Hz, 1H), 3.64 – 3.52 (m, 3H), 3.50 – 3.40 (m, 8H), 3.39 – 3.32 (m, 5H), 3.29 – 3.25 (m, 2H), 3.18 – 3.08 (m, 1H), 1.63 – 1.51

(m, 6H), 1.45 (s, 18H), 1.41 – 1.24 (m, 42H), 0.90 (t, *J* = 6.9 Hz, 3H). ESI-MS: *m/z* [M + Na]$^+$ calc'd for $C_{48}H_{93}N_5O_{10}Na^+$: 922.68, found: 922.8

Compound 48: A solution of **47** (0.21 g, 0.23 mmol) in methanol (6.0 ml) was treated with a catalytic amount of $Pd(OH)_2/C$ (10 % wt.) and stirred under H_2 gas atmosphere for 1 h. The resulting solution was filtered, concentrated in *vacuo* and purified by flash chromatography (dichloromethane/methanol, 5:1, v/v) to give **48** (0.16 g, 80 %). ^{1}H NMR (500 MHz, MeOD): δ = 4.28 (d, *J* = 8.1 Hz, 1H, H-1), 3.81 (dd, *J* = 10.5, 4.6 Hz, 1H), 3.64 – 3.52 (m, 3H), 3.50 – 3.40 (m, 8H), 3.39 – 3.32 (m, 5H), 3.29 – 3.25 (m, 2H), 3.18 – 3.08 (m, 1H), 1.63 – 1.51 (m, 6H), 1.45 (s, 18H), 1.41 – 1.24 (m, 42H), 0.90 (t, *J* = 6.9 Hz, 3H). ESI-MS: *m/z* [M + H]$^+$ calc'd for $C_{48}H_{95}N_3O_{10}H^+$: 874.70, found: 874.7

Compound 49: A solution of chlorambucil, **2** (0.067 g, 0.22 mmol) in dry DMF (5.0 ml) was pre-activated with TBTU (0.07 g, 0.22 mmol) and diisopropylethylamine (0.2 ml), and stirred for 20 mins at RT. Compound **48** (0.16 g, 0.18 mmol) was then added and stirred for 5 h at RT. The resulting mixture was concentrated in *vacuo* and purified by flash chromatography (hexanes/ethyl acetate, 1:1, v/v) to yield **49** (0.15 g, 73 %). ^{1}H NMR (500 MHz, MeOD): δ = 7.09 – 7.01 (m, 2H), 6.71 – 6.64 (m, 2H), 4.66 (d, *J* = 8.4 Hz, 1H, H-1), 4.01 (dd, *J* = 10.6, 4.9 Hz, 1H), 3.77 (dd, *J* = 10.7, 3.0 Hz, 1H), 3.74 – 3.44 (m, 18H), 3.27 – 3.11 (m, 4H), 3.09 – 3.04 (m, 2H), 2.99 – 2.85 (m, 1H), 2.51 (t, *J* = 7.6 Hz, 1H), 2.16 (t, *J* = 7.6 Hz, 2H), 1.90 – 1.80 (m, 2H), 1.74 – 1.65 (m, 2H), 1.56 (t, *J* = 6.8 Hz, 2H), 1.48 (t, *J* = 6.8 Hz, 2H), 1.45 (s, 18H), 1.29 (m, 44H), 0.89 (t, *J* = 6.9 Hz, 3H). ESI-MS: *m/z* [M + H]$^+$ calc'd for $C_{62}H_{112}Cl_2N_4O_{11}Na^+$: 1181.76, found: 1181.9

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-{6'-*N*-[12-*N*-(4-[bis(2-chlorethyl)amino] benzenebutanoyl)-aminododecyl]-2',6'-diamino-2',6'-dideoxy-glucopyranoside}-*sn*-glycerol (33): A solution of **49** (0.10 g, 0.086 mmol) in DCM (5.0 ml) was treated with trifluoroacetic acid (2 ml) and stirred for 1 h. The reaction was then concentrated in *vacuo*

and purified by flash chromatography (dichloromethane/methanol, 5:1, v/v) to give **33** (0 07g, 85 %). $[\alpha]_D^{25}$ = 7.3° (*c* = 0.1, MeOH); ^{1}H NMR (500 MHz, MeOD): δ = 7.09 – 7.01 (m, 2H), 6.71 – 6.64 (m, 2H), 4.66 (d, *J* = 8.4 Hz, 1H, H-1), 4.01 (dd, *J* = 10.6, 4.9 Hz, 1H), 3.77 (dd, *J* = 10.7, 3.0 Hz, 1H), 3.74 – 3.44 (m, 18H), 3.27 – 3.11 (m, 4H), 3.09 – 3.04 (m, 2H), 2.99 – 2.85 (m, 1H), 2.51 (t, *J* = 7.6 Hz, 1H), 2.16 (t, *J* = 7.6 Hz, 2H), 1.90 – 1.80 (m, 2H), 1.74 – 1.65 (m, 2H), 1.56 (t, *J* = 6.8 Hz, 2H), 1.48 (t, *J* = 6.8 Hz, 2H), 1.29 (m, 44H), 0.89 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, MeOD): δ = 174.54 (C-amide), 144.56, 130.37, 129.15, 112.14, 99.03 (C-1), 78.84, 72.15, 72.05, 71.86, 71.36, 69.39, 69.30, 56.97, 55.81, 53.16, 40.27, 38.89, 35.13, 33.79, 31.64, 29.35, 29.34, 29.31, 29.24, 29.22, 29.19, 29.17, 29.06, 29.04, 28.99, 28.95, 28.81, 27.64, 26.55, 26.16, 25.79, 25.69, 22.30, 13.01. HRMS: m/z [M + Na]+ calc'd for $C_{52}H_{96}Cl_2N_4O_7Na^+$: 981.6554, found: 981.660

6.2 Biological

6.2.1 Cell Culture

MDA-MB-231, BT474, MiaPaCa2, DU145 and PC3 cell lines were grown from frozen stocks of cell lines that were originally obtained from ATCC (Manassas, VA, USA). JIMT1 cells were originally obtained from DSMZ (Braunschweig, Germany). MDA-MB-231, JIMT1 and DU145 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), PC3 cells grown in F12K medium while BT474 cells were grown in DMEM/F12K medium. The cells were grown in media supplemented with 10 % fetal bovine serum (FBS), penicillin (100 UmL^{-1}) and streptomycin (0.1 $mgmL^{-1}$). MiaPaCa2 was grown in DMEM supplemented with FBS to a concentration of 10 % and horse serum to a final concentration of 2.5 %.

6.2.2 Cytotoxicity Assay

Cell viability was determined with the CellTitre 96 AQueous One solution (MTS assay kit, Promega). Equal numbers of cancer cells (7500 – 9000) in media (100 μL) were dispersed into 96-well plates. As blanks, media without cells (100 μL) were also placed in some wells and treated similarly to the cell-containing wells. After an incubation period of 24 h, a solution of test compound (100 μL) in medium at twice the desired concentration was added to each well. The treated cells were then incubated further for 48 hours, after which 20 % v/v MTS reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was added to each well. The plates were then incubated for 1 – 4 h on a Nutator mixer in a 5 % CO_2 incubator. The optical density (OD) was read at 490 nm on a SpectraMax M2 plate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The values of blank were subtracted from each value and the viability values of the treated samples relative to the controls with vehicle were calculated. The values for the plots are the means ± standard deviation.

6.2.3 Statistical Analysis

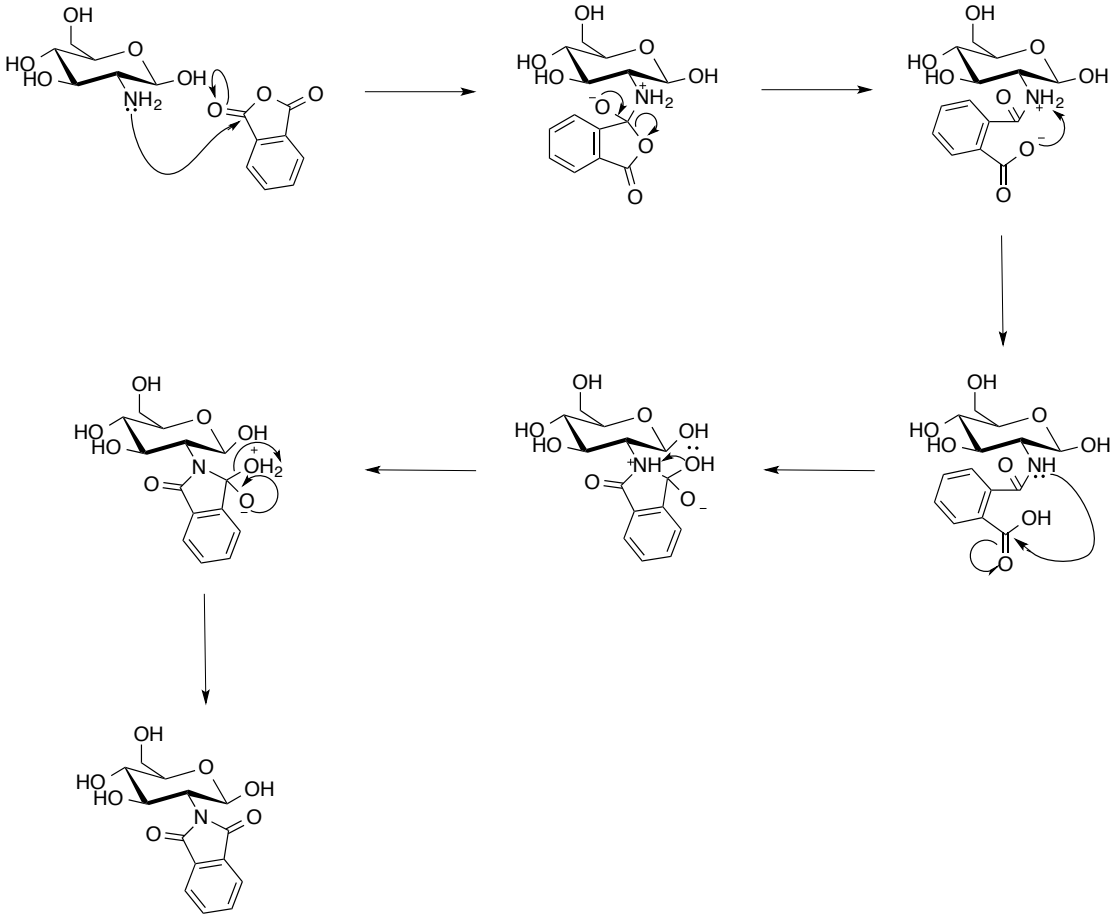
Statistical analysis was performed using a two-tailed paired student's test in order to evaluate the null hypothesis. The cut-off level for statistical significance was set to 5 %. $P < 0.05$ when compared to vehicle treated cells (blank) under same conditions.

CHAPTER SEVEN: REACTION MECHANISMS

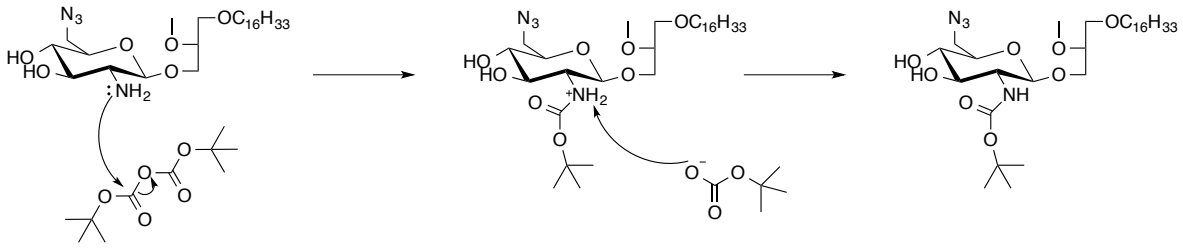
7 GENERAL MECHANISMS OF REACTIONS

7.1 Functional Group Protection

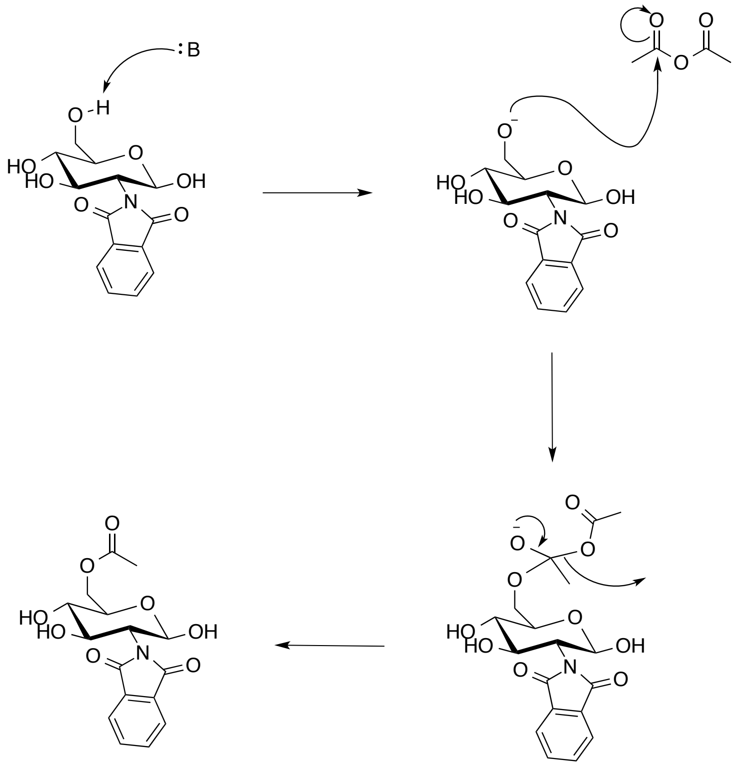
- Protection of free amine with phthalimido



- Protection of free amine with boc anhydride

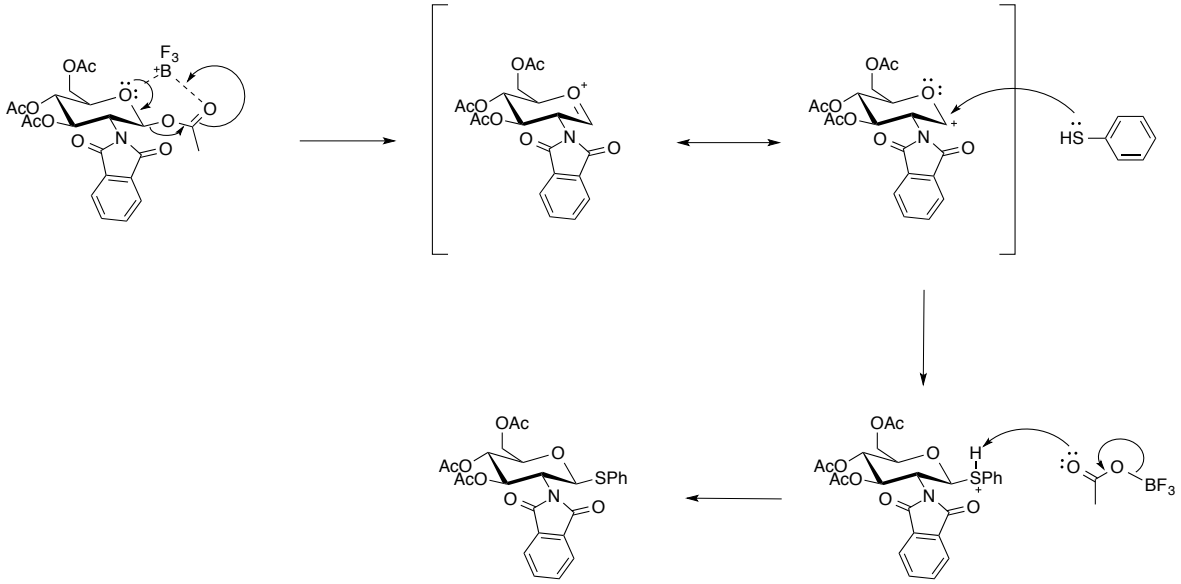


- Acetylation of hydroxyl group

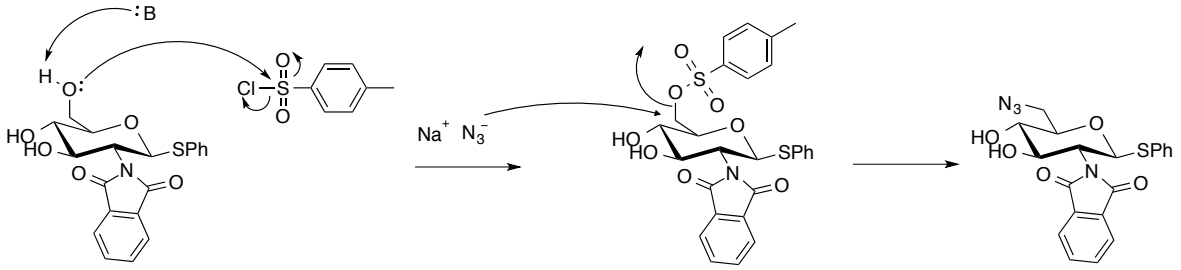


7.2 Functional Group Modifications

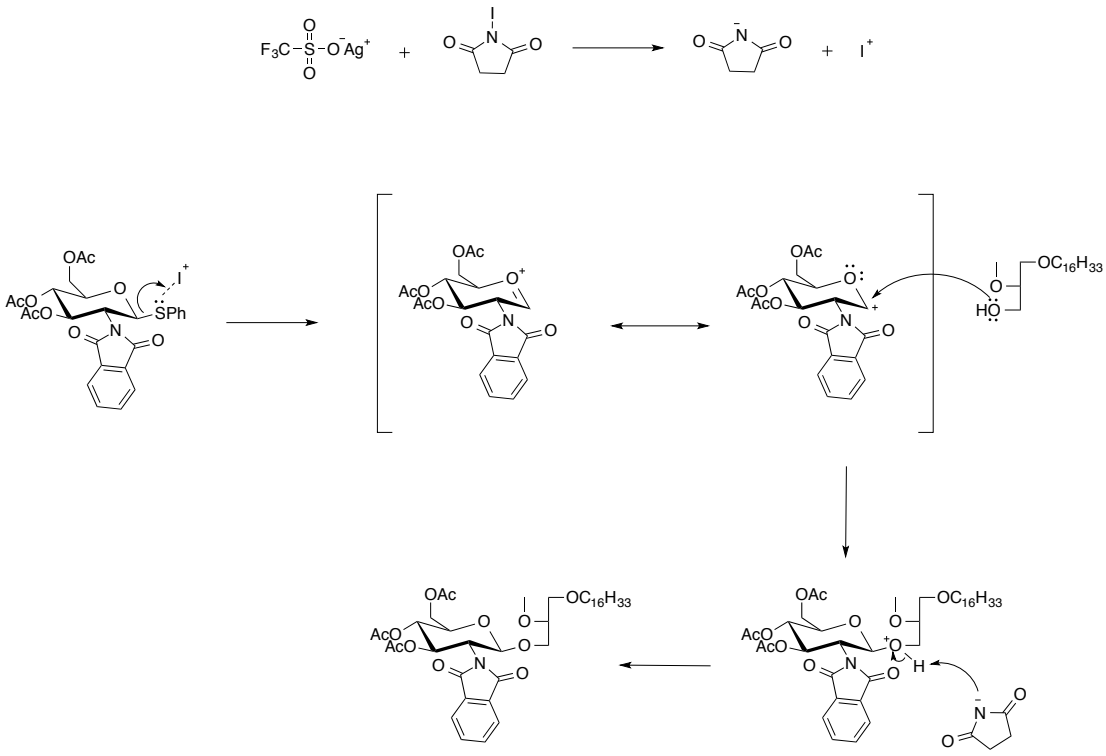
- Conversion of anomeric acetate to thiophenyl group



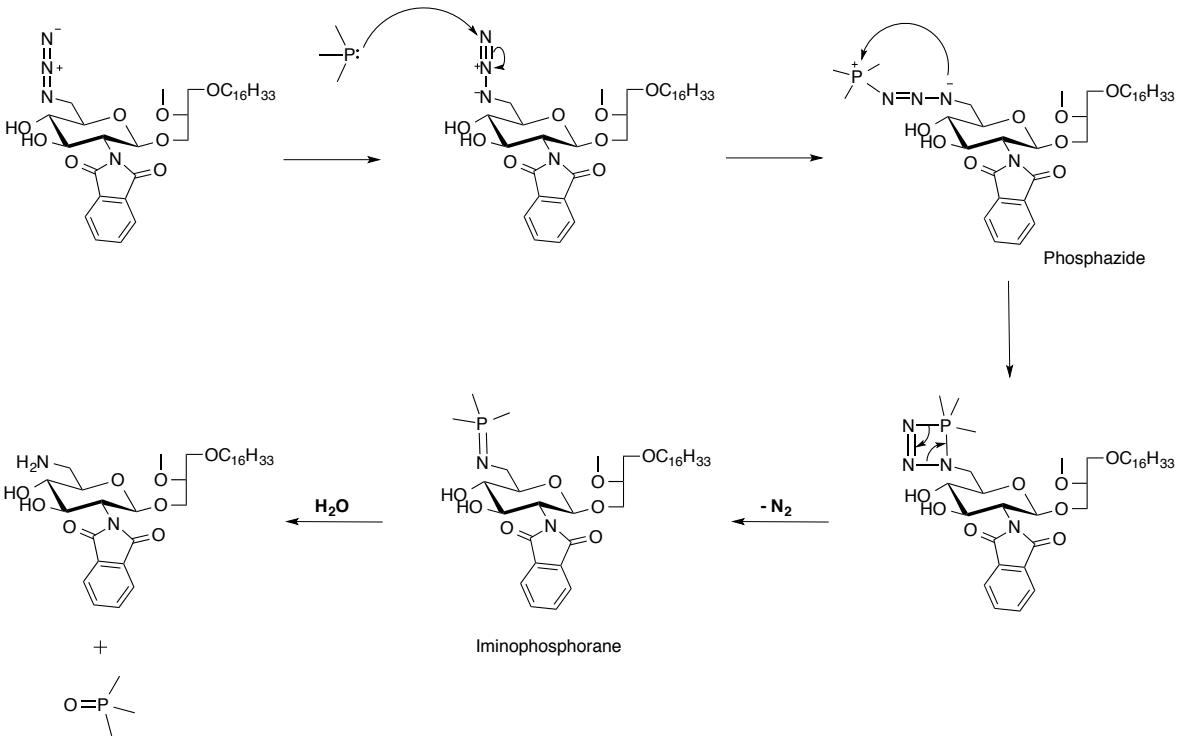
- Conversion of hydroxyl to azide



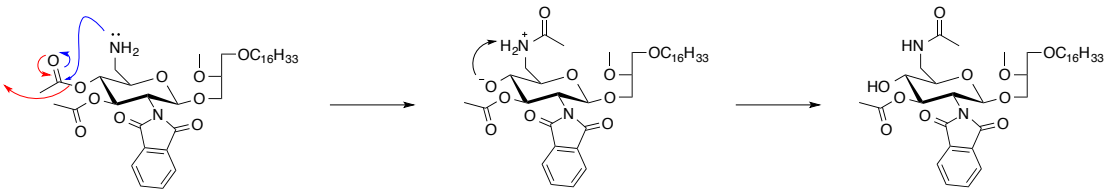
- Glycosylation



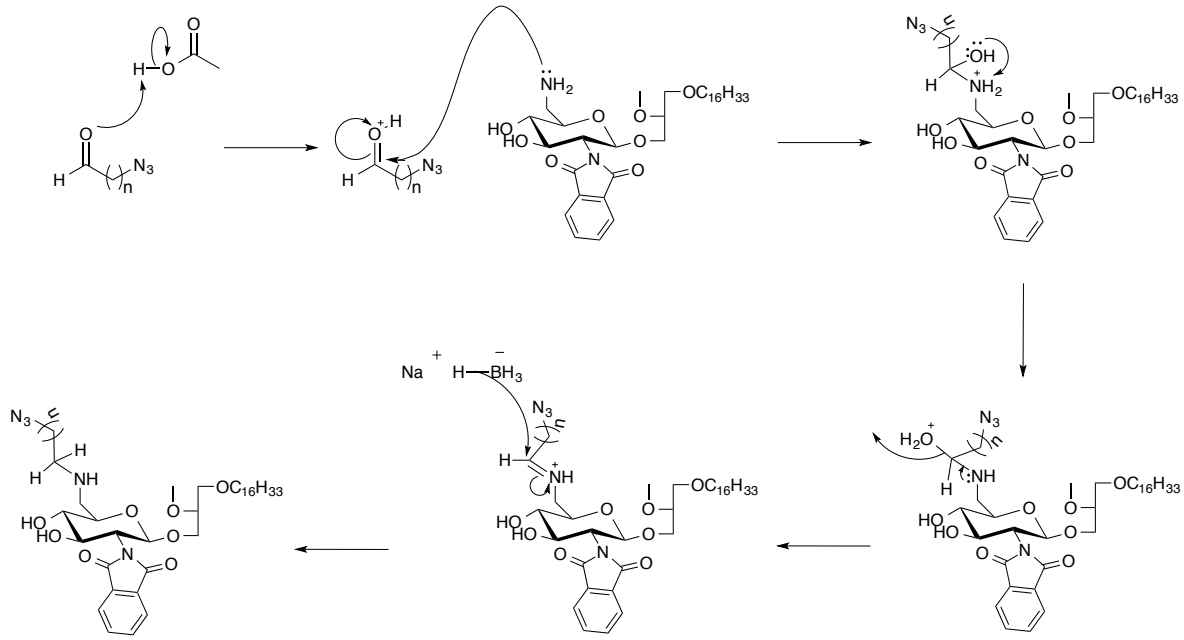
- Reduction of azide to amine (Staudinger's reaction)



- *In situ* migration of acetate group

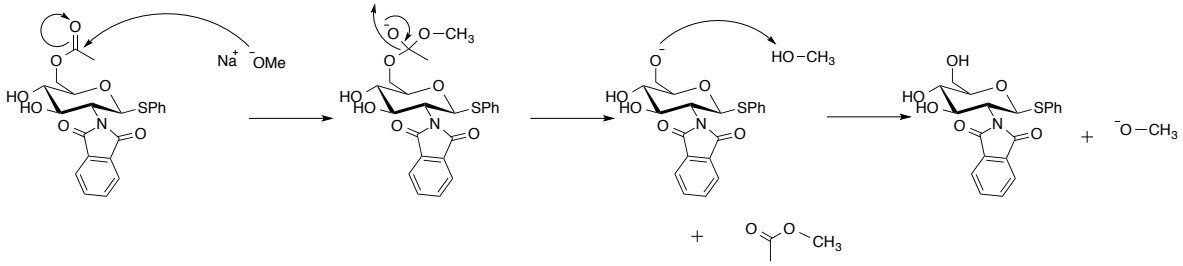


- Reductive Amination

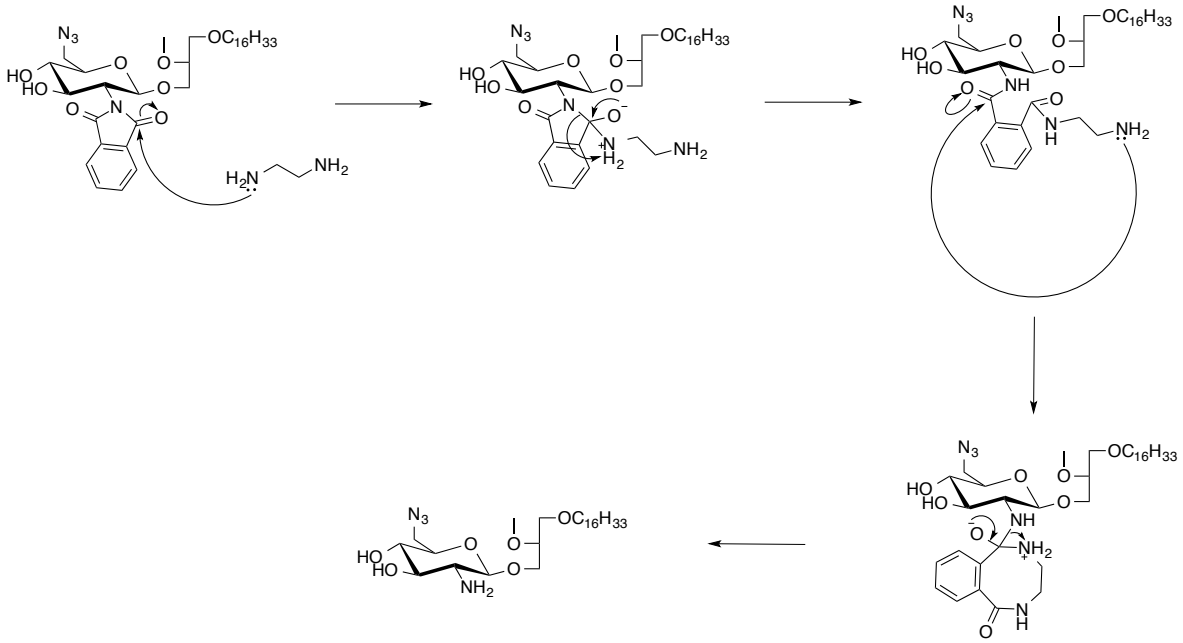


7.3 Functional Group Deprotection

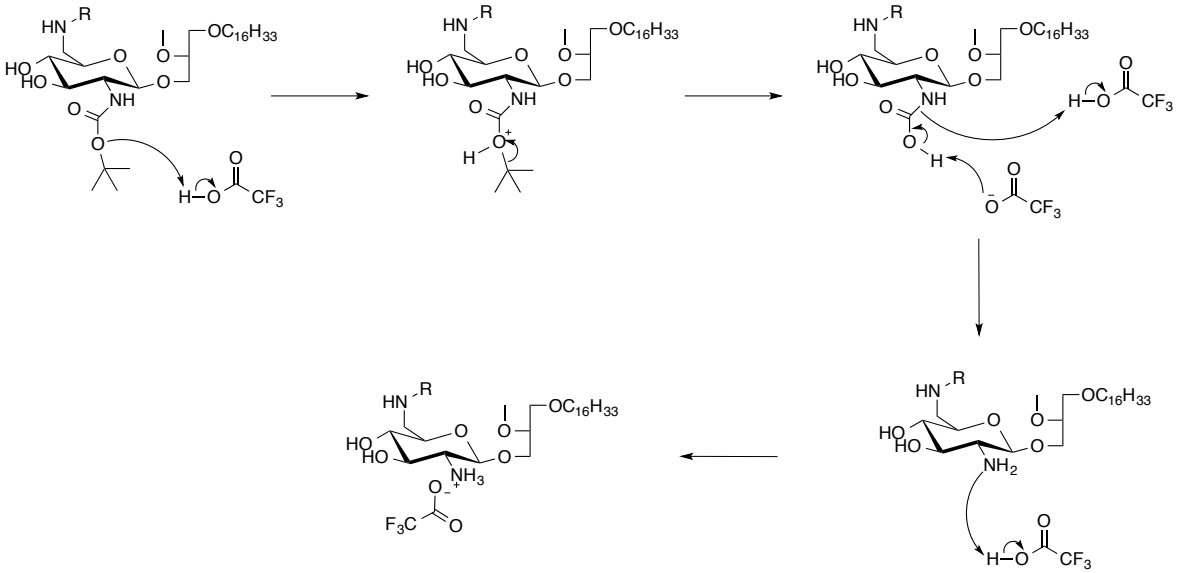
- Deacteylation using sodium methoxide



- Removal of phthalimido group



- Removal of boc-protecting group



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Appendix I

Compd	Concentration (μM)							
	0	**2.5**	**5.0**	**7.5**	**10.0**	**12.5**	**15.0**	**20.0**
2	100 ± 7.27	96.79 ± 8.77	101.76 ± 13.69	99.51 ± 5.93	104.96 ± 10.59	107.04 ± 10.70	101.69 ± 6.89	107.99 ± 7.17
3	100 ± 5.64	80.34 ± 4.98	64.17 ± 4.63	56.21 ± 5.18	37.00 ± 6.06	19.50 ± 4.32	9.02 ± 2.93	3.46 ± 1.51
4	100 ± 8.33	93.34 ± 11.29	73.5 ± 10.00	49.20 ± 9.04	34.88 ± 7.21	18.53 ± 4.18	10.50 ± 5.83	2.39 ± 3.38
5	100 ± 11.81	111.24 ± 8.99	103.47 ± 15.06	112.79 ± 10.78	114.45 ± 8.71	128.85 ± 10.04	99.01 ± 14.72	110.68 ± 7.18
6	100 ± 12.02	116.44 ± 18.61	101.98 ± 5.85	100.89 ± 7.57	73.43 ± 11.9	12.69 ± 7.02	4.66 ± 4.66	1.48 ± 1.35
7	100 ± 11.91	99.17 ± 8.44	107.71 ± 10.65	104.95 ± 9.62	104.29 ± 9.76	103.67 ± 10.87	77.84 ± 12.57	67.57 ± 8.58
31a	100 ± 6.58	113.04 ± 16.05	99.46 ± 11.88	66.05 ± 9.81	7.84 ± 4.89	-0.99 ± 1.11	0.02 ± 1.53	1.32 ± 0.36
31b	100 ± 6.47	92.78 ± 6.62	91.48 ± 4.26	85.92 ± 3.97	78.68 ± 5.14	53.58 ± 6.08	18.10 ± 4.36	2.64 ± 1.81
32a	100 ± 8.34	58.98 ± 8.41	-3.05 ± 1.25	-1.43 ± 0.81	0.04 ± 0.96	0.13 ± 0.70	0.31 ± 0.98	1.42 ± 1.68
32b	100 ± 6.18	98.08 ± 3.67	99.70 ± 5.28	97.79 ± 11.52	85.79 ± 3.10	68.33 ± 5.46	30.96 ± 6.14	2.20 ± 1.51
33	100 ± 18.89	85.62 ± 9.92	64.63 ± 12.88	66.63 ± 10.75	70.38 ± 8.96	66.03 ± 11.5	55.64 ± 11.27	33.73 ± 15.61
34	100 ± 2.18	105.78 ± 9.55	120.32 ± 17.50	127.07 ± 16.39	111.71 ± 13.87	107.03 ± 13.79	130.14 ± 19.68	87.78 ± 5.83

The effect of **2-7** and **31-34** on the % viability (± standard deviation) of JIMT1 cells at different concentrations, relative to the untreated control (set at 100%)

Appendix II

Compd	Concentration (μM)							
	0	**2.5**	**5.0**	**7.5**	**10.0**	**12.5**	**15.0**	**20.0**
2	100 ± 11.06	112.08 ± 15.45	102.48 ± 19.22	121.18 ± 22.43	116.94 ± 14.33	97.71 ± 18.44	94.17 ± 8.00	93.87 ± 15.52
3	100 ± 9.26	79.09 ± 10.11	58.12 ± 8.44	35.93 ± 3.97	20.19 ± 6.44	10.94 ± 3.19	7.71 ± 3.03	5.57 ± 2.40
4	100 ± 10.29	82.71 ± 9.75	74.62 ± 6.97	65.45 ± 12.38	60.83 ± 12.81	35.94 ± 1.69	20.55 ± 4.03	6.84 ± 3.89
5	100 ± 16.71	100.09 ± 9.41	99.43 ± 9.06	102.78 ± 14.03	115.03 ± 10.66	104.69 ± 9.02	108.92 ± 5.06	92.19 ± 20.77
6	100 ± 8.23	106.6 ± 9.56	99.02 ± 12.48	104.79 ± 14.33	96.8 ± 12.14	97.35 ± 11.22	51.4 ± 6.03	2.58 ± 1.50
7	100 ± 9.57	86.54 ± 15.88	104.13 ± 7.44	102.94 ± 14.07	95.14 ± 20.14	115.43 ± 12.66	88.55 ± 13.28	99.02 ± 4.57
31a	100 ± 15.00	100.9 ± 10.40	97.37 ± 6.86	57.34 ± 9.75	2.98 ± 7.41	-0.74 ± 1.52	-0.32 ± 1.08	0.78 ± 0.93
31b	100 ± 13.39	107.66 ± 7.71	99.12 ± 11.10	99.66 ± 10.53	90.47 ± 8.76	86.28 ± 4.94	80.33 ± 9.35	35.57 ± 7.06
32a	100 ± 12.92	90.01 ± 8.21	7.66 ± 4.09	-0.83 ± 1.22	0.11 ± 1.09	0.54 ± 0.54	1.10 ± 1.13	2.07 ± 1.43
32b	100 ± 12.59	97.17 ± 7.73	92.95 ± 10.17	99.61 ± 12.32	89.72 ± 8.74	78.50 ± 10.83	63.88 ± 13.49	12.35 ± 12.58
33	100 ± 7.18	96.87 ± 7.28	96.58 ± 11.22	102.06 ± 14.59	91.31 ± 12.49	93.50 ± 9.96	83.01 ± 8.14	71.34 ± 2.10
34	100 ± 21.27	109.95 ± 18.53	122.95 ± 23.53	123.05 ± 20.76	132.24 ± 30.14	117.7 ± 29.46	100.26 ± 4.28	96.69 ± 8.69

The effect of **2-7** and **31-34** on the % viability (± standard deviation) of DU145 cells at different concentrations, relative to the untreated control (set at 100%)

Appendix III

Compd	Concentration (μM)							
	0	**2.5**	**5.0**	**7.5**	**10.0**	**12.5**	**15.0**	**20.0**
2	100 ± 8.45	95.86 ± 6.85	104.31 ± 15.09	105.65 ± 10.14	104.37 ± 17.46	102.74 ± 12.22	88.98 ± 5.83	87.39 ± 4.62
3	100 ± 10.48	92.39 ± 7.67	79.96 ± 11.79	60.73 ± 5.64	28.75 ± 5.76	13.98 ± 7.73	6.58 ± 2.45	2.5 ± 1.76
4	100 ± 7.16	111.09 ± 12.22	84.25 ± 6.77	80.04 ± 7.21	57.68 ± 8.48	30.66 ± 6.82	11.65 ± 5.45	1.75 ± 0.71
5	100 ± 5.89	105.18 ± 8.45	96.18 ± 7.53	111.04 ± 14.24	110.01 ± 14.34	108.08 ± 11.83	95.00 ± 7.53	95.06 ± 5.00
6	100 ± 5.46	98.48 ± 12.02	93.82 ± 17.94	75.90 ± 8.71	73.58 ± 18.05	60.47 ± 17.03	36.72 ± 10.84	1.28 ± 1.14
7	100 ± 12.83	95.89 ± 14.25	97.32 ± 12.47	82.17 ± 12.96	87.78 ± 6.49	75.31 ± 12.36	69.88 ± 13.67	63.06 ± 8.61
31a	100 ± 4.38	89.51 ± 3.53	58.23 ± 6.44	34.67 ± 3.59	4.12 ± 3.99	-0.05 ± 0.95	0.70 ± 0.87	1.14 ± 0.90
31b	100 ± 3.02	97.99 ± 4.06	95.70 ± 6.83	88.51 ± 8.96	82.51 ± 2.78	75.05 ± 5.97	68.39 ± 7.48	29.75 ± 6.85
32a	100 ± 4.21	43.62 ± 6.03	-0.20 ± 1.32	0.32 ± 1.25	1.27 ± 0.69	1.47 ± 0.64	1.77 ± 0.49	2.51 ± 0.87
32b	100 ± 4.51	92.51 ± 7.03	87.09 ± 4.43	89.01 ± 9.88	90.98 ± 8.87	81.46 ± 4.53	81.62 ± 4.88	6.59 ± 2.68
33	100 ± 3.30	83.67 ± 4.58	68.43 ± 2.51	60.9 ± 4.07	51.80 ± 3.17	44.68 ± 5.27	36.61 ± 4.11	25.05 ± 6.95
34	100 ± 4.22	91.18 ± 6.48	98.44 ± 11.39	105.40 ± 6.23	107.66 ± 4.39	105.97 ± 5.20	104.67 ± 5.18	103.21 ± 5.44

The effect of **2-7** and **31-34** on the % viability (± standard deviation) of MDA-MB-231 cells at different concentrations, relative to the untreated control (set at 100%)

Appendix IV

Compd	Concentration (μM)							
	0	**2.5**	**5.0**	**7.5**	**10.0**	**12.5**	**15.0**	**20.0**
2	100 ± 3.83	103.77 ± 5.63	106.74 ± 10.83	110.95 ± 3.41	112.84 ± 3.40	109.98 ± 1.02	90.39 ± 14.46	106.59 ± 3.05
3	100 ± 3.72	118.79 ± 4.06	117.35 ± 3.13	110.54 ± 3.33	87.57 ± 6.42	45.7 ± 3.78	16.22 ± 3.05	6.42 ± 2.46
4	100 ± 3.12	106.23 ± 3.48	113.76 ± 6.22	98.78 ± 4.14	67.91 ± 2.34	40.25 ± 2.79	17.27 ± 3.92	5.03 ± 1.52
5	100 ± 2.87	98.11 ± 3.01	94.88 ± 5.97	100.98 ± 4.05	103.36 ± 3.46	107.31 ± 3.35	107.52 ± 5.39	109.56 ± 3.44
6	100 ± 2.92	98.4 ± 2.63	99.72 ± 3.80	94.35 ± 5.01	84.1 ± 8.14	63.71 ± 4.38	45.94 ± 6.63	11.57 ± 6.08
7	100 ± 11.19	99.39 ± 9.46	90.72 ± 7.32	86.54 ± 8.86	87.68 ± 5.11	70.13 ± 7.44	63.9 ± 8.09	54.08 ± 4.60
31a	100 ± 4.80	94.79 ± 7.85	102.6 ± 16.65	65.5 ± 11.55	28.89 ± 5.04	5.38 ± 3.34	-0.69 ± 1.66	1.83 ± 1.86
31b	100 ± 10.20	97.97 ± 4.96	94.47 ± 3.33	85.9 ± 5.15	76.5 ± 4.70	68.00 ± 7.84	48.53 ± 11.11	16.93 ± 2.86
32a	100 ± 2.88	89.61 ± 13.97	19.53 ± 8.47	0.92 ± 2.98	0.35 ± 0.73	0.62 ± 0.33	0.48 ± 1.37	1.32 ± 1.17
32b	100 ± 2.76	93.57 ± 2.64	91.52 ± 3.54	81.95 ± 1.57	63.23 ± 6.70	51.67 ± 4.05	29.81 ± 8.06	11.4 ± 8.79
33	100 ± 5.48	92.20 ± 9.42	89.93 ± 10.35	88.02 ± 9.19	90.63 ± 5.53	89.58 ± 8.84	73.22 ± 9.32	46.13 ± 1.92
34	100 ± 9.36	111.08 ± 7.25	103.93 ± 22.92	110.93 ± 18.32	109.41 ± 9.66	104.19 ± 4.34	86.57 ± 13.98	97.09 ± 9.22

The effect of **2-7** and **31-34** on the % viability (± standard deviation) of PC3 cells at different concentrations, relative to the untreated control (set at 100%)

Appendix V

Compd	Concentration (μM)							
	0	**2.5**	**5.0**	**7.5**	**10.0**	**12.5**	**15.0**	**20.0**
2	100 ± 16.56	91.54 ± 20.97	109.78 ± 13.63	110.54 ± 12.60	100.76 ± 18.81	94.25 ± 17.54	96.37 ± 13.98	91.90 ± 11.73
3	100 ± 17.49	110.92 ± 24.96	84.71 ± 7.51	77.32 ± 10.88	65.34 ± 2.73	51.64 ± 4.18	40.51 ± 2.26	46.71 ± 2.74
4	100 ± 10.67	79.77 ± 12.57	88.78 ± 14.23	82.47 ± 12.61	72.28 ± 3.82	49.38 ± 5.42	30.96 ± 3.10	24.05 ± 5.16
5	100 ± 19.01	106.74 ± 22.63	91.76 ± 12.17	103.48 ± 18.75	105.90 ± 18.93	121.69 ± 19.09	117.41 ± 13.77	87.70 ± 6.67
6	100 ± 23.05	97.53 ± 22.47	78.84 ± 10.83	63.05 ± 11.76	56.04 ± 11.66	60.10 ± 10.63	45.36 ± 3.59	16.85 ± 3.02
7	100 ± 21.03	99.95 ± 26.76	100.14 ± 22.13	106.86 ± 20.62	102.52 ± 21.48	101.33 ± 18.08	80.55 ± 15.18	66.21 ± 7.36
31a	100 ± 7.26	123.83 ± 15.40	100.15 ± 13.70	105.73 ± 11.43	97.09 ± 10.52	72.13 ± 8.58	17.3 ± 2.66	6.05 ± 0.61
31b	100 ± 12.40	139.42 ± 22.91	148.46 ± 30.36	132.98 ± 23.79	139.38 ± 16.41	138.24 ± 20.64	123.73 ± 18.08	63.54 ± 7.92
32a	100 ± 8.47	57.91 ± 16.31	36.44 ± 16.51	8.28 ± 1.42	6.56 ± 2.98	6.67 ± 2.52	5.92 ± 2.30	8.01 ± 2.63
32b	100 ± 8.14	111.98 ± 14.24	112.95 ± 11.12	107.57 ± 3.71	109.41 ± 1.64	112.4 ± 8.76	84.35 ± 7.66	80.33 ± 4.66
33	100 ± 12.04	65.8 ± 2.39	63.27 ± 6.66	60.84 ± 4.94	61.78 ± 7.55	52.15 ± 8.65	43.82 ± 5.25	38.44 ± 2.82
34	100 ± 7.74	99.14 ± 7.84	98.73 ± 7.84	101.06 ± 8.43	96.26 ± 7.35	96.76 ± 9.15	100.68 ± 4.39	102.22 ± 5.23

The effect of **2-7** and **31-34** on the % viability (± standard deviation) of BT474 cells at different concentrations, relative to the untreated control (set at 100%)

Appendix VI

Compd	Concentration (μM)							
	0	**2.5**	**5.0**	**7.5**	**10.0**	**12.5**	**15.0**	**20.0**
2	100 ± 17.56	108.67 ± 5.45	106.86 ± 3.81	108.44 ± 8.78	96.36 ± 12.45	106.67 ± 10.3	96.89 ± 7.94	100.18 ± 16.76
3	100 ± 6.71	99.34 ± 7.40	93.47 ± 5.92	89.03 ± 7.31	87.07 ± 9.09	83.69 ± 7.48	60.27 ± 5.21	24.1 ± 8.66
4	100 ± 13.62	84.85 ± 8.34	80.43 ± 9.01	82.24 ± 9.32	87.74 ± 8.76	74.89 ± 12.55	69.90 ± 7.29	53.12 ± 9.57
5	100 ± 10.25	107.89 ± 6.68	106.25 ± 14.89	113.33 ± 8.08	112.04 ± 8.87	114.6 ± 8.85	109.54 ± 7.83	110.38 ± 9.95
6	100 ± 11.09	95.51 ± 7.12	88.34 ± 11.93	84.71 ± 9.46	50.85 ± 8.25	42.65 ± 5.38	45.76 ± 7.54	24.33 ± 2.99
7	100 ± 11.63	95.31 ± 13.09	89.97 ± 18.46	89.97 ± 12.75	92.07 ± 12.23	86.53 ± 9.24	68.16 ± 8.37	54.56 ± 9.38
31a	100 ± 9.47	97.97 ± 13.64	80.91 ± 24.86	60.33 ± 9.81	37.83 ± 9.86	8.30 ± 6.21	0.82 ± 1.82	2.02 ± 1.44
31b	100 ± 9.79	87.74 ± 11.34	82.5 ± 9.89	74.82 ± 11.29	65.48 ± 12.91	57.63 ± 7.62	48.74 ± 12.05	34.84 ± 3.66
32a	100 ± 12.57	96.66 ± 9.70	67.35 ± 3.04	22.70 ± 6.22	-0.02 ± 1.66	-0.21 ± 1.03	0.33 ± 0.62	1.65 ± 0.88
32b	100 ± 8.26	104.12 ± 8.91	93.86 ± 9.42	96.11 ± 7.85	89.19 ± 7.97	89.20 ± 6.24	68.51 ± 14.83	40.82 ± 8.09
33	100 ± 9.90	99.79 ± 10.15	95.05 ± 10.32	89.24 ± 9.11	94.30 ± 11.65	79.99 ± 12.04	80.65 ± 10.53	70.94 ± 4.00
34	100 ± 13.42	111.84 ± 9.69	116.10 ± 17.28	117.5 ± 9.48	117.43 ± 12.62	108.95 ± 19.21	116.66 ± 6.35	99.57 ± 9.34

The effect of **2-7** and **31-34** on the % viability (± standard deviation) of MiaPaCa2 cells at different concentrations, relative to the untreated control (set at 100%)

Appendix VII

Cell lines	Concentration (μM)							
	0	1.5	3.0	4.5	6.0	9.0	12.0	15.0
BT474	100 ± 4.81	52.38 ± 5.23	36.04 ± 2.71	3.66 ± 2.89	4.44 ± 2.07	4.27 ± 1.13	3.22 ± 0.59	3.51 ± 0.48
PC3	100 ± 6.17	68.11 ± 9.85	13.31 ± 7.68	3.46 ± 4.36	-0.70 ± 1.69	0.90 ± 1.13	1.03 ± 0.76	0.91 ± 1.59
MiaPaCa2	100 ± 9.03	96.48 ± 9.50	68.46 ± 16.12	39.62 ± 8.47	14.67 ± 8.52	-2.6 ± 1.16	-0.30 ± 1.01	2.04 ± 0.61
JIMT1	100 ± 6.75	105.17 ± 10.35	68.02 ± 16.11	10.99 ± 9.56	-0.05 ± 3.45	0.35 ± 1.42	0.97 ± 1.19	1.49 ± 1.01
DU145	100 ± 14.96	88.78 ± 21.13	82.08 ± 7.42	16.55 ± 8.92	-0.38 ± 1.54	0.24 ± 1.59	0.56 ± 1.49	0.94 ± 1.17
MDA-MB-231	100 ± 12.29	50.88 ± 14.77	23.38 ± 4.18	1.89 ± 4.17	-0.08 ± 1.24	2.03 ± 0.96	2.31 ± 0.48	2.1 ± 1.04
BT549	100 ± 11.98	85.65 ± 14.54	34.04 ± 21.53	2.96 ± 12.72	1.07 ± 1.34	1.26 ± 1.82	1.70 ± 2.81	1.69 ± 1.05
MDA-MB-453	100 ± 7.51	80.25 ± 3.79	86.42 ± 12.84	28.92 ± 6.27	7.88 ± 3.50	5.19 ± 2.07	4.16 ± 2.44	4.44 ± 1.33
MDA-MB-468	100 ± 5.13	66.95 ± 3.30	19.41 ± 5.08	3.17 ± 1.85	-0.33 ± 1.54	1.17 ± 1.17	1.39 ± 0.65	1.05 ± 0.77
Hs578t	100 ± 5.13	66.95 ± 3.30	19.41 ± 5.08	3.17 ± 1.85	-0.33 ± 1.54	1.17 ± 1.17	1.39 ± 0.65	1.05 ± 0.77
U87	100 ± 5.91	77.28 ± 1.83	37.59 ± 2.70	-5.41 ± 1.25	-2.21 ± 1.28	-1.27 ± 1.47	-0.92 ± 0.77	-0.36 ± 0.99
U251	100 ± 8.82	102.73 ± 15.63	34.38 ± 4.94	-1.00 ± 0.89	0.29 ± 0.51	0.83 ± 1.51	1.05 ± 1.31	1.10 ± 0.83
A2780cp	100 ± 8.53	98.20 ± 10.31	84.93 ± 7.27	20.26 ± 8.12	5.82 ± 7.09	0.36 ± 1.05	0.43 ± 1.85	0.89 ± 1.89
A2780s	100 ± 7.76	94.79 ± 6.49	22.45 ± 5.41	-1.00 ± 1.37	-0.03 ± 2.28	0.60 ± 1.23	0.66 ± 0.80	1.05 ± 0.89

The effect of **32a** on the % viability (± standard deviation) of epithelial cancer cell lines at different concentrations, relative to the untreated control (set at 100%)

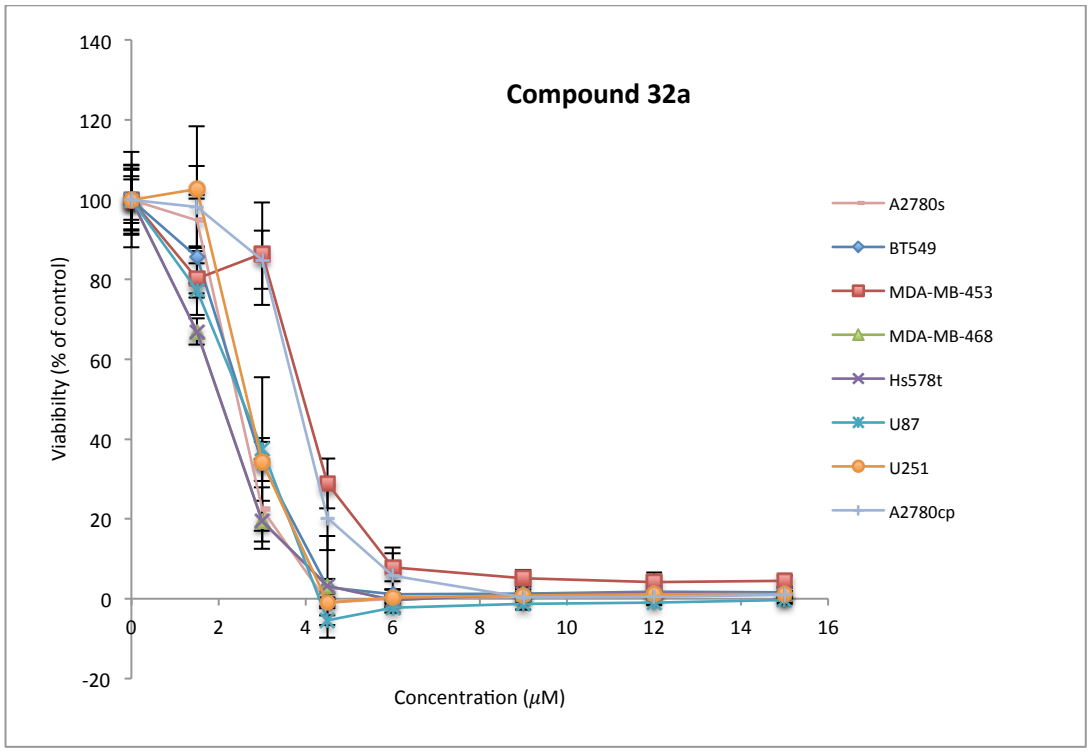
Appendix VIII

	DU145		MDA-MB-231		JIMT1		MiaPaCa2		BT474		PC3	
CODE	***CC_{50}***	***CC_{90}***	***CC_{50}***	***CC_{90}***	***CC_{50}***	***CC_{90}***	***CC_{50}***	***CC_{90}***	***CC_{50}***	***CC_{90}***	***CC_{50}***	***CC_{90}***
1	10	15.0	7.1	ND	9.0	16.0	9.0	18.0	8.0	13.0	13.5	28.0
2	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150
3	6.0	12.5	8.5	13.5	8.5	14.5	16.0	>20	12.5	>20	12.0	17.5
4	11.0	19.5	10.5	15.5	7.5	15.5	20.0	>20	12.5	>20	11.5	17.5
5	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
6	15.0	19.5	13.5	18.5	11.0	12.5	10.0	>20	13.5	>20	14.5	20.0
7	>15	>15	>15	>15	>15	>15	15.0	>15	>15	>15	15.0	>15
31a	7.5	9.5	5.5	9.5	8.0	9.5	8.5	12	13.5	16.5	8.5	11.5
31b	18.5	>20	17.5	>20	12.5	16.5	14.5	>20	>20	>20	14.5	>20
32a	3.8	4.8	1.5	3.8	3.4	4.6	4.0	6.6	1.6	4.2	2.0	3.2
32b	16.5	20.0	13.5	19.5	13.5	18.5	18.5	>20	>20	>20	12.5	20.0
33	>20	>20	10.5	>20	16.0	>20	>20	>20	13.0	>20	19.0	>20
34	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
37	16.5	>30	ND	ND	27.0	>30	18.0	>30	ND	ND	24.0	>30

Cytotoxicity of compounds **1-7** and **31-34** against human epithelial cancer cell lines

CC_{50} and CC_{90} values are the concentrations required to decrease cell viability by 50 and 90 % respectively, relative to the untreated control

Appendix IX



Effect of compound **32a** on the viability of epithelial cancer cell lines

Appendix X

Cell lines	Derived from tumors of:	
JIMT1	Trastuzumab-resistant, PR and ER negative breast carcinoma	**BREAST CANCER**
BT474	HER and ER positive solid invasive ductal breast carcinoma	
MDA-MB-231	ER negative breast cancer	
BT549	Invasive ductal breast carcinoma	
MDA-MB-453	Triple negative breast cancer (estrogen, progesterone and hormonal epidermal growth factor receptors absent)	
MDA-MB-468		
Hs578t		
PC3	Highly metastatic hormone-resistant prostatic adenocarcinoma cells	**PROSTATE CANCER**
DU145	Moderately metastatic hormone-resistant prostatic adenocarcinoma cells	
MiaPaCa2	TGF-β resistant tumor of the pancreas	**PANCREATIC CANCER**
U87	Malignant glioma cancer	**BRAIN CANCER**
U251	Gliobastoma-derived brain carcinoma	
A2780cp	Cisplatin-resistant ovarian cancer	**OVARIAN CANCER**
A2780s	Ovarian carcinoma	

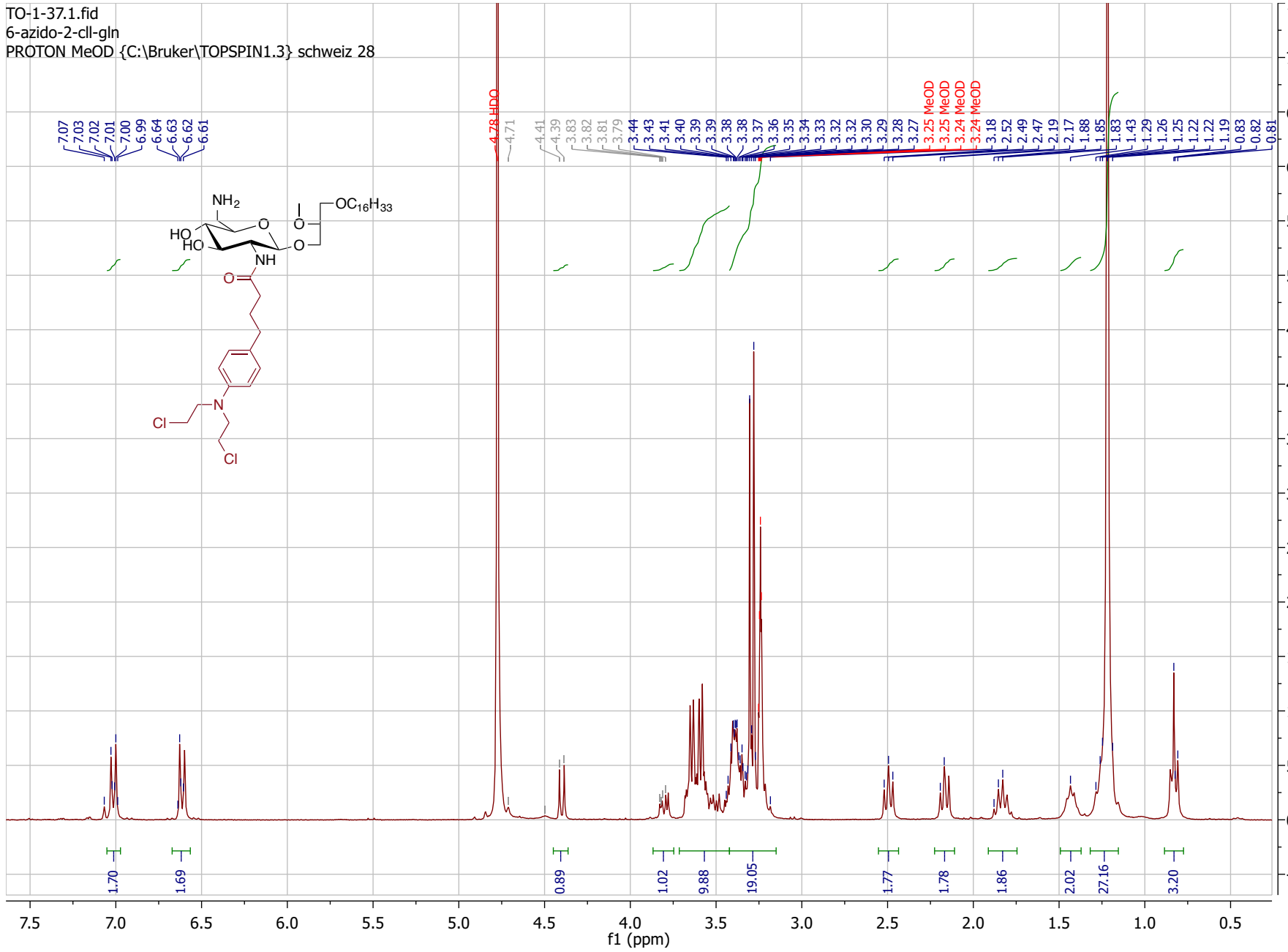
Cancer cell lines used in the study

(PR, progesterone receptor; ER, estrogen receptor;

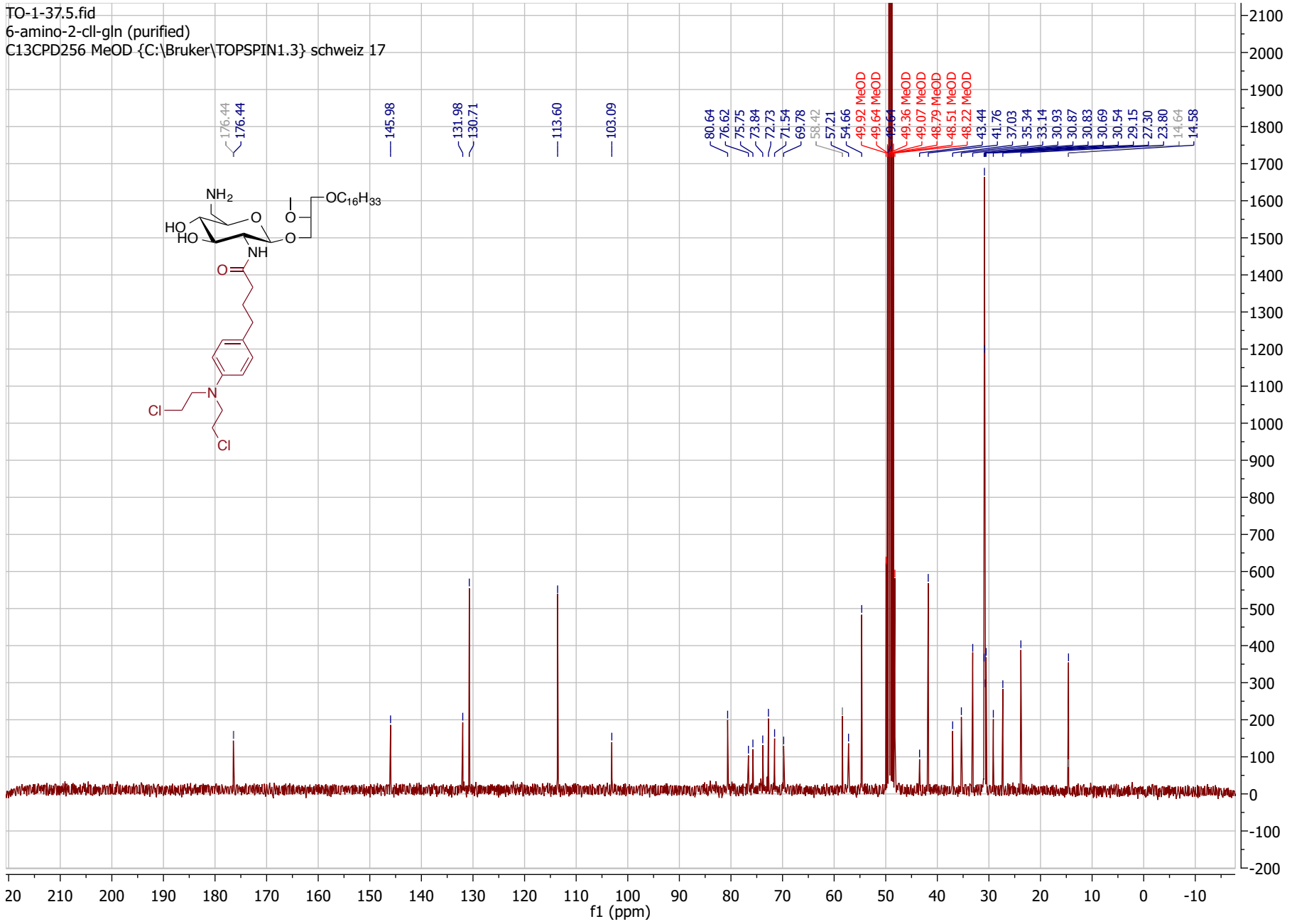
HER, hormonal epidermal receptor; TGF, tumor growth factor)

SUPPORTING INFORMATION

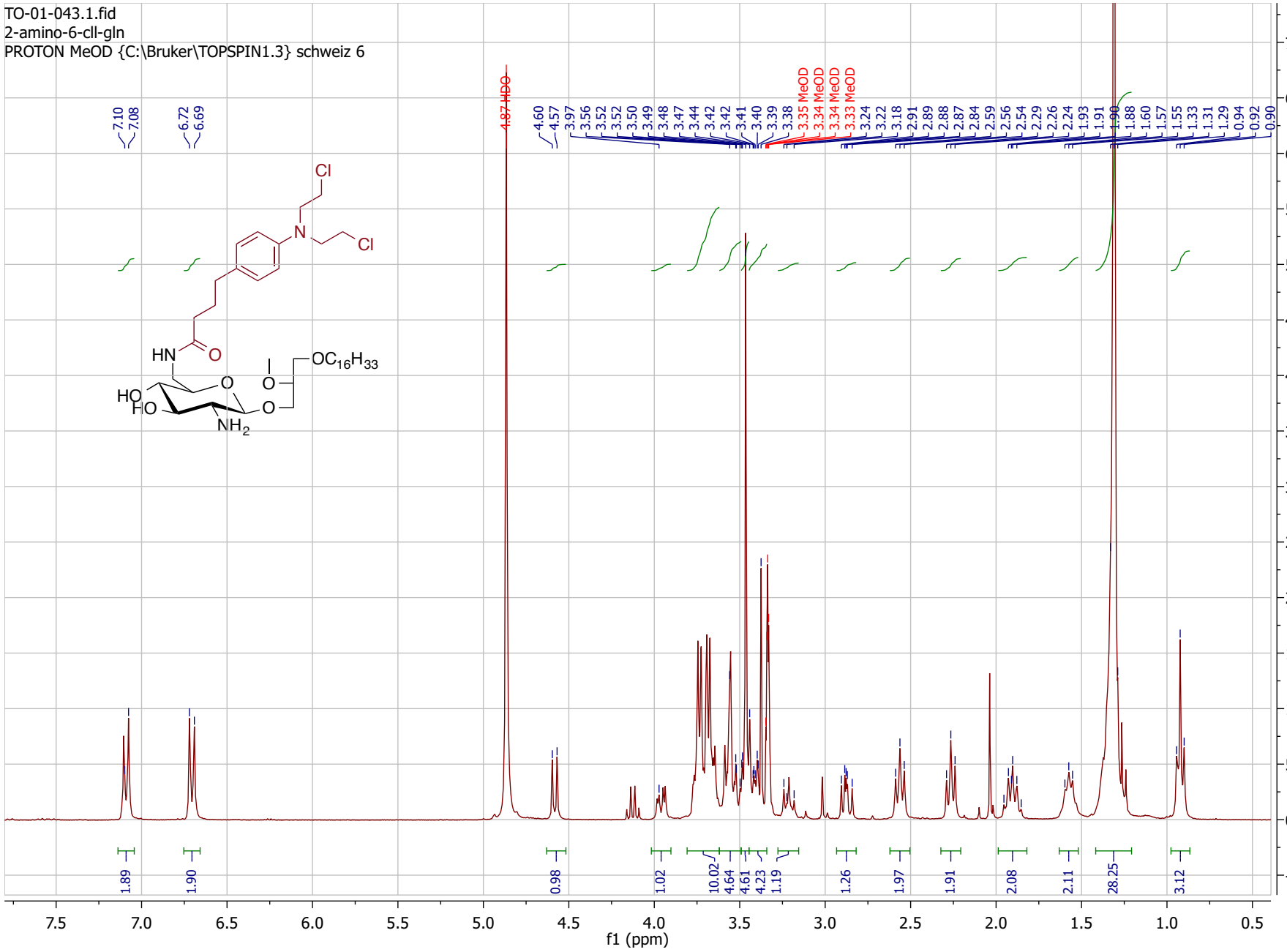
^{1}H and ^{13}C NMR spectra of all final compounds are attached.



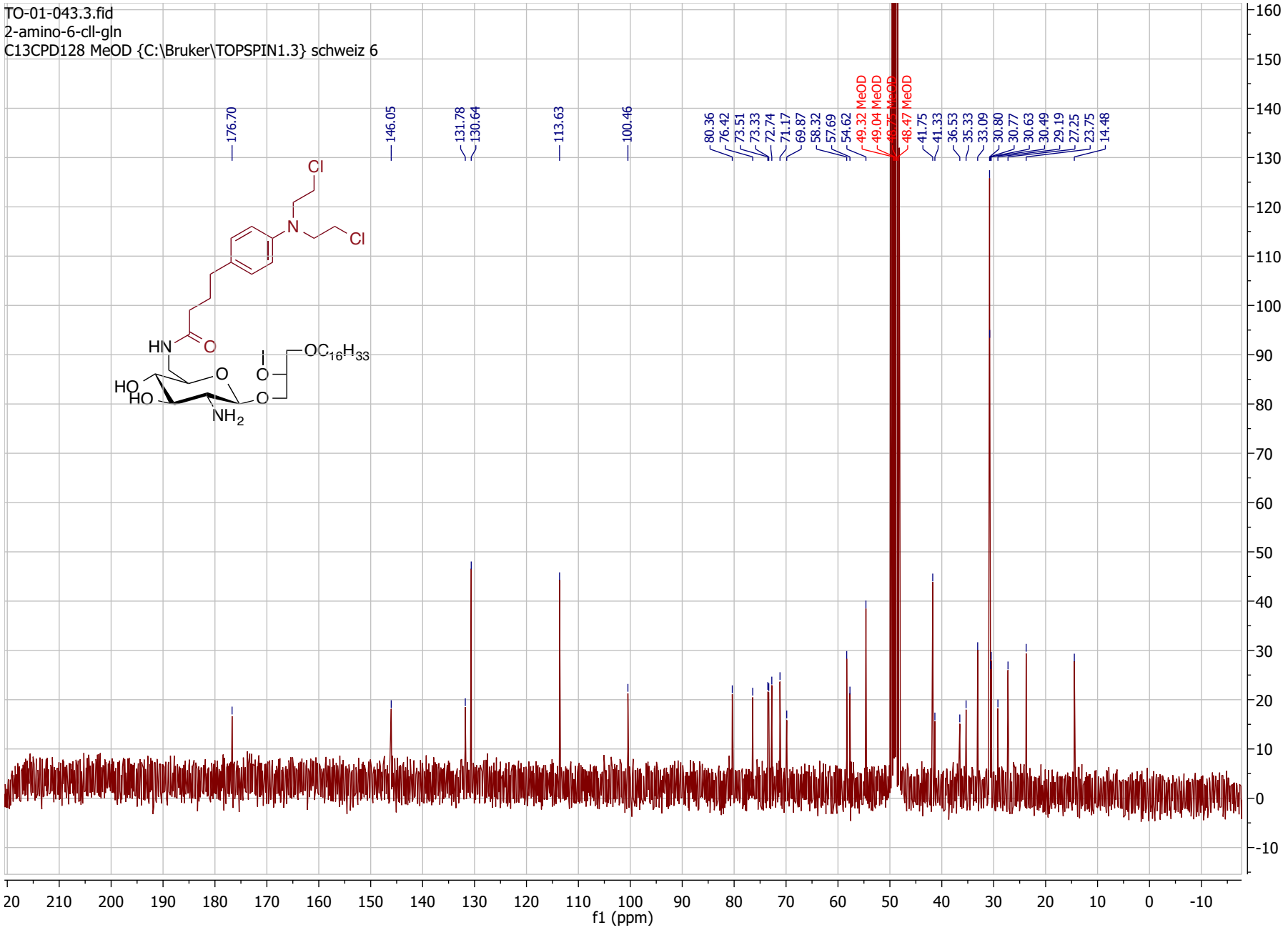
^{1}H NMR of Compound **3**



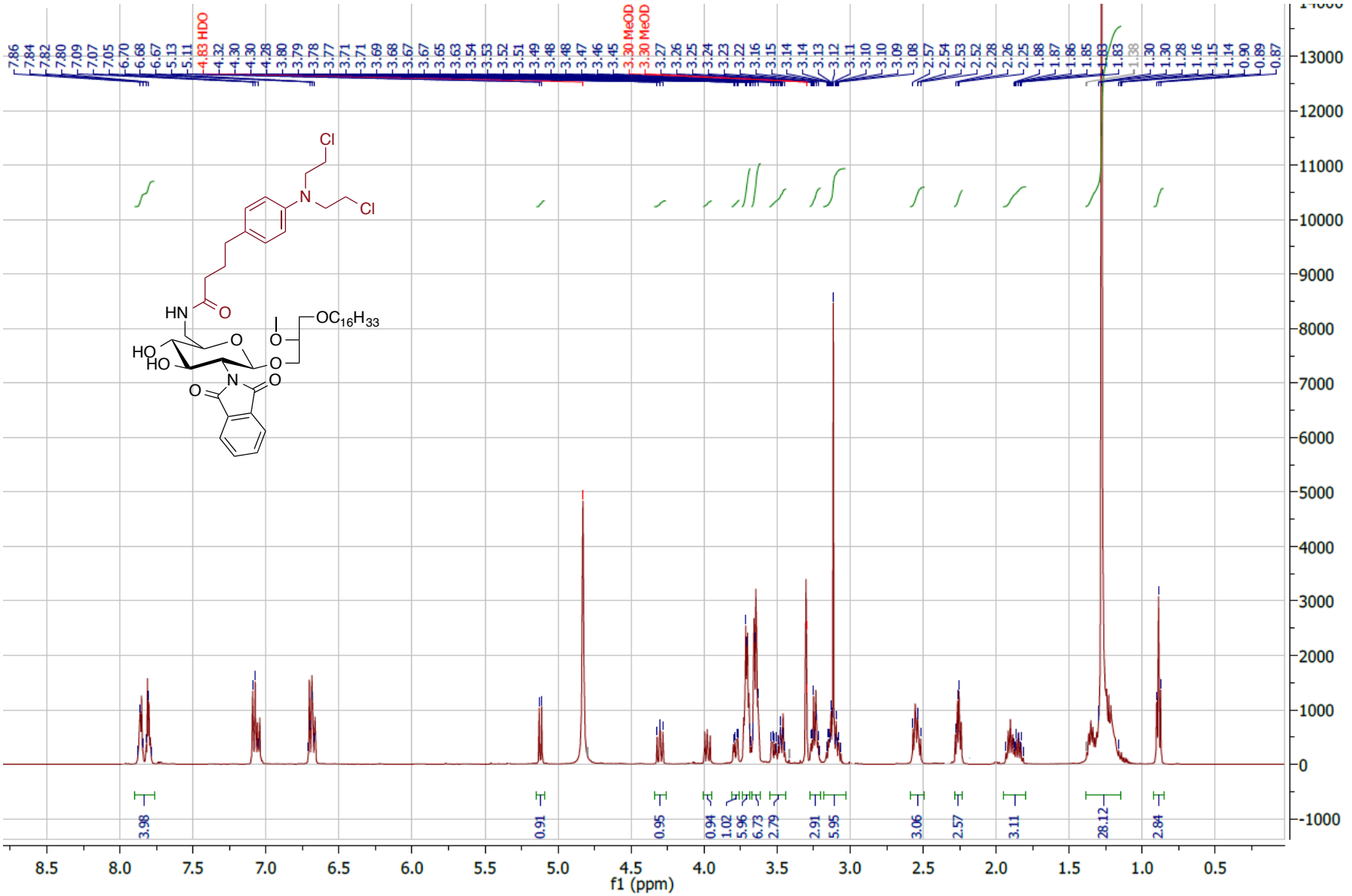
^{13}C NMR of Compound **3**



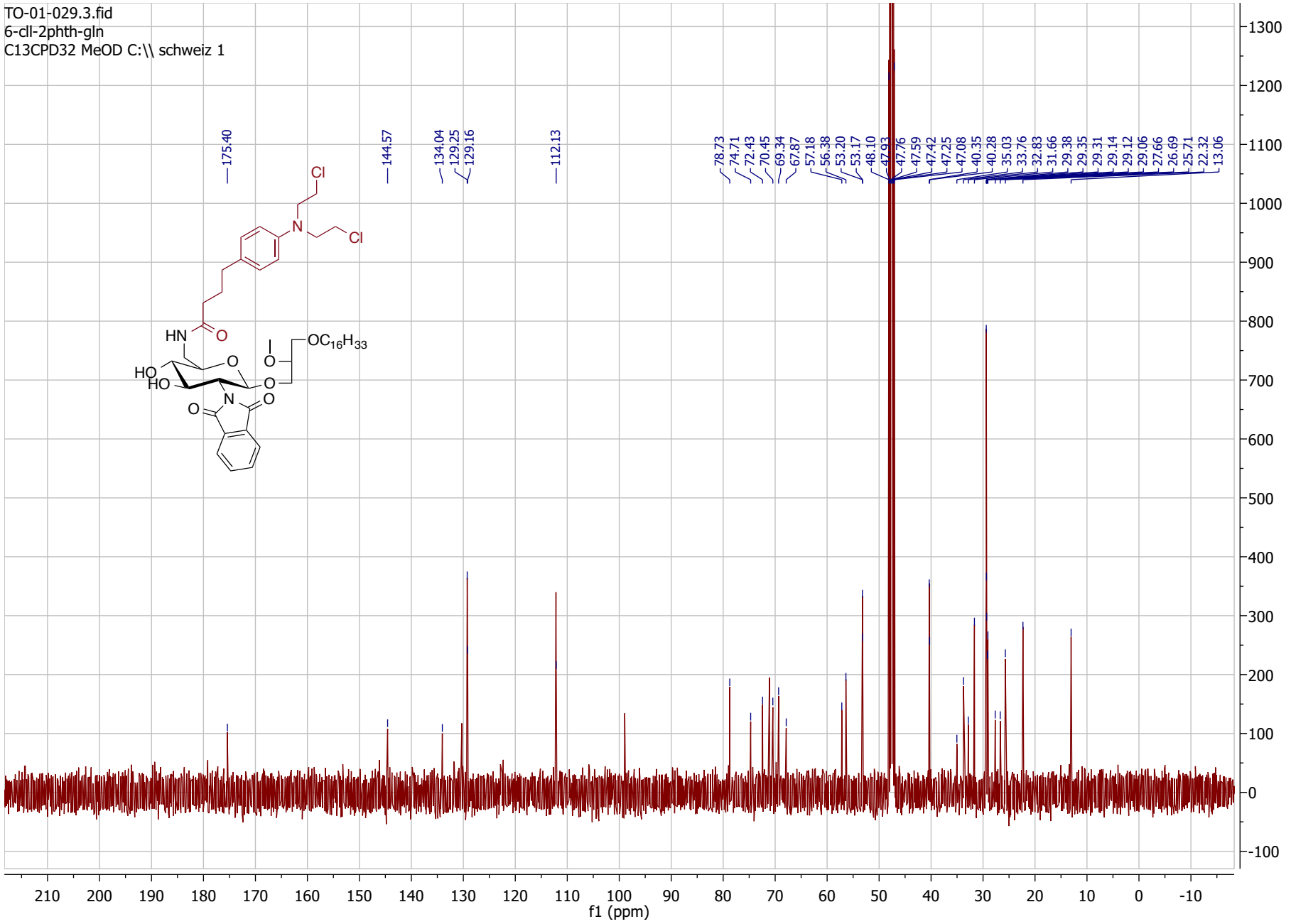
^{1}H NMR of Compound **4**



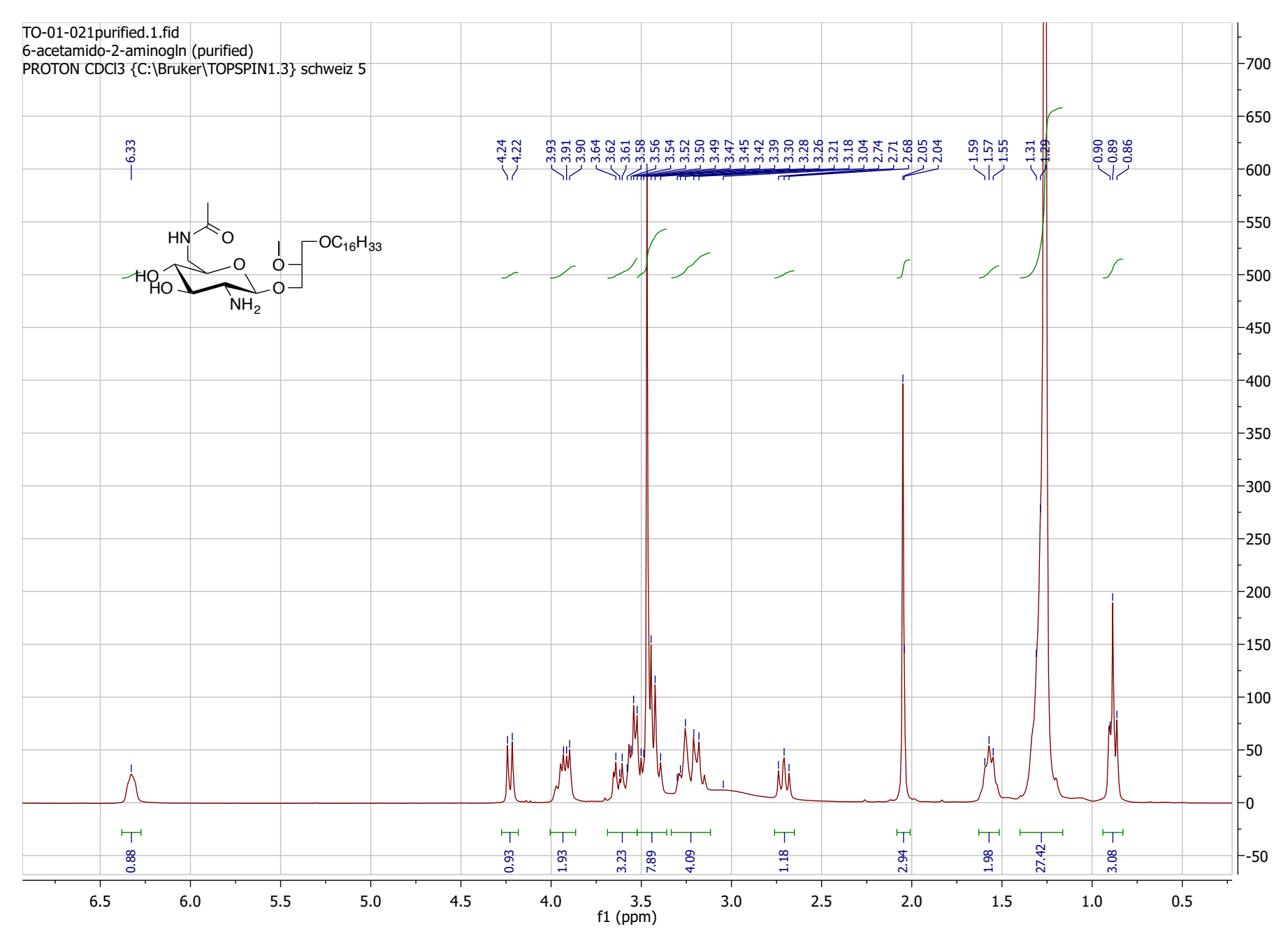
^{13}C NMR of Compound **4**



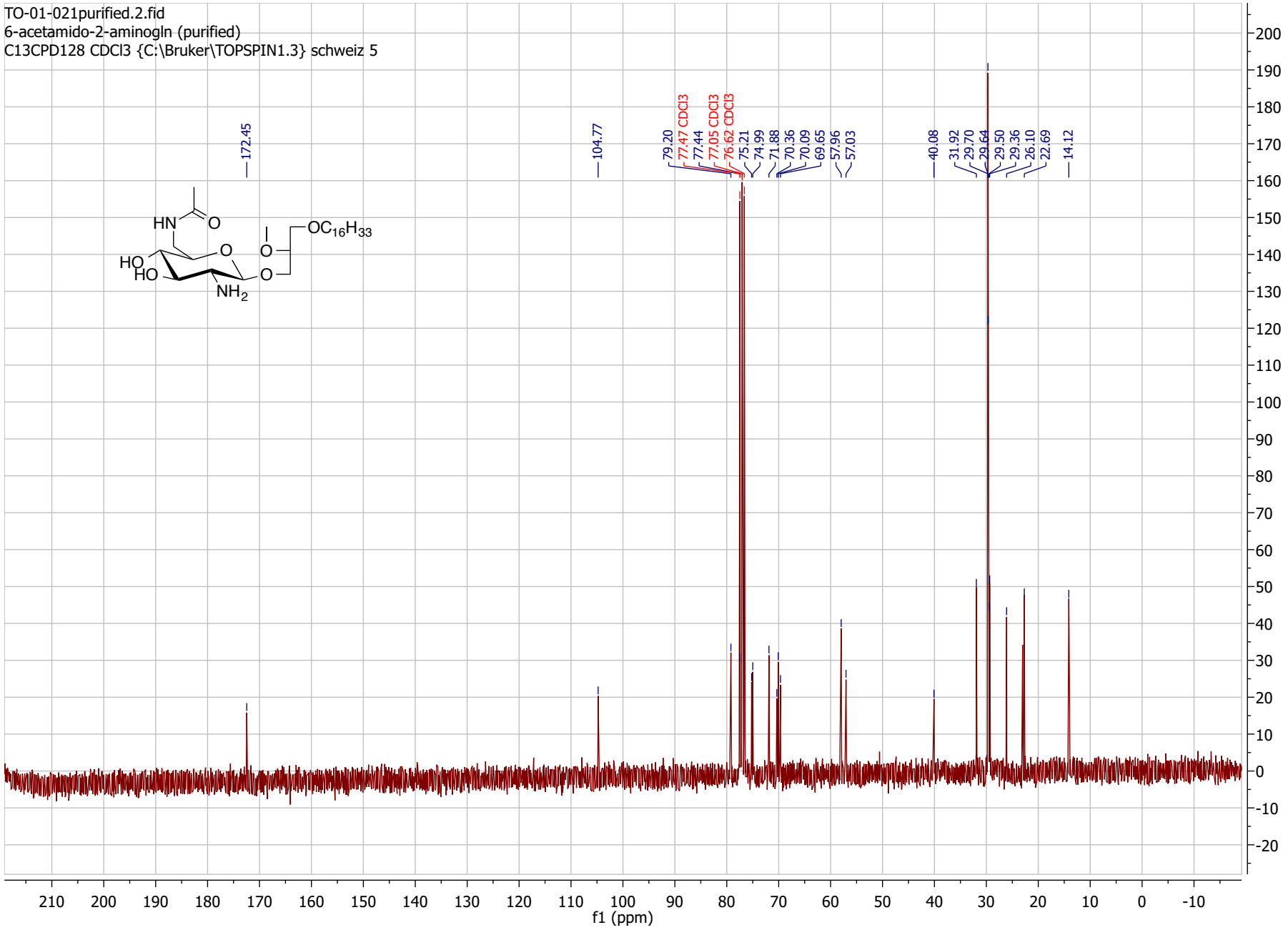
^{1}H NMR of Compound **5**



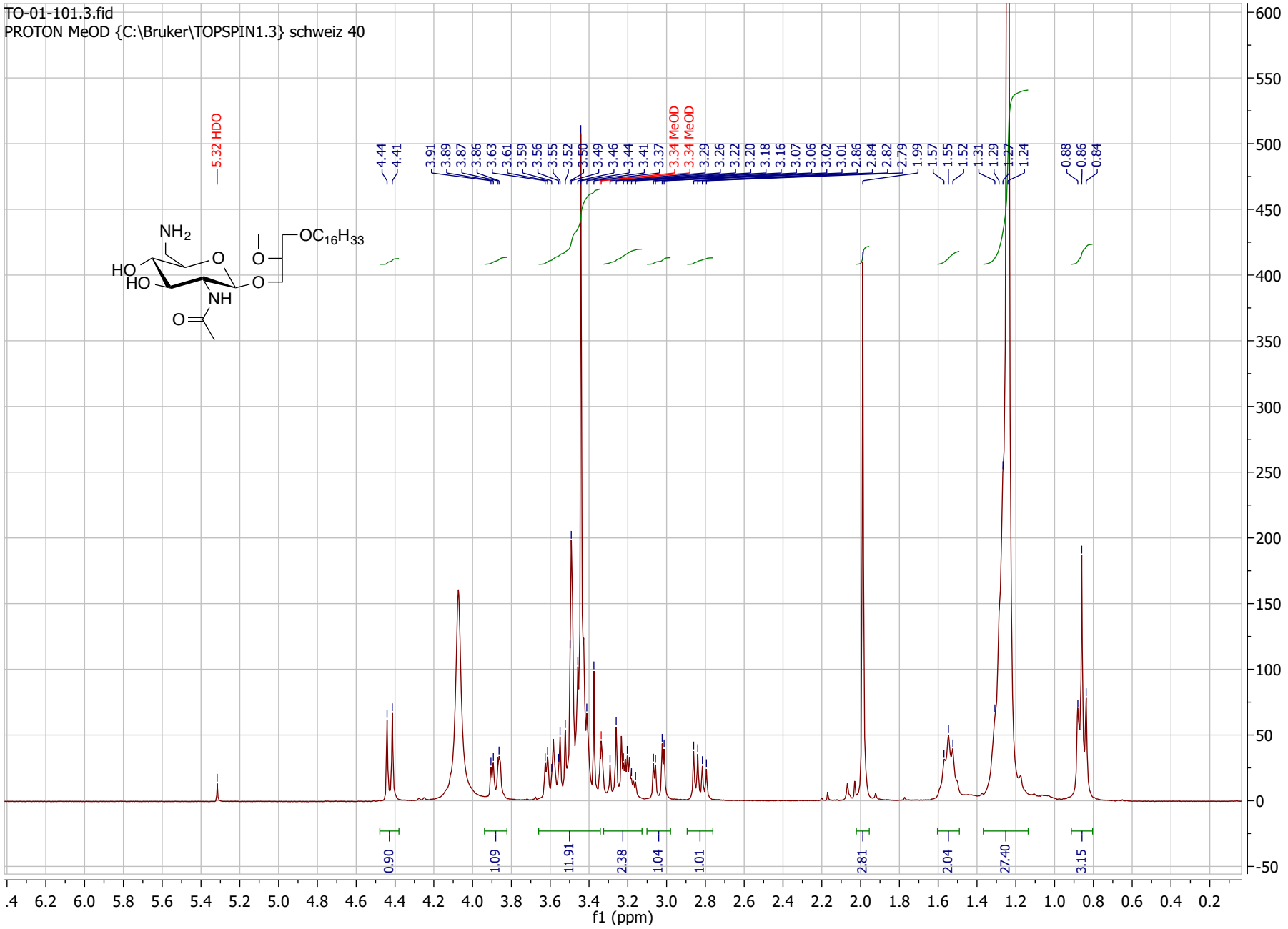
^{13}C NMR of Compound **5**



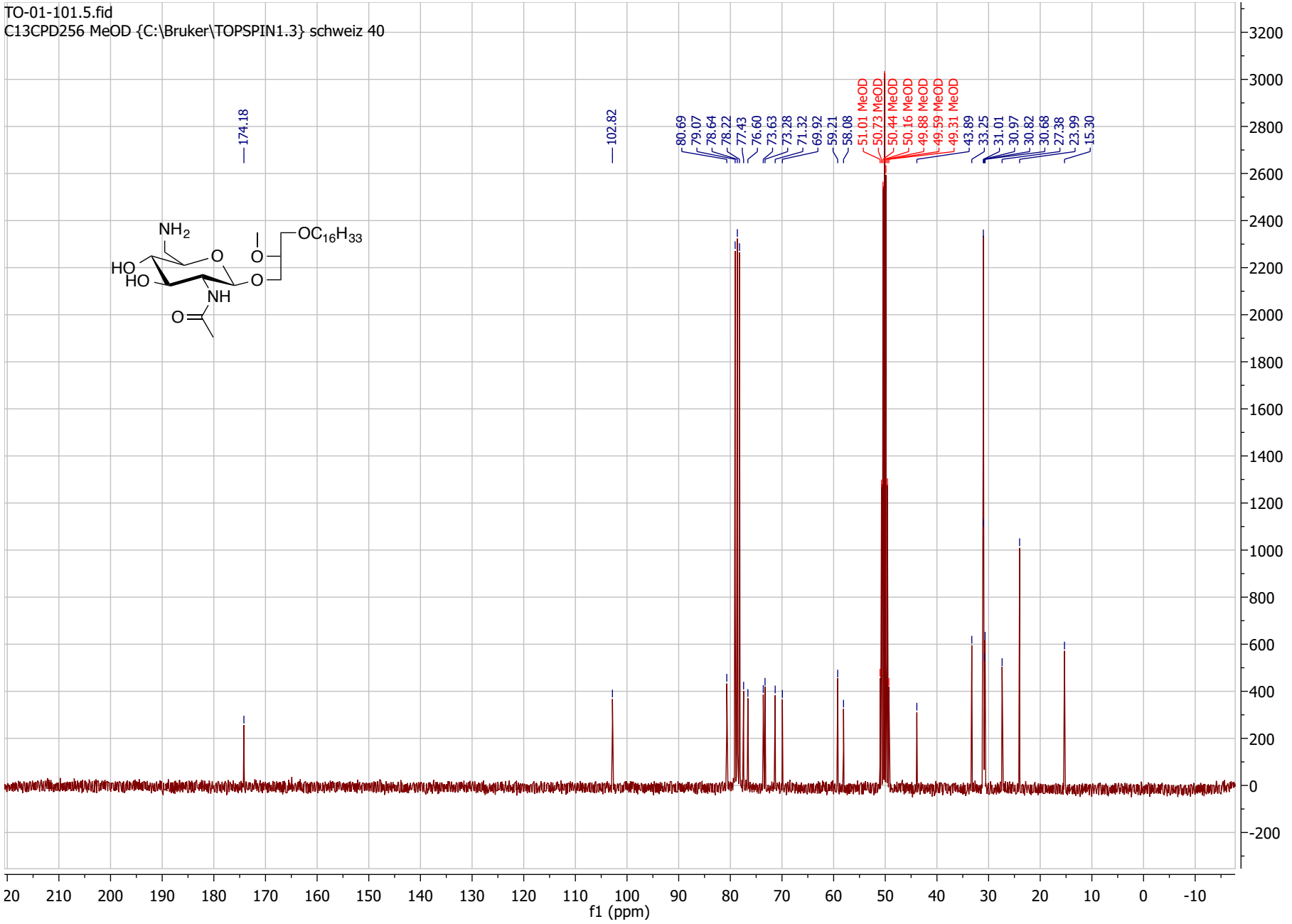
^{1}H NMR of Compound **6**



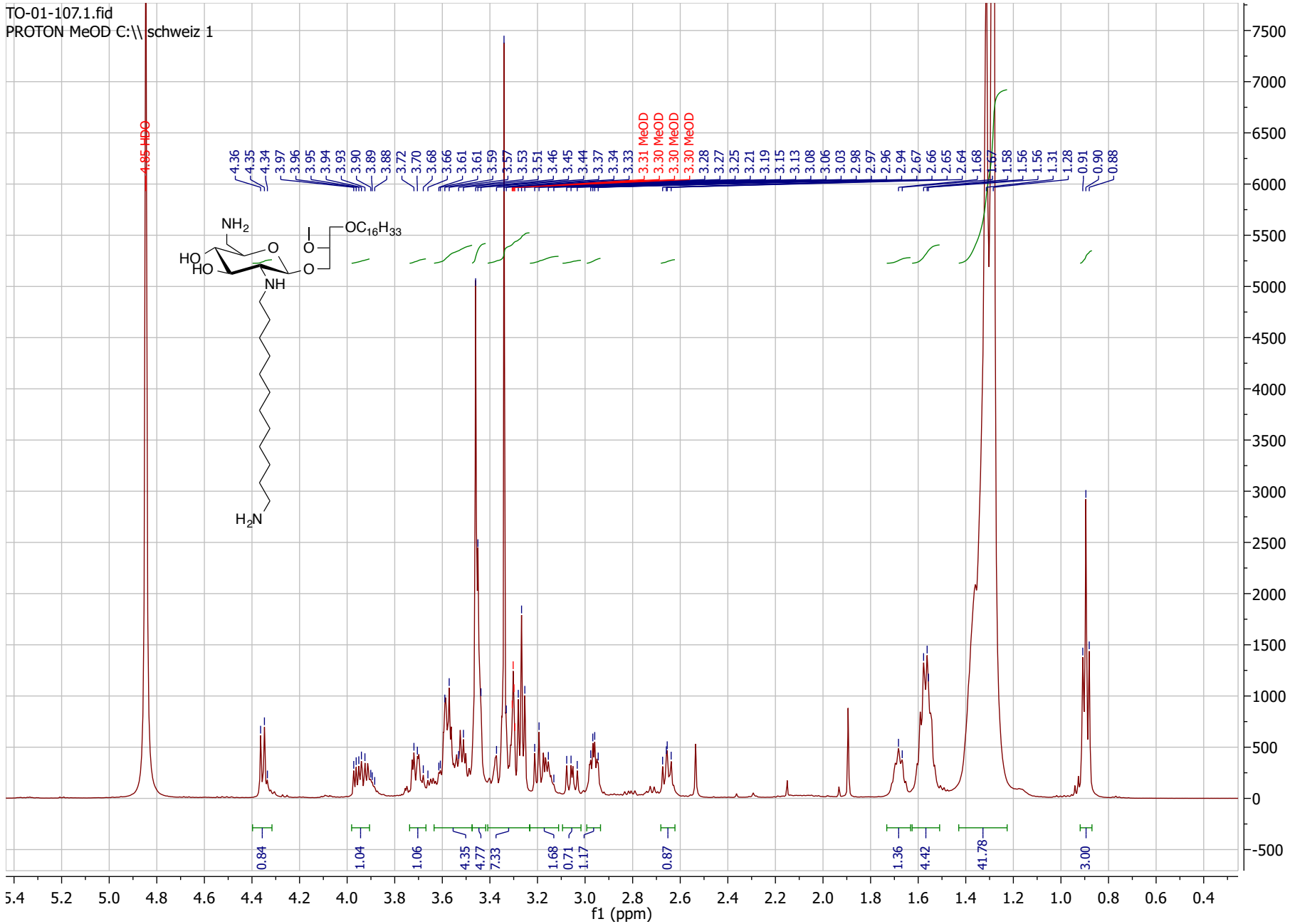
^{13}C NMR of Compound **6**



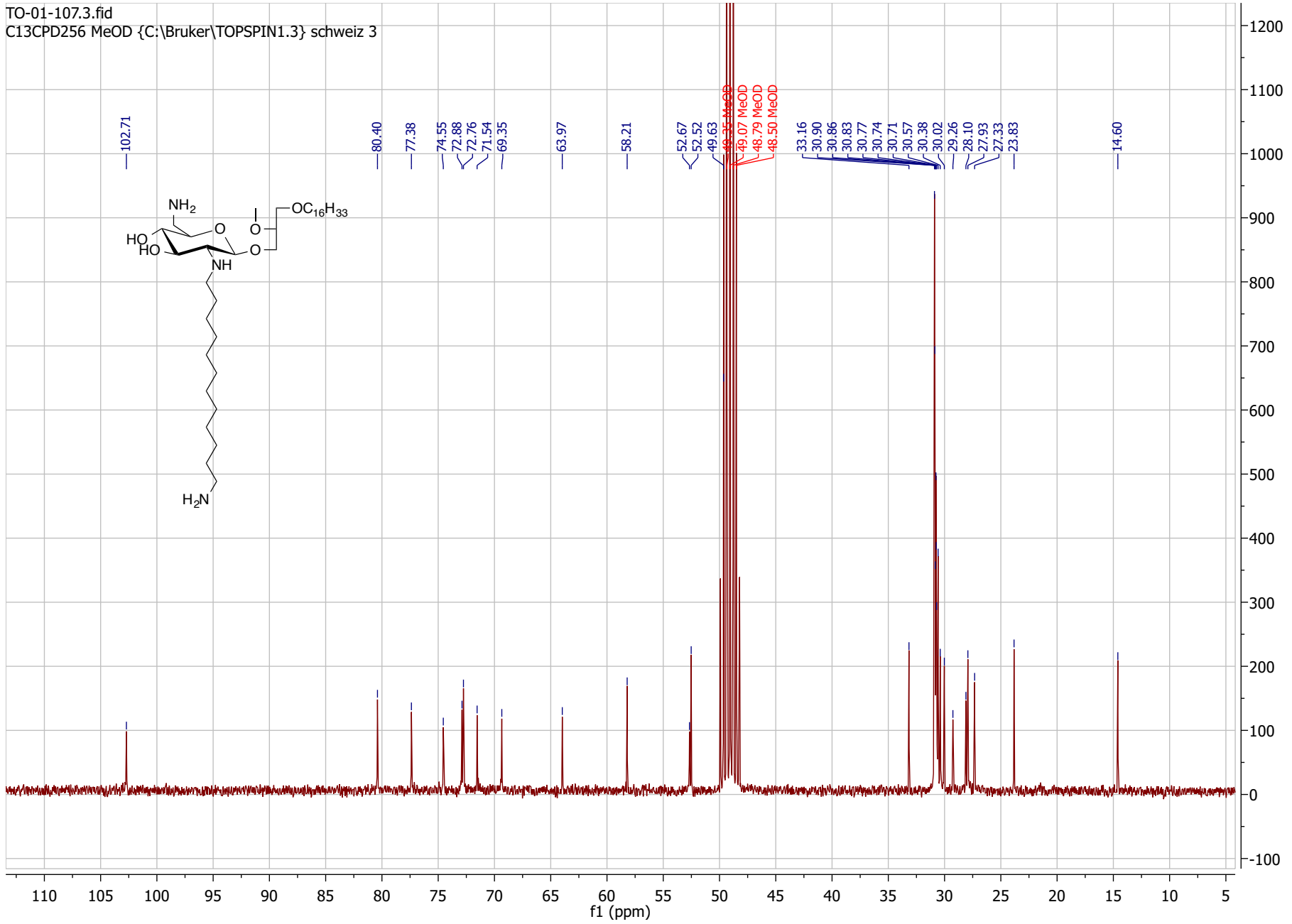
^{1}H NMR of Compound **7**



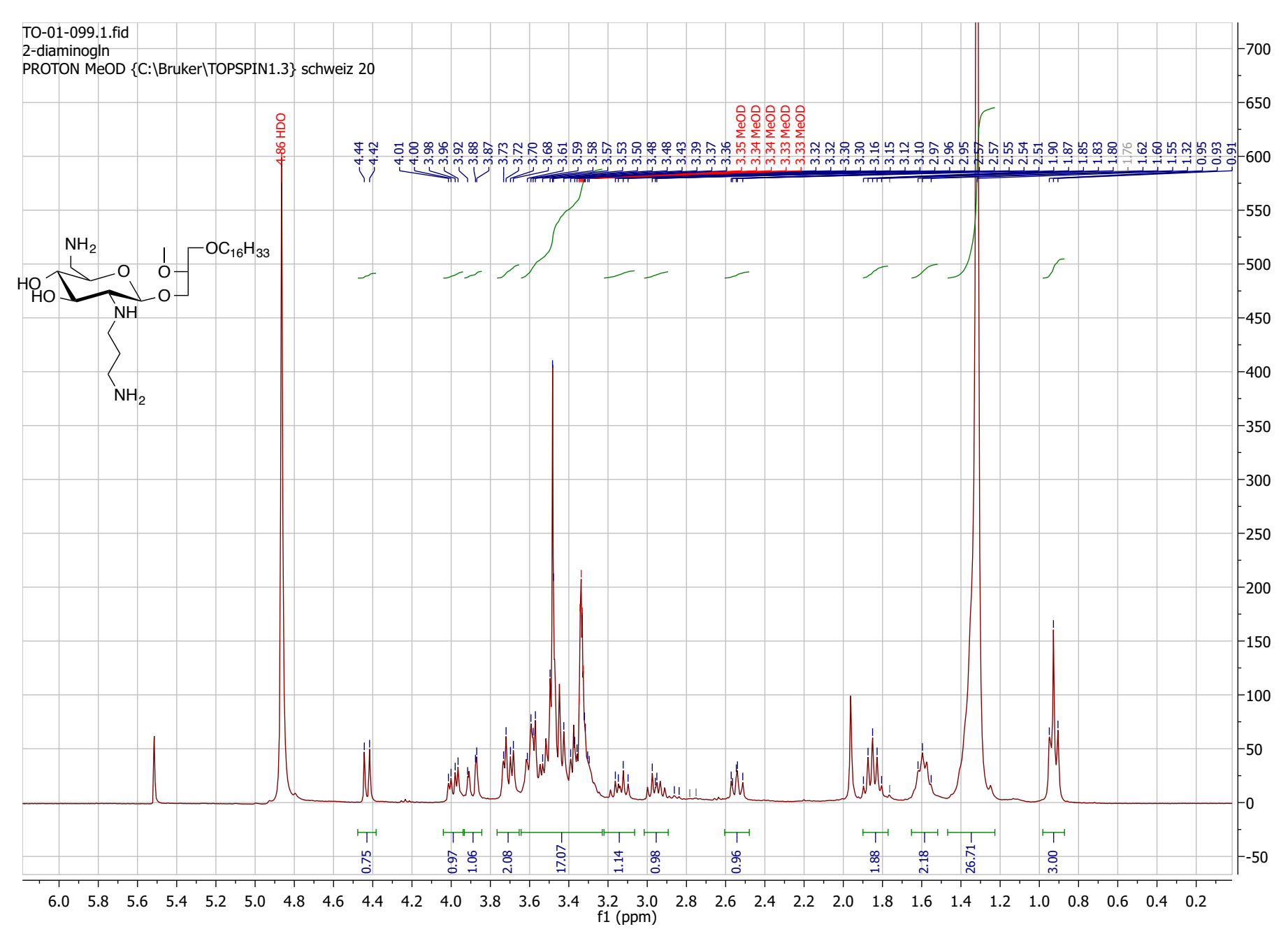
^{13}C NMR of Compound **7**



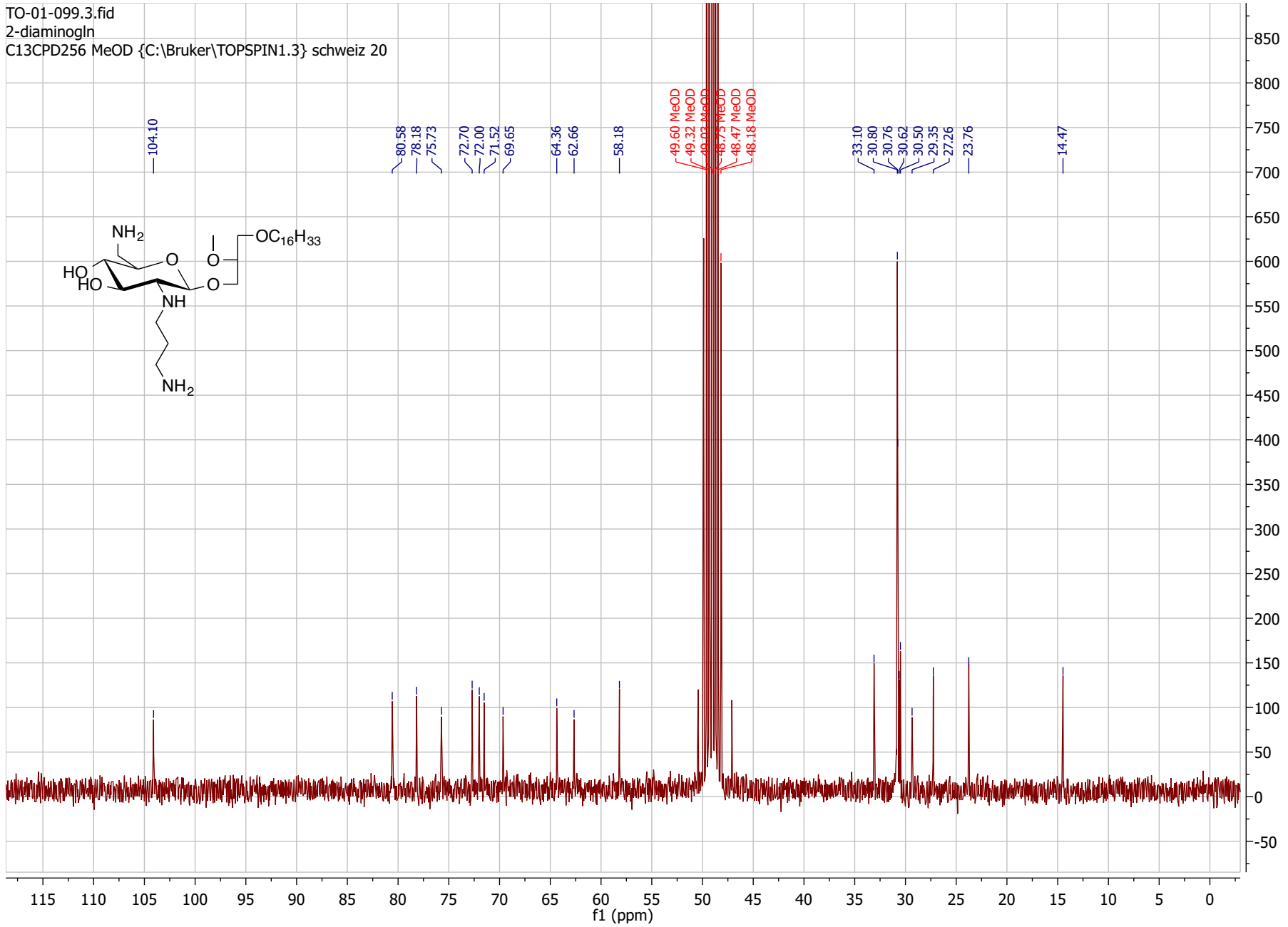
[1]H NMR of Compound **31a**



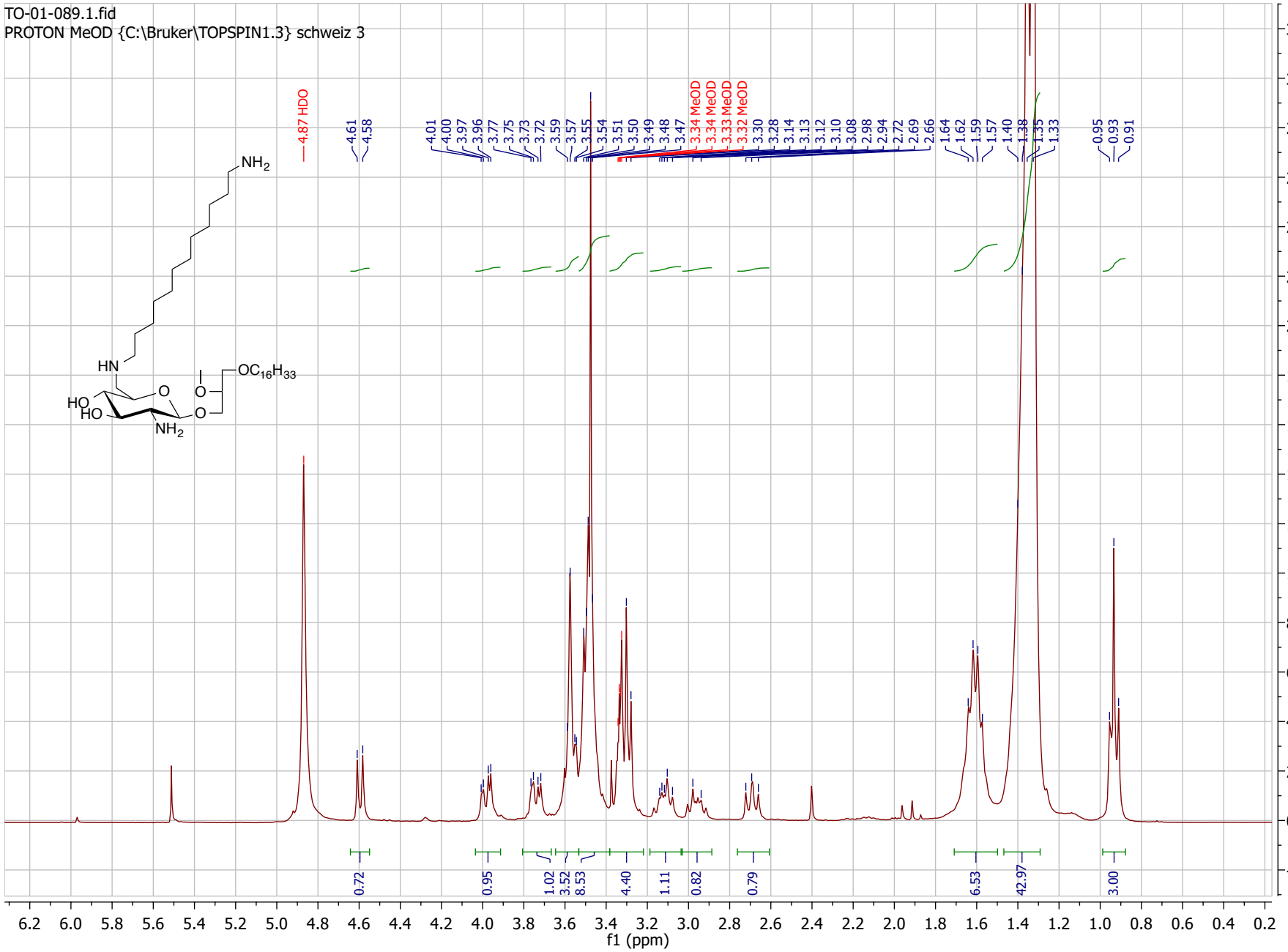
^{13}C NMR of Compound **31a**



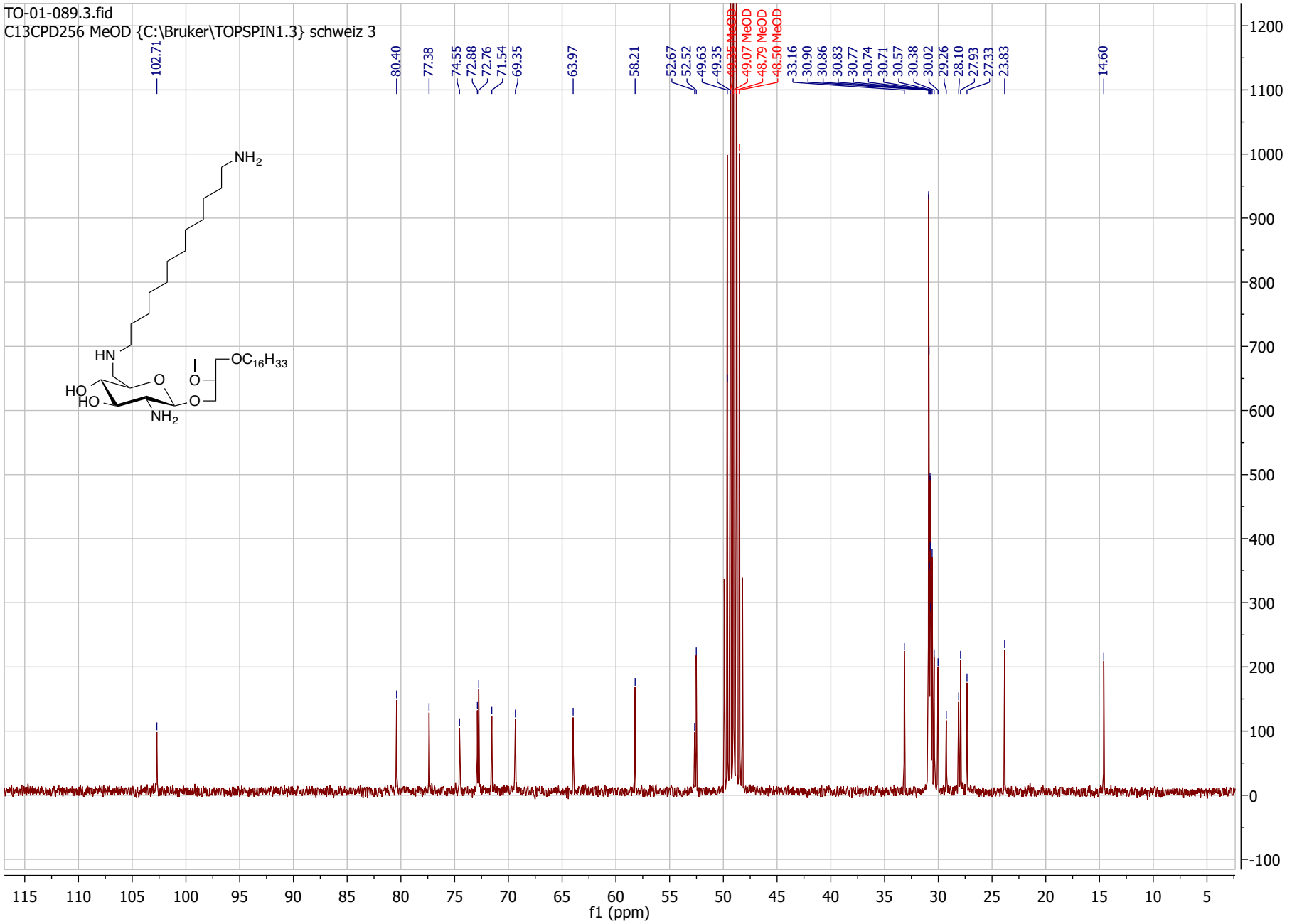
^{1}H NMR of Compound **31b**



^{13}C NMR of Compound **31b**



^{1}H NMR of Compound **32a**



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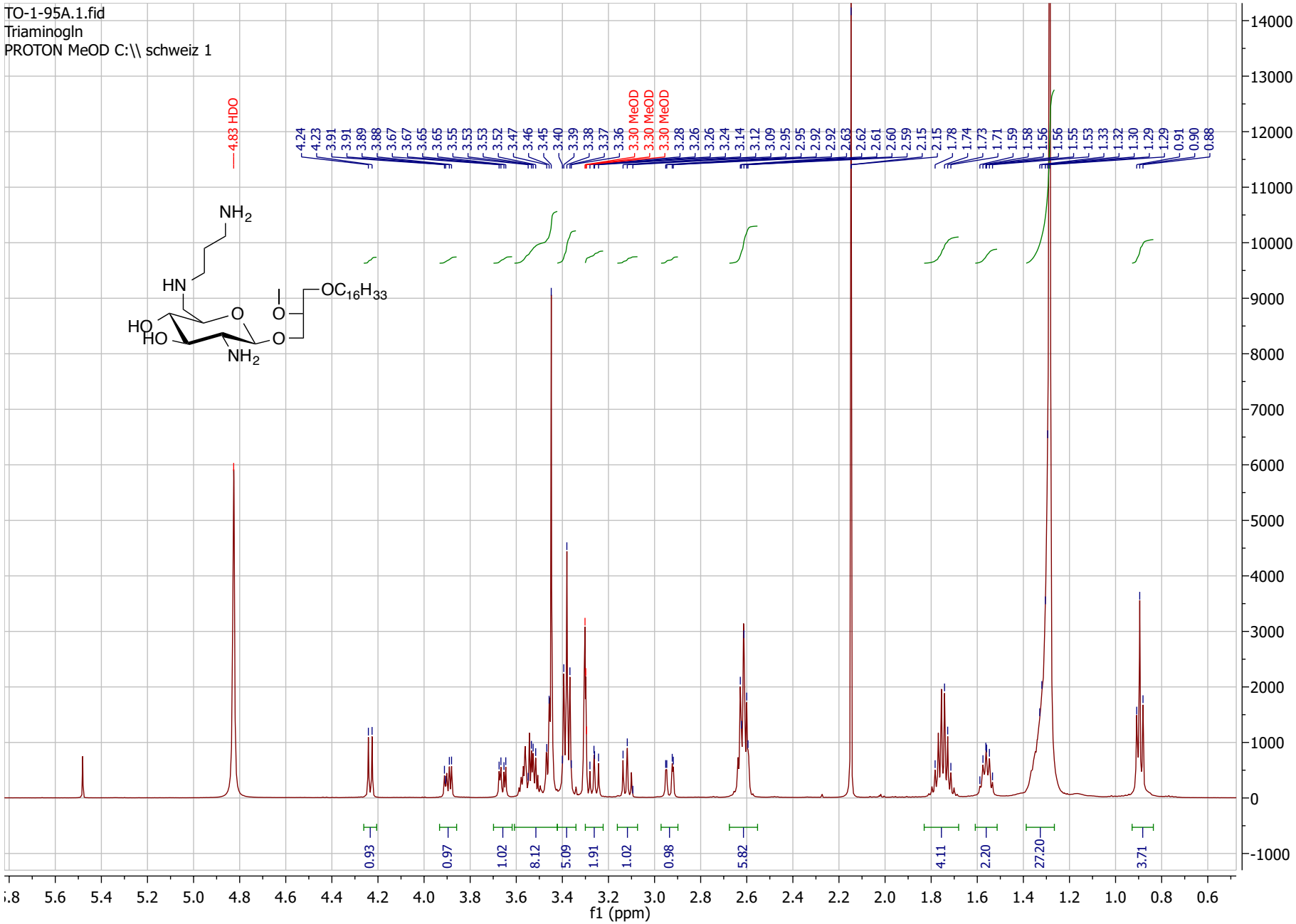

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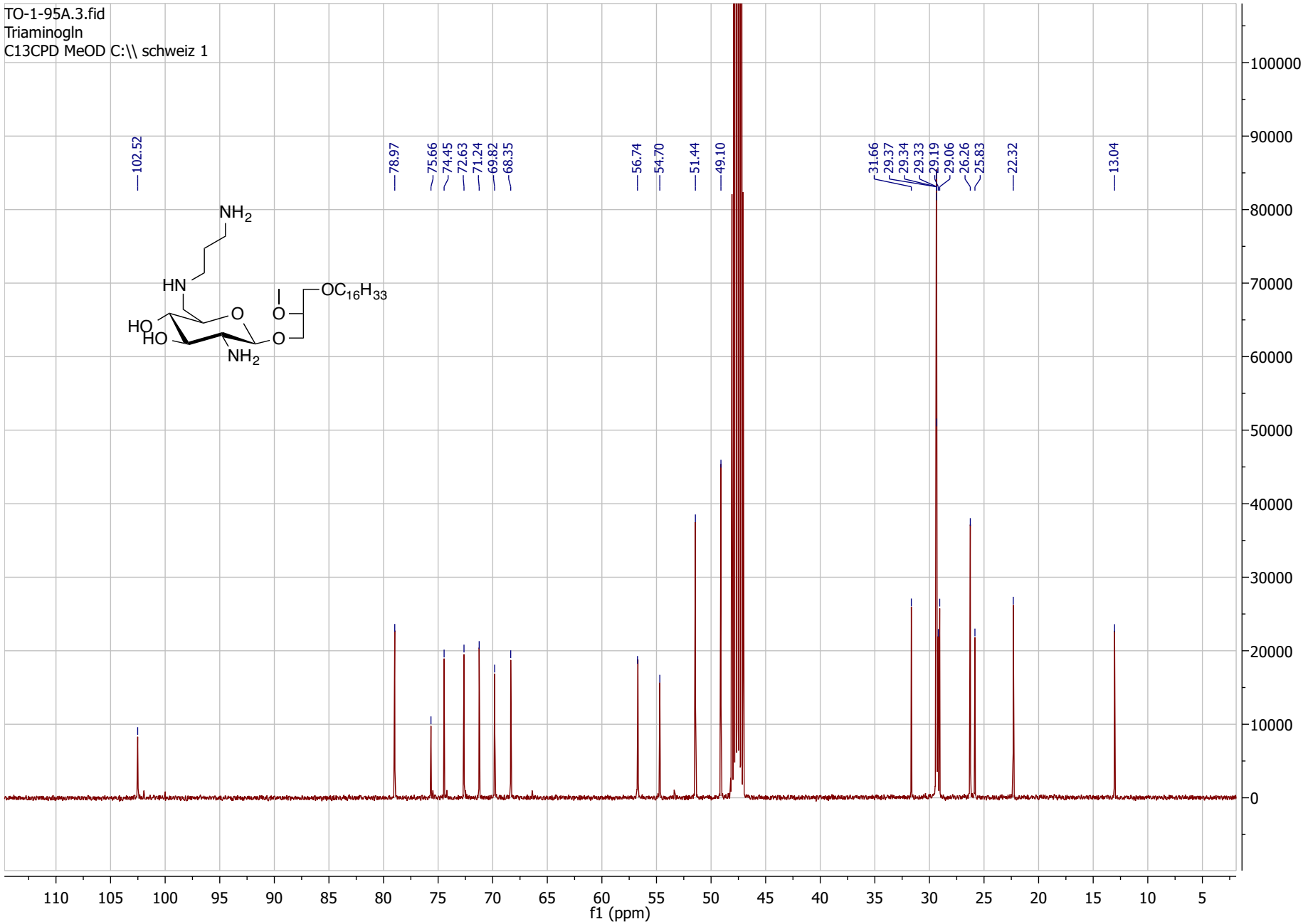

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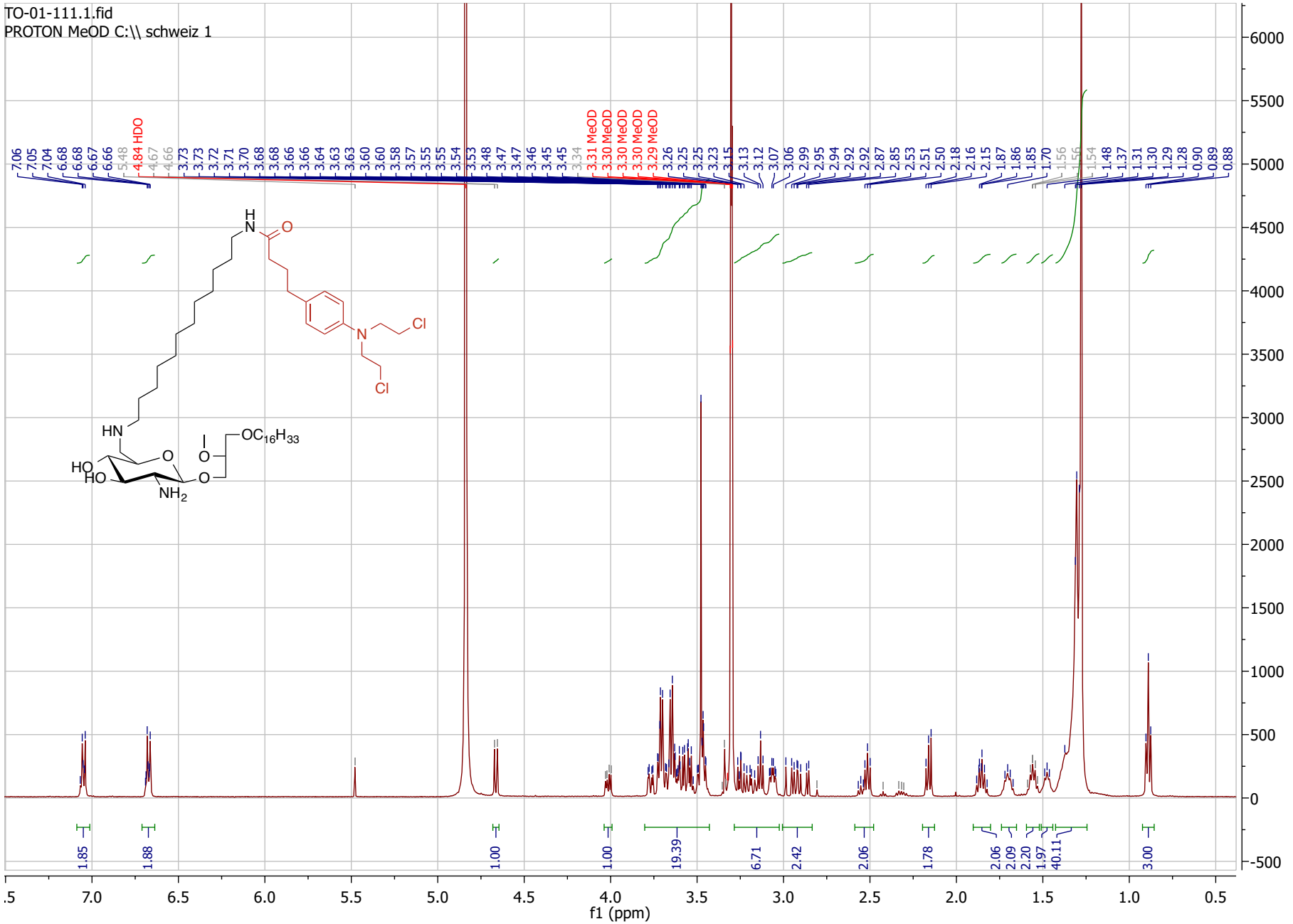
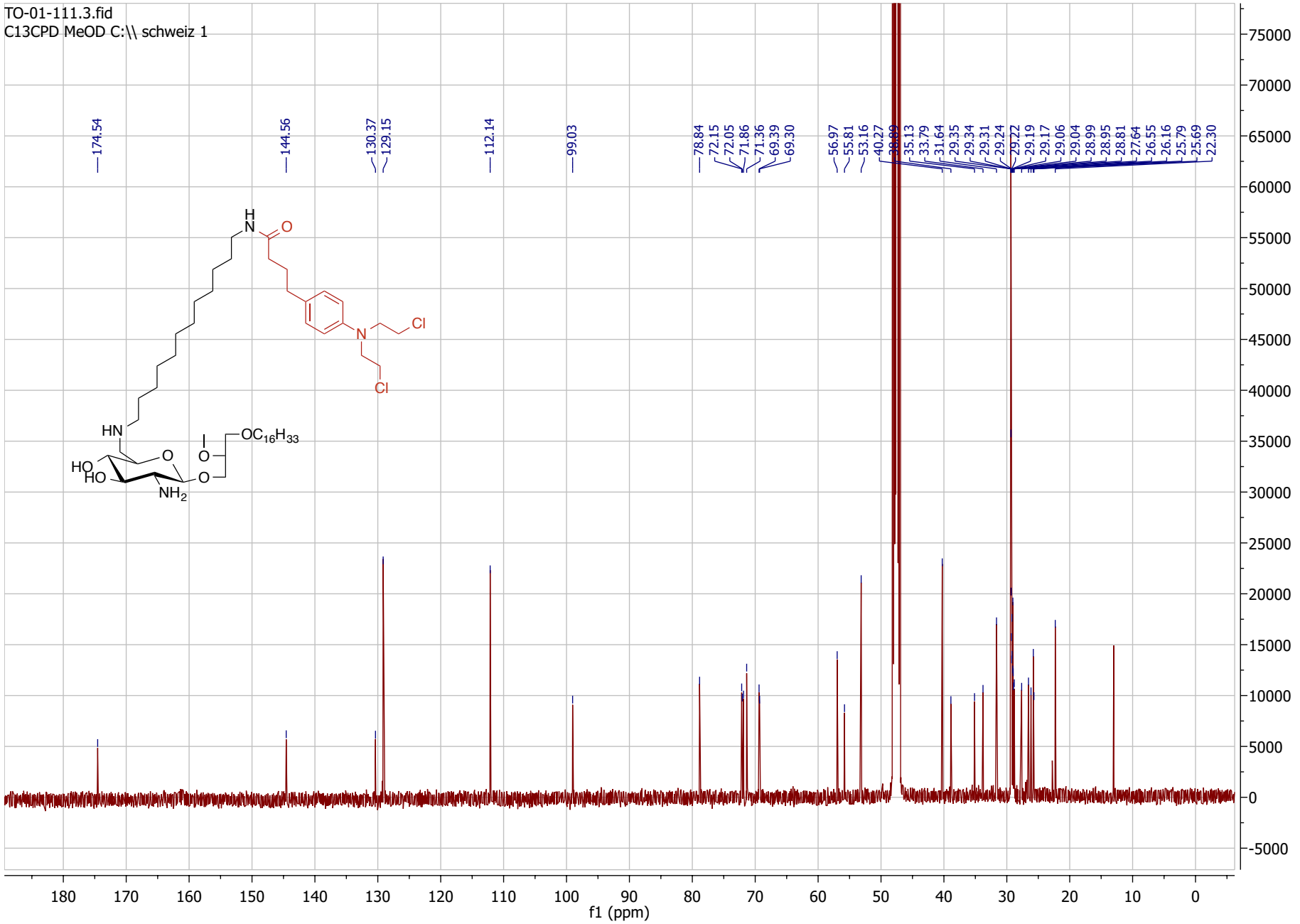
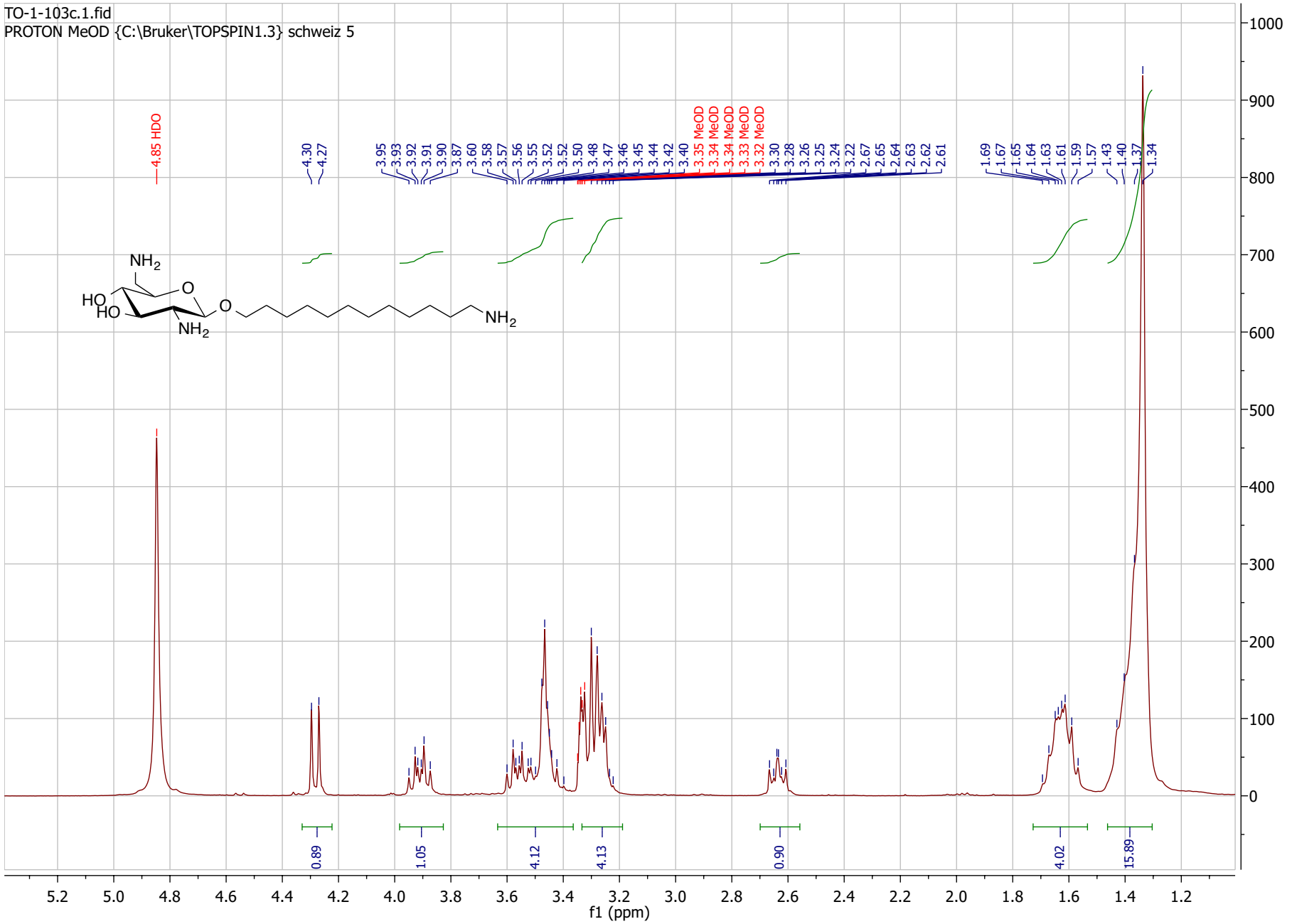


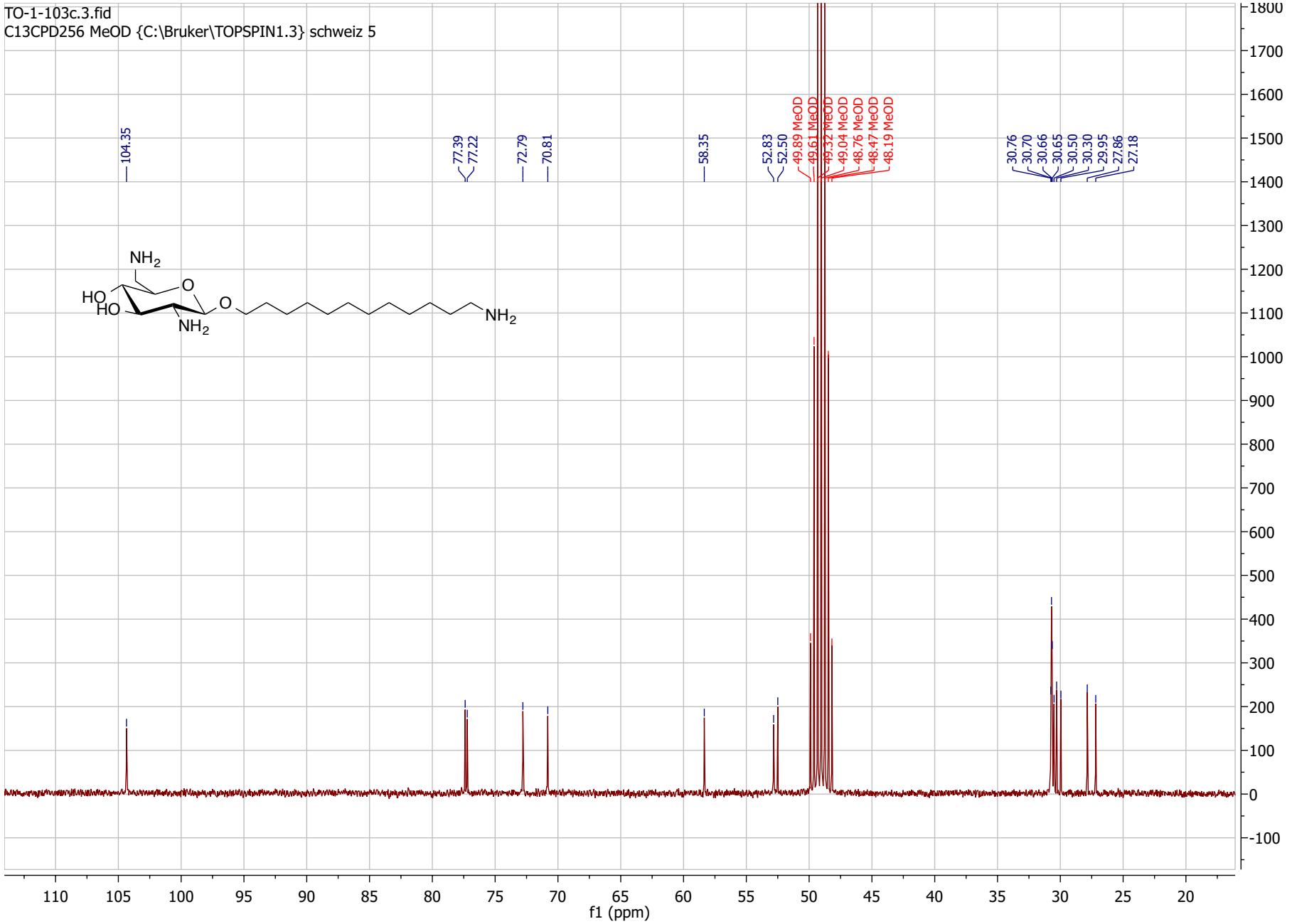
^{1}H NMR of Compound **33**



^{13}C NMR of Compound **33**



^{1}H NMR of Compound **34**



^{13}C NMR of Compound **34**