Cytotoxic Methylthioadenosine Analogues

by

Thomas Doerksen
B.Sc., The King's University, 2015

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in the Department of Chemistry

Supervisory Committee

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Abstract

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The gene for methylthioadenosine phosphorylase (MTAP) is absent in almost 30% of cancers, opening a door for selective chemotherapy. One strategy to target the absence of MTAP involves the design of a cytotoxic methylthioadenosine (MTA) analogue. Non-cancerous cells would break down the cytotoxic analogue, since they contain MTAP, but cancerous cells would not, since they do not have MTAP. However, before such a compound can be made, we need to better understand the types of substrates accommodated by MTAP. The purpose of this thesis was therefore to explore a series of MTA analogues, probing the structure-function relationships between MTAP and specific structural modifications of MTA.

Nine phenylthioadenosine (PTA) derivatives bearing *ortho*-, *meta*-, or *para*-methyl carboxylate, carboxylate, and hydroxymethyl substituents were synthesized and tested for cytotoxicity and as substrates for MTAP. The biological results of these nine compounds suggested that addition of substituents to the *ortho*-position was not tolerated by MTAP, and substituents similar to the hydroxymethyl might be accommodated by MTAP. None of the compounds were cytotoxic. This informed the design of ten more PTA derivatives, most of which were synthesized and tested for cytotoxicity and as

substrates for MTAP. The range of functionalities included an amine, an acetamide, a urea, an isovaleramide, and an *N*-nitrosourea group inspired by the known anticancer agent lomustine. The amine derivatives of PTA were the best substrates of all MTA analogues tested (including PTA). The *meta*-amine derivative and the *meta*-isovaleramide showed minor cytotoxicity. Finally, the urea derivatives were moderate substrates of MTAP, and this pointed towards the future creation of other nitrosoureas as potential cytotoxic MTAP substrates.

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List of Abbreviations and Symbols

^{1}H NMR	proton nuclear magnetic resonance
^{13}C NMR	carbon-13 nuclear magnetic resonance
AcOH	acetic acid
ATP	adenosine triphosphate
AMP	adenosine monophosphate
AU	absorbance units
BOC	*tert*-butyloxycarbonyl
CF_3CO_2H	trifluoroacetic acid
CH_2Cl_2	dichloromethane
cm^{-1}	wavenumber
DMF	dimethylformamide
DMSO	dimethylsulfoxide
ENU	*N*-ethyl-*N*-nitrosourea
EtOAc	ethyl acetate
Eq	equivalents
FBS	fetal bovine serum
h	hour(s)
H_2SO_4	sulphuric acid
HCl	hydrochloric acid
HR-MS	high resolution mass spectrometry
Hz	Hertz, s^{-1}
IR	infrared
J	coupling constant
K_{cat}	turnover number
K_m	Michaelis-Menten constant
KOCN	potassium cyanate
$LiAlH_4$	lithium aluminum hydride
LC	liquid chromatography
M	molar

MAT	methinine adenosyltransferase
Me_3Si-I	trimethylsilyl iodide
MeCN	acetonitrile
MeOH	methanol
mg	milligrams
min	minutes
mmol	millmoles
MTA	methylthioadenosine
MTAP	methylthioadenosine phosphorylase
MTRP	methylthioribose-1-phosphate
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$NaBH_4$	sodium borohydride
$NaHCO_3$	sodium bicarbonate
NaH	sodium hydride
NaOH	sodium hydroxide
NH_4OH	ammonium hydroxide
OH	alcohol group
PBS	phosphate buffered saline
PTA	phenylthioadenosine
r.t.	room temperature
SAM	*S*-adenosyl methionine
t-BuOK	potassium *tert*-butoxide
THF	tetrahydrofuran
TsCl	tosyl chloride

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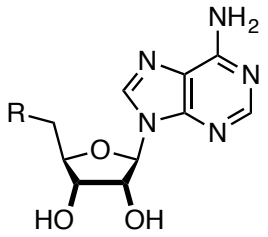
Dr. Peter Constabel from the biology department at the University of Victoria acted as the external member to the supervisory committee.

The cytotoxicity experiments were performed by Dr. Hongwei Cheng, at the BC cancer agency in Vancouver.

Chapter 1 Undermining A Shadowy Disease



1.1 Cancer

With the discomfort of a recurring toothache, the throb of a deep bruise, or the agony of an inflamed appendix, the broken body is impossible to ignore. Yet for all the attention disease demands, the confusion and the suffering, it does not give up a remedy willingly. Diseases have such a variety of conditions and contexts that even the definition of 'disease' is disputed among philosophers and scientists today (1–6). Some of this intellectual bickering has an unfortunate root in Platonic philosophy, where diseases exist as ideals in the realm of the Forms and where they manifest as a shadow of an ideal in the particularity of a patient (7). "Platonic physicians" match a patient's symptoms to the conditions of an ideal disease and treat accordingly. This is not too different from how medical practitioners behave today. In some cases, such as an appendectomy, platonic reasoning is efficacious; no one questions that all burst appendices should be removed. But there is one notorious disease that is far too shadowy to exist in Plato's realm of Forms, as if the shadows were cast by a multitude of ideals. It is a disease that has evaded a simple etiology and, for the majority of history, when surgical excision failed, has evaded a cure altogether (8–10).

That disease is cancer, the dark half of humanity's aspiration to immortality. It can begin with an environmental force—high energy radiation, reactive chemicals, viruses, or bacteria—which cause multiple mutations in the cell's DNA, twisting the programmed

order into an unchecked proliferation (11–13). It may also have its origins in obesity (14–15), excessive intake of alcohol or tobacco (17–18), suppression of the immune system (19–20), or even an individual's genetics (21–22). Whatever its origin, the cell perpetuates obliviously, growing and dividing—with no concern for its surrounding mortal community. It produces its own growth signals, ignores anti-growth commands, evades apoptosis, unceasingly replicates, constructs its own blood vessels, and often invades other regions of its host, thereby establishing new colonies (13). Death comes swiftly. In Canada, cancer claims the lives of almost a third of the population (23).

In an effort to move away from the Platonism of the past, cancer has become an umbrella term for hundreds of complex diseases (13, 24–27). Physicians consider a cancer to be significantly different based on its mutational origin, its home organ, and its home tissue (13). In other words, cancer has many origins and can have very unique behaviours. Now it is obvious that there cannot be a single 'cure' for cancer. It is no surprise then that developing drugs for cancer therapy has been an incredible challenge. In a process that takes 10 to 20 years—through drug discovery and preclinical testing, through phase I, II, and III clinical trials, through the FDA review, and then scaling up for manufacturing—tens of thousands of potential medicines are reduced to a single drug (27–29). This can cost hundreds of millions of US dollars (27–29). Even then, there are no guarantees.

Have we made progress in the war against cancer? Rather than repeat statistics of changes in cancer survival rates, I recognize there are multifarious factors that contribute to such statistics (30–31) and that some consider the statistics from pharmaceutical industries to be skewed (32–34). The fact that scientists and philosophers can even

entertain discussions on how much bias has skewed results, suggests that if progress is being made, it is not revolutionary. The war continues in increments: scientists offer varied solutions, medical practitioners understand the diseases in further nuance. They form a collective force that digs at the foundation of the castle of the shadowy tyrant. We hope that one day, in the distant future, cancer will clarify into an array of different diseases that can be treated and eradicated.

1.2 Chemotherapy

Finding themselves in the middle of a war, it is natural (and advisable) for scientists and doctors to ask: "What weapons do we have?" For localized cancers, the answer can be either surgery or radiation therapy (35). There are issues with both of these: both surgery and radiation therapy can fail and cause serious side effects (36–38). Beyond these—to tidy up after a surgery, to attempt where radiation failed, or to fight a non-localized cancer—the most promising weapon is chemotherapy (39–43). (There is another promising field of research called targeted therapy, the use of antibodies to specifically target cancerous cells, but that is not the focus of this thesis (35).)

By virtue of their unchecked proliferation, cancer cells differ from normal human cells; and difference is the gateway to selection. In principle, if enough biochemical differences can be discovered between these cells, then selective destruction of the cancer cells is possible. Early chemotherapy has focused on the fuzzy distinction of cell growth (35). The hope was that cancer cells could be targeted by virtue of their rapid growth and division. While this theoretical basis dominated early chemotherapy, it has developed into a broader discipline: the use of cytotoxic compounds to systemically treat cancer

(35).

There are many classes of chemotherapeutics, including alkylating drugs (44), antimetabolites (45), mitotic inhibitors (46), topoisomerase inhibitors (47), and antitumor antibiotics (48–50). A few examples are shown in the **Figure 1.1**. Alkylating drugs, such as cisplatin, nitrogen mustard, and lomustine, react strongly with the genetic material of cells, introducing unnatural alkyl chains onto DNA and inhibiting normal transcription (44). This has a larger effect on more rapidly dividing cells. Antimetabolites compete with important biomolecules, where in this case 2-fluoroadenine competes with adenine and methotrexate competes with folate (45). Antimetabolites inhibit regular metabolism usually relevant to the synthesis of DNA. This is one way to prevent cell growth and it has a more toxic effect on cells that are dividing more rapidly and producing more DNA (44–45). The last example in **Figure 1.1** is taxol, which is a mitotic inhibitor (46). By

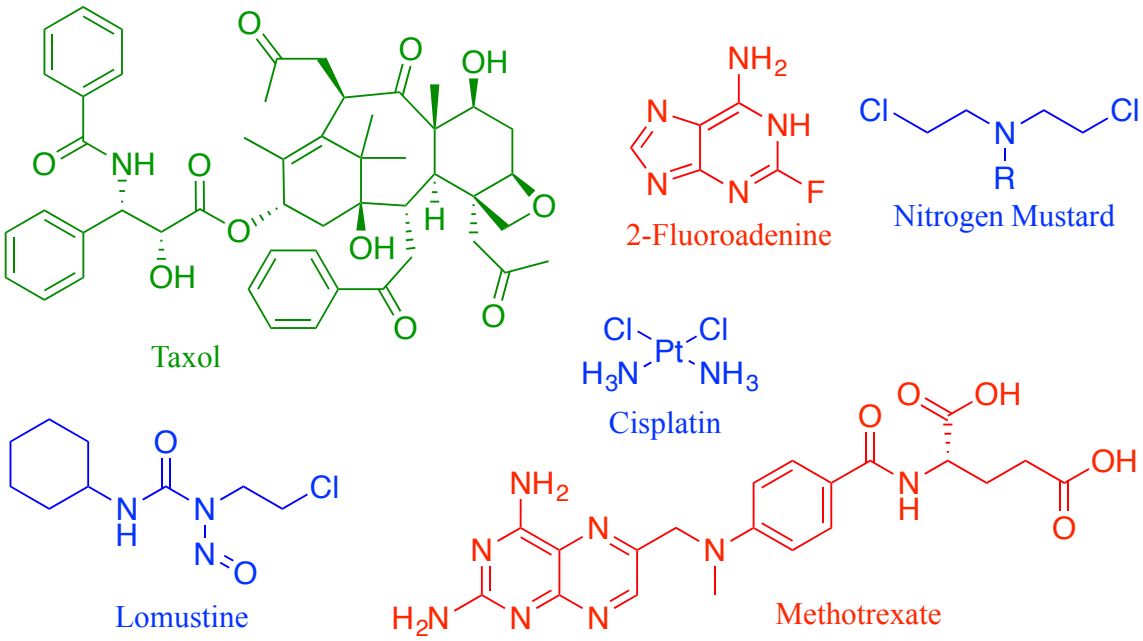


Figure 1.1 Chemotherapeutics. Alkylating drugs in blue, antimetabolites in red, and mitotic inhibitor in green.

binding to tubulin, a structural protein that constitutes microtubules, taxol prevents the disassembly of microtubules, which is an essential step in cell division (46). All of these examples, each of which are part of mainstream chemotherapy, preferentially impact rapidly dividing cells, but do not target cancer *per se*.

Chemotherapy is not a cure for all cancers. The growth of tumours slows with increasing size (this is called Gompertzian growth kinetics), and so for larger tumours (indeed the most dangerous tumours) the selectivity of mainstream chemotherapy is lacking (51). Cancers can also develop resistance to chemotherapeutics, already equipped with such a weapon as the *P-glycoprotein*, a versatile protein that can pump drugs out of the cell (52). In the case of antimetabolites, cancerous cells can decrease the expression of certain membrane proteins to prevent the uptake of a drug (such as methotrexate or 2-fluoroadenine) (53). Even when chemotherapy is effective, it can cause great harm to the patient, with extreme side-effects such as alopecia (patchy balding) or myelosupression, which can cause anemia or drastically weaken the immune system (54). Many of the side effects can be attributed to the indiscriminate nature of classic chemotherapeutic agents, targeting *all* rapidly dividing cells, such as hair or bone marrow (54). Chemotherapy's difficulty then is that its cell-growth distinction lies on a gradient (it is not binary) and so is not truly selective for cancer.

Concrete biochemical distinction, rather, could provide concrete results. If cancer cells contain or exclude specific biochemical pathways, for purposes such as tumour growth and development, this provides an 'either-or' mechanism to search and selectively destroy only the cancer cells in a patient's body (54–55). This facile model seems terribly close to Plato's form of a disease, which we know does not truly exist for cancer. It may

be the case however that such variability in the disease is more a difficulty for the medical practitioner in determining how the patient should be treated based on a medical toolkit, and that the task of scientists is in fact to hold Plato's ideals in their mind, searching for generalizations of cancer that could produce widely applicable methods of cancer therapy. At the same time, less generalized tools, which can only be used for very specific cancers, would also be helpful to the medical practitioner. Thus the goal of chemotherapeutic research is to add tools, however widely applicable, to the doctor's toolkit.

Most commonly conceived, chemotherapeutics selectively destroy cancers by way of specific targets present in the cancer cells, such as DNA or tubulin. This focus has difficulties, since most targets present in a cancer cell are also present in non-cancer cells. Traditional chemotherapeutics differentiate cancer and non-cancer cells based on their degree of growth, and so the selectivity of these drugs is also a matter of degree. To avoid this shortcoming, to create drugs that can be more selective for cancer, we might remove our focus from targets of high importance in cancer cells (such as DNA, adenine, folate, or tubulin) and instead focus on targets *absent* in cancer cells. Arguably, the genetic absence of a target can provide a more concrete selectivity than the mere abundance of another target. This leaves us with two questions: how can we target an *absence*? and are there any examples of such a dramatic absence in cancers?

1.3 MTAP deficient cancers

There is a biochemical trait that is found in nearly 30% of late stage cancers: the absence of a protein found in normal human tissues, called methylthioadenosine

phosphorylase (MTAP) (56–76). MTAP can be absent from non-small cell lung cancer (56, 63, 70), leukemia (61, 65, 71), glioma (62), mesothelioma (68), melanoma (72), osteosarcoma (73), soft tissue sarcoma (74), pancreatic cancer (75), and periampullary cancer (76), to name just a few. The absence of MTAP appears to be a phenomenon that is spread across all forms of cancer; wherever investigators have looked, there seems to be a significant percentage of cases that lack MTAP.

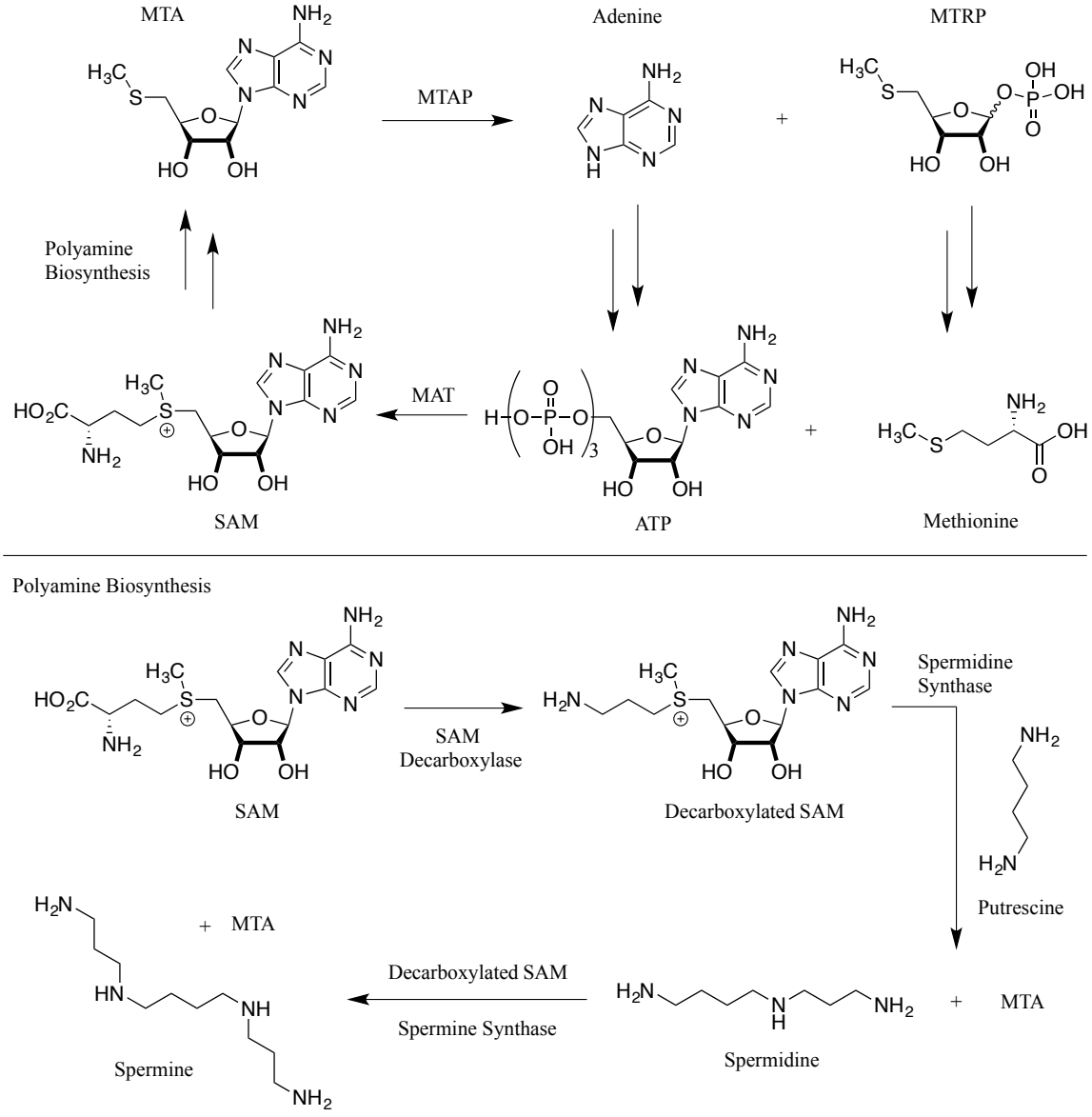
There might be a few reasons for the absence of MTAP. When cancers mutate to promote their own immortality—i.e. to survive—they tend to remove genes that inhibit cell growth (13). When they cut out these genes they frequently cut messily, unconcerned if they also cut out a nonessential protein from their genome; this is called codeletion (68). This frequently happens in the case of MTAP, whose gene is located near a tumour suppressor gene, called cyclin-dependent kinase inhibitor 2A (CDKN2A) (57, 77–93). MTAP-CDKN2A codeletion has been correlated to a drop in patient survival (82, 94–96). Another explanation of MTAP's absence involves the methylation of the MTAP promoter gene, not deleting the MTAP gene, but preventing its expression (82, 97). There is also the possibility that MTAP is itself a tumour suppressor gene (91). Whether these explanations are correct or not, the most important point is that MTAP is absent in nearly 30% of cancers, and this should provide, in certain cases, a concrete distinction between cancerous and non-cancerous cells, allowing for selective therapy. A successful method of selective therapy requires an understanding of MTAP's biochemical role.

1.4 MTAP

In all mammalian cells, short-chain polyamines are essential compounds, linked to

DNA synthesis and the cell cycle (98). The polyamines putrescine, spermidine, and spermine can be obtained through one's diet, or else synthesized within the cell (98). Their synthesis begins with the cell's primary methylating agent, *S*-adenosyl methionine (SAM) (98). See the bottom of **Figure 1.2** for the polyamine biosynthesis pathway. Instead of acting as a methylating agent in this case, SAM is first decarboxylated (98). The propylamine substituent on SAM is then added to the amine building block,

Figure 1.2 The MTAP biochemical pathway.



putrescine, to form spermidine (98). Each time decarboxylated SAM is used to form a longer-chain polyamine, methylthioadenosine (MTA) is formed as a byproduct (see the top of **Figure 1.2**) (98, 104). But MTA is more than waste material: it regulates the polyamine pathway as an inhibitor of many proteins in the pathway (96, 99–102). MTAP, the enzyme of interest, recycles MTA by cleaving it into adenine and methylthioribose-1-phosphate (MTRP) (**Figure 1.2**) (57, 102–104). Through another series of biochemical reactions, involving many more enzymes and other molecules, adenine can be added to a ribose sugar to eventually form adenosine triphosphate (ATP) and MTRP is eventually transformed into methionine (56, 57, 59, 104–108). ATP and methionine are multifunctional compounds, but in this context they can react together with the help of methionine adenosyltransferase (MAT) to form *S*-adenosyl methionine (SAM). SAM is the key player in the polyamine biosynthetic pathway, completing the cycle (98).

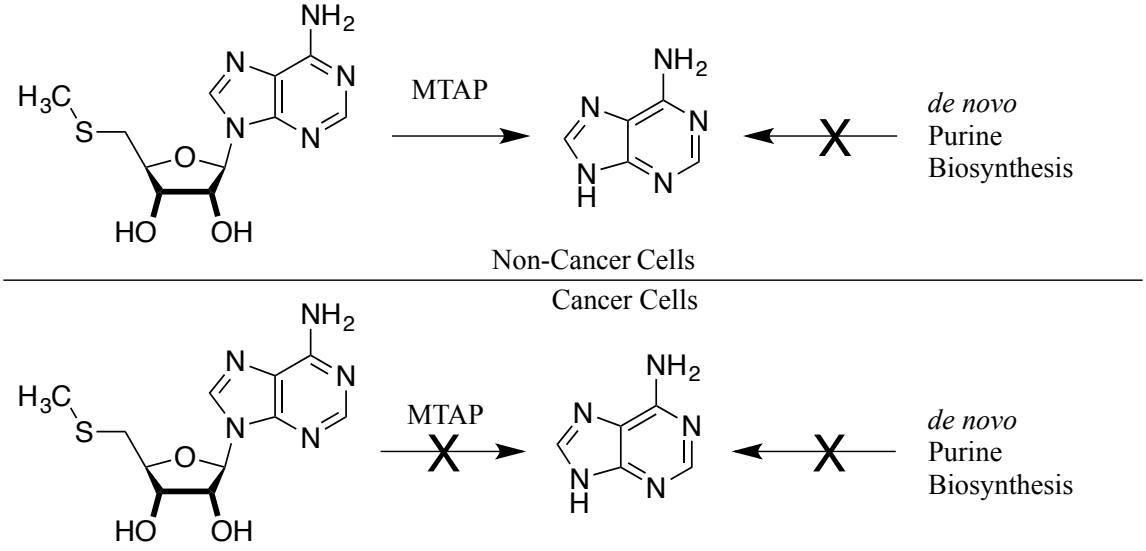
1.5 Targeting Strategies

MTAP plays an important role in the recycling of MTA for polyamine biosynthesis, as well as the production of free adenine (98). Since polyamines and adenine can be obtained through the diet, this is not an essential pathway and it is clear how cancer cells can survive without MTAP; cancer cells take up polyamines from the surroundings and consequently do not need MTAP to recycle any waste products of the polyamine biosynthesis pathway. Almost a third of cancers have accidentally deleted MTAP from their genome and in their haste to divide and grow, they cut corners for efficiency. This might be their downfall.

There have been only a few strategies to fight MTAP-deficient cancers. One such approach involved co-administration of MTA and a *de novo* purine biosynthesis inhibitor

(**Figure 1.3**), such as methotrexate, 6-mercaptopurine, asaserine or L-alanosine (56, 59, 68, 74, 76, 104, 109, 110). Besides *de novo* synthesis, the MTAP protein is the only known doorway to free-cellular adenine (62, 68, 73, 74, 79, 93, 111–113). Inhibiting *de novo* synthesis of purines (adenine and guanine) would leave only one biochemical route to adenine: recycling MTA with MTAP. Cancers deficient in MTAP will not have the option of recycling MTA for adenine, and if purine synthesis is inhibited they will become adenine deficient. Adenine is a crucial component of RNA and DNA, and so limiting this compound would limit the production of RNA and DNA, drastically preventing cell growth of the cancer cells. Since the treatment involves the co-administration of MTA, non-cancer cells will have access to adenine through MTAP, they will not become adenine deficient, and so the non-cancer cells will be unaffected by the treatment. The logic of this strategy is quite sound, but there has been difficulty with its application, giving poor results (66, 104, 111). Bertino *et al.* speculate that the issue might be pharmacodynamic, such that the *de novo* purine synthesis inhibitors are unable to enter the cancer tumours (60).

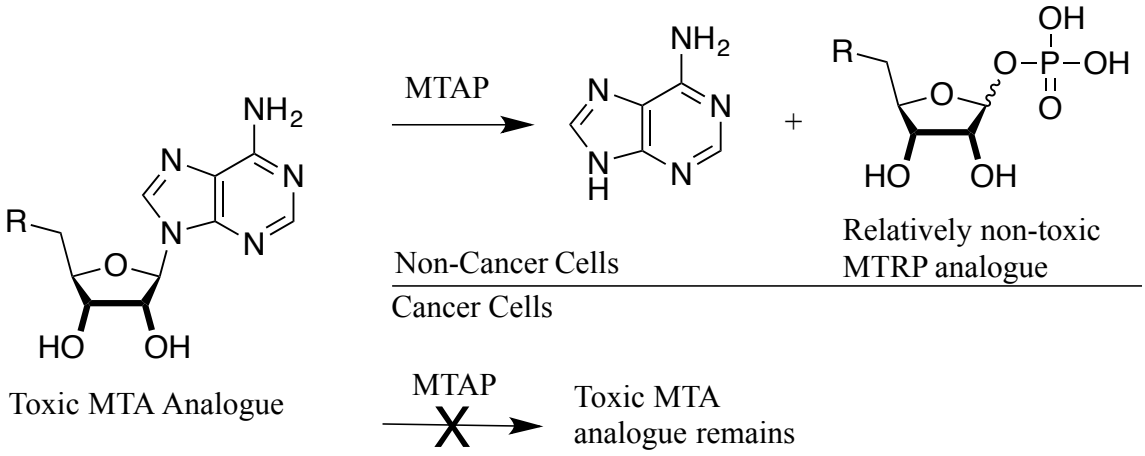
Figure 1.3 A Previous strategy to target MTAP-deficient cancers using a *de novo* purine synthesis inhibitor co-administered with MTA.



Another strategy involved the use of toxic purine analogues including diaminopurine (DAP), 6-methylpurine (MeP), and 2-fluoroadenine (F-Ade) (111). These analogues are toxic to both cancer and non-cancer cells, but would be co-administered with an agent such as MTA that would protect the non-cancer cells (111). Since MTAP is present in normal human tissues, MTA would provide a source of adenine, and this would compete with the toxic adenine analogues for phosphoribosylation (the next important step for making use of free-cellular adenine) (111). Cancer cells deficient in MTAP would be unable to obtain adenine from MTA and so would only have the toxic adenine analogues for phosphoribosylation, effectively killing the cancer (111). DAP initially showed good results in a few clinical trials but was abandoned after toxic side effects were noted (111, 114). Application of MeP and F-Ade for treatment of MTAP-deficient cancers has shown good preclinical results so far (111, 115, 116).

We propose a very different strategy to target the absence of MTAP. It involves the design of a cytotoxic MTA analogue (**Figure 1.4**). This MTA analogue should be toxic, but MTAP should still be able to break it up into two general components, resembling the adenine and MTRP from above, and these should either be non-toxic or should be easily evacuated from the cell. When a patient is given a dose of this toxic MTA analogue,

Figure 1.4 The Cytotoxic MTA Analogue Strategy.



ideally two things will happen. First, the cancer cells will be unable to break up the toxic MTA analogue because they lack MTAP and they will be unable to easily remove it from the cell, effectively killing the cancer. Second, the non-cancer cells, which still contain MTAP, will cleave the toxic MTA analogue into adenine and a relatively less toxic MTRP analogue. The non-cancer cells can then evacuate the MTRP analogue, thus keeping the rest of the body free from poison. There are two reasons that the MTRP analogue would be less toxic than the MTA analogue. First, the MTRP analogue product contains a polar phosphate group, and this should increase its water solubility, which in turn allows it to be more easily evacuated from the cells and excreted from the body. Secondly, the MTA analogue will likely be toxic because of its similarity to adenosine (which has an alcohol in place of the methylthio-substituent), a crucial biomolecule that plays a part in many areas of the cell, most noticeably RNA. The MTRP analogue doesn't have a structural similarity to *as* important a biomolecule, and should be much less toxic. Ideally, a toxic MTA analogue will thus be toxic only to cancer cells that lack MTAP and should lack serious toxicity for the rest of human body. The MTA analogue must fulfill two conditions: (1) it must be cytotoxic; (2) it must be a substrate of MTAP, comparable to MTA. Before creating such a molecule, we need to better understand the extent to which structural modifications of MTA are tolerated by MTAP. The goal of this thesis was then to explore a series of MTA analogues, determining which structural changes affected their ability to be processed by MTAP.

1.6 MTAP Protein Analysis and Substrates

A previous study of MTAP by a group at Pfizer has provided a few important clues

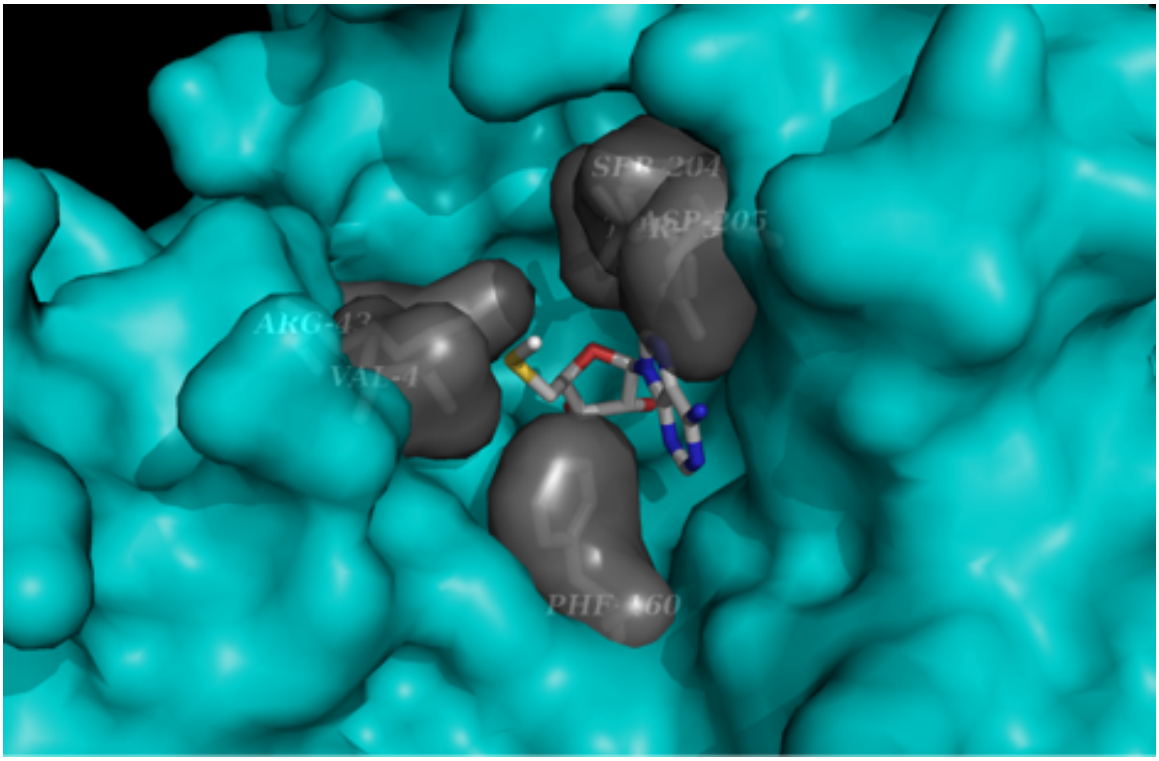


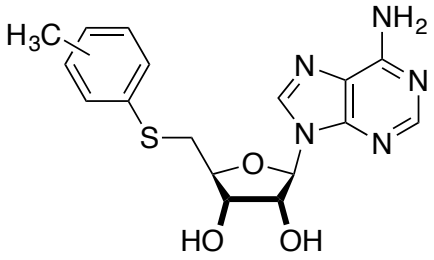
Figure 1.5 Crystal structure of MTA bound to the active site of MTAP.

towards a cytotoxic MTA analogue (103). In the crystal structure of MTAP bound to MTA (117), they noted a large pocket near the C5′ carbon of MTA, not occupied by either protein or substrate. A single perspective of this binding interaction is shown in **Figure 1.5** (data from 117).

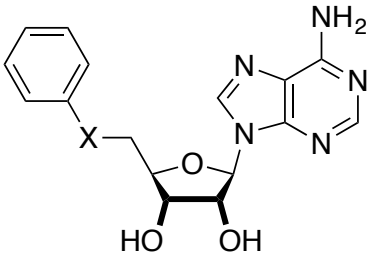
This pocket is surrounded by residues including valine, phenylalanine, arginine, and serine. Noting this, the Pfizer group explored a variety of different substrates, replacing the methylthio-substituent of MTA with a series of other functionalities such as alkyl chains, phenyl rings, pyridyl groups, 5-membered heterocycles, quinolines, benzyls, and saturated carbon rings (103). The pocket was large enough to incorporate all of these substituents, with a wide range of activities (measured as K_{cat} and K_m values). An MTA analogue, phenylthioadenosine (PTA, shown in **Figure 1.6**), was a moderately effective substrate relative to MTA, confirming that MTA analogues with large hydrophobic

substituents bonded to the sulfur atom in place of the methyl group can be MTAP substrates (103). They also appended methyl substituents to the phenyl ring at varying positions to see how much this would affect reactivity. Their results showed that adding a methyl group at the *ortho*-position drastically worsened the compound as a substrate, while adding it to the *meta*-position very slightly improved it as a substrate (103). This result is important for later compounds that I synthesized and tested. Kung *et al.* also explored the substrate potential for a set of PTA-related compounds, replacing the sulfur atom with carbon, nitrogen, and oxygen linkers (**Figure 1.6**). They found that replacing the sulfur with a methylene group, called 'compound 34' (**Figure 1.6**) was an MTAP substrate comparable to MTA (103).

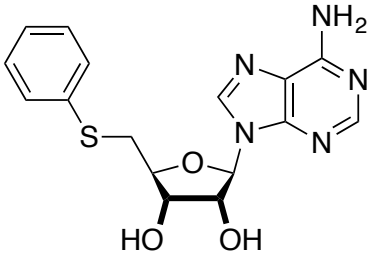
Figure 1.6 MTAP substrates explored by Kung *et al.* (103) with relative activities.



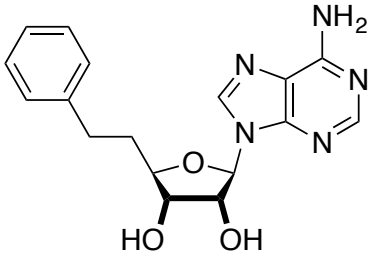
meta > none > para > ortho



X = CH_2 > NH > S > O



Phenylthioadenosine (PTA)



'Compound 34'

Compound 34 was the best potential cytotoxic MTA analogue constructed so far, since it was a good substrate of MTAP and since it had a significant structural variation from MTA. To test this, a previous PhD student in the Wulff lab at the University of

Victoria, Mike Brant, synthesized compound 34 and tested it as an MTAP substrate *in vitro* and *in vivo*, finding it mildly cytotoxic, but also finding it to be a poor substrate for MTAP. This was an unexpected result, and is likely due to different recombinant MTAP proteins used between the labs, which would have slightly different sequences and structures. If compound 34 was so sensitive to different recombinant proteins, it was unlikely that it would be useful as an effective *in vivo* treatment.

Taking a step back and continuing explorations of potential MTAP substrates, Jordan Friedmann, a BSc student, synthesized PTA and confirmed it to be a moderate substrate of MTAP, as well as being mildly cytotoxic. Since PTA differs from compound 34 only by the replacement of a carbon with a sulfur, this suggested that the sulfur atom was essential for good MTAP substrates, at least for this specific recombinant protein. This also confirmed the possibility of incorporating large hydrophobic substituents at the thiol position. PTA was then a model substrate compound for MTAP and was the inspiration for further research.

1.7 Research Direction

The goal of this study was to explore analogues of PTA, measuring their acceptance by MTAP and their cytotoxicity. Knowledge of their activity would inform what structural changes on PTA are tolerated by MTAP, pointing towards future MTA analogues. Knowledge of their cytotoxicity would point towards future *cytotoxic* MTA analogues.

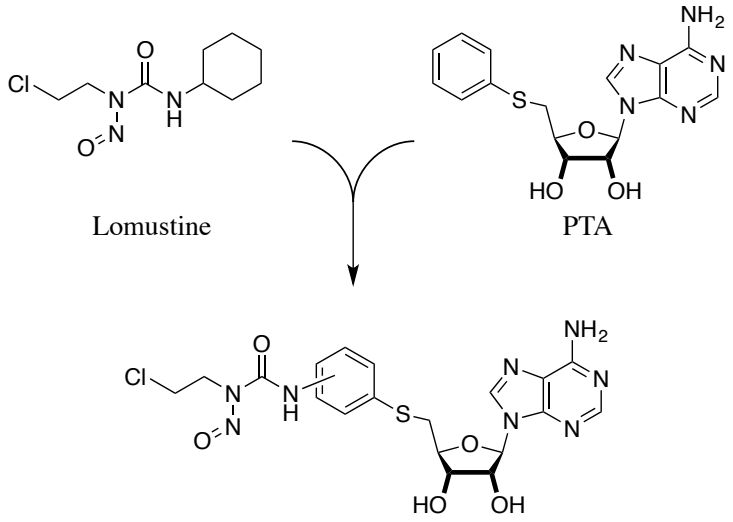
To increase cytotoxicity, it was important to hypothesize *how* PTA derivatives could be cytotoxic. MTA shows a striking similarity to adenosine, a significant

component of RNA and ATP—these are two of the most important biological molecules in every cell. Whereas adenosine contains a 5′-alcohol group, MTA has a methylthio-substituent. The antimetabolites can be cytotoxic by virtue of their structural similarity to important biomolecules (45). Similarly, if a PTA derivative were to contain an alcohol in the C5′ region, as in adenosine, it is possible that this derivative would have enough structural similarity to adenosine and could compete with adenosine, rendering it cytotoxic. Furthermore, the 5′-alcohol of adenosine can be phosphorylated by adenosine kinase to make adenosine monophosphate, which in turn can be phosphorylated over two steps to form ATP (118–120). RNA polymerase then adds ATP to a growing chain of RNA (118, 121). If the alcohol of the PTA derivative were to be phosphorylated in the same manner as adenosine, then it could also eventually be incorporated into RNA. Adenosine analogues can accumulate in their triphosphate form and inhibit RNA synthesis (122). In general, nucleoside analogues are common cytotoxic agents (123). It is possible, then, that PTA derivatives with an alcohol in the C5′ region could be cytotoxic for the same reasons.

PTA derivatives could also be cytotoxic if they contained a well-known toxic functional group appended to the phenyl ring. Nitrosoureas are common functional groups used in chemotherapeutics and known to have strong cytotoxic effects (124–125). Lomustine is an example of such a chemotherapeutic (**Figure 1.1**). I hypothesized that a Lomustine-PTA fusion could be a very good cytotoxic MTA analogue (**Figure 1.7**). This compound contains the cytotoxic functional group of lomustine, which suggests that the compound will be cytotoxic. It also might still be a good substrate for MTAP since the added functionality is relatively far from the active site of the protein.

It was uncertain whether the lomustine-PTA fusion would still be a substrate of MTAP, so I did not begin with the synthesis of this compound, but instead began with the synthesis of a series of simpler PTA derivatives. A range of different substrates with

Figure 1.7 Potential cytotoxic MTA analogue: a lomustine-PTA fusion.



differing functionalities, shapes, and activities, would flesh out the important structural-function relationships between MTAP and possible substrates.

1.8 Objectives

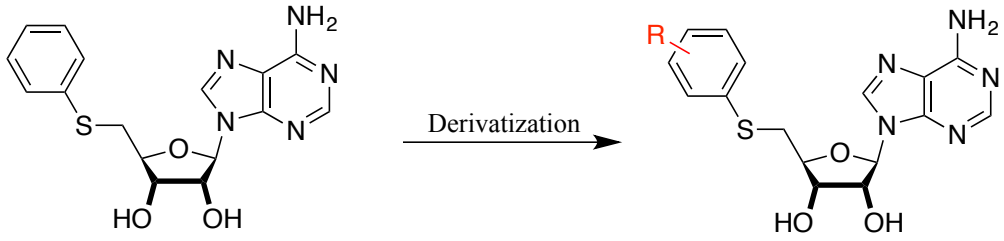
To explore the possibility of a cytotoxic MTA analogue, I designed and synthesized two series of PTA derivatives. In **Chapter 2**, I performed a reconnaissance of MTAP's active site around the phenyl ring of PTA. I added three functional groups, each to three different positions on the phenyl ring, and I tested each of these compounds as substrates for MTAP. This reconnaissance informed and motivated the content of **Chapter 3**, in which I describe the synthesis and biological testing of a second set of PTA derivatives. This further probed MTAP's active site and presented a new direction for future work.

Chapter 2 A Reconnaissance of MTAP's Active Site

2.1 Introduction

In my initial reconnaissance of MTAP's active site, I explored nine derivatives of PTA, which contained various substituents appended to the phenyl ring (**Figure 2.1**). After design, synthesis, and characterization, I tested the derivatives as substrates for MTAP, and Dr. Hongwei Cheng from the BC Cancer Agency tested the derivatives for cytotoxicity.

Figure 2.1 Derivatives of PTA.



PTA *ortho*, *meta*, *para*

A few research groups have synthesized derivatives of PTA by adding substituents to the phenyl ring, although the summation of their work forms an incomplete library (103, 126–129). The substituents include: all of the halogens at various positions (126, 128); methoxy substituents at all positions on the ring, including multiple substitutions (103, 126); amines at all positions (126–127); an acetamido group at the *para*-position (129); hydroxyl groups at all positions, methyl groups at all positions (103); multiple methyl groups, multiple methoxy groups, isopropyl groups at the *meta*-position, isobutyl at the *para*-position, trifluoromethyl groups at the *meta*- and *para*-position, methyl ester groups at all positions, carboxylic acids at the *meta*- and *para*-positions, and finally a primary alcohol at the *meta*-position (126). A portion of these substrates have been tested

for K_m and K_{cat} with MTAP. It was clear that the PTA derivatives analyzed so far were not designed and synthesized *systematically*, and that there are numerous lacunae in the research of PTA derivatives so far (such as the remaining regioisomers of many functional groups).

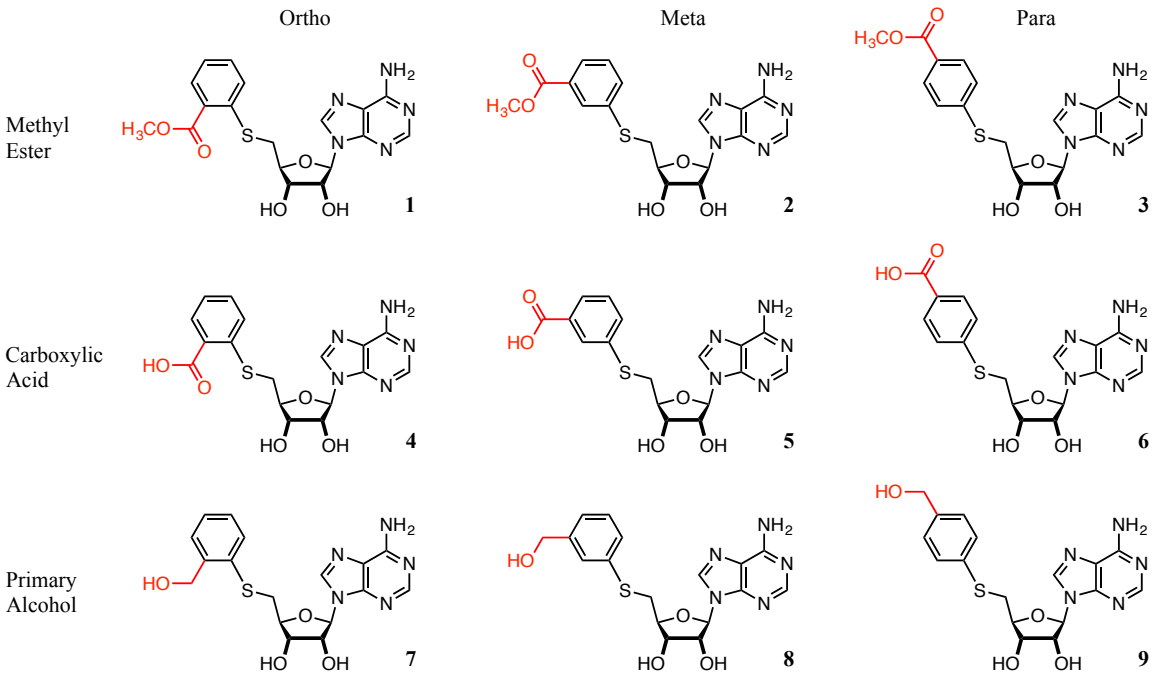
I decided to design, synthesize, and test a series of PTA derivatives that would fill in the cracks of previous research. The purpose was to test these derivatives as substrates for MTAP and for their cytotoxicity. With data from a thorough set of compounds—i.e., a set that included *all* the regioisomers of *each* functional group appended to the phenyl ring—I could then form a solid hypothesis for future work. Testing a thorough set of compounds as substrates for MTAP would probe the structure-function relationships between MTAP and possible substrates, revealing the shape and electronic preferences of MTAP's active site around PTA's phenyl ring. Nine compounds were designed, synthesized, characterized, and tested for their cytotoxicity and as substrates for MTAP. These results led to the design of a series of other PTA derivatives, the topic of the next chapter.

2.2 Design of Compounds 1–9

Like previous work, the PTA derivatives I designed contain a functional group appended to various positions on the phenyl ring. For the first set of derivatives, a methyl ester, a carboxylic acid, and a primary alcohol, were each added to the *ortho*-, *meta*-, and *para*-position of the phenyl ring. These nine compounds are shown in **Figure 2.2**.

Compounds **1–9** were designed to complement the literature and to explore their novel use as cytotoxic agents. Bloom *et al.* already synthesized a subset of these

Figure 2.2 Compounds 1–9, derivatives of PTA (added functionalities in red).



compounds, intending them as *anti*-toxicity agents in a strategy for fighting MTAP deficient cancers (126). In their strategy, *de novo* inosinate synthesis inhibitors were co-administered with anti-toxicity agents (126). Kung *et al.* also investigated MTA analogues (and PTA derivatives), with their intended purpose as anti-toxicity agents (similar to **Figure 1.3**) (103). The previous strategies of both Bloom *et al.* and Kung *et al.* were in some sense the opposite of my thesis, since I have created similar PTA derivatives as *cytotoxic* agents rather than anti-toxicity agents. The derivatives that I designed were not all previously synthesized and tested, and the compounds that *were* synthesized previously were not tested for cytotoxicity (126). The purpose of this work was thus to fill in the cracks of the previous studies and to test the complete set of derivatives for their potential role as cytotoxic MTA analogues.

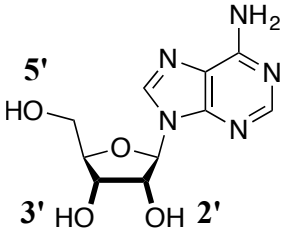
Compounds **1–9** were designed to test for tolerated functionalities around the

phenyl ring. The LogP values, an approximation of lipophilicity, were 0.66 for compounds **1–3**, –3.66 for **4–6** (carboxylate), and –0.44 for **7–9** (calculated by ChemDraw). This probed an initial range of polarities for PTA derivatives. The derivatives also probed the *shape* of MTAP's active site around the phenyl ring of PTA. In **Chapter 1**, I discussed the findings of Kung *et al.*, who investigated the addition of methyl substituents to the phenyl ring, and found that addition to the *ortho*-position made for a bad MTAP substrate, addition to the *para*-position made for a slightly worse substrate than PTA, and addition to the *meta*-position made for a slightly better substrate than PTA (103). I hypothesized that for each new functional group tested, addition to the *meta*-position (compounds **2**, **5**, and **8**) should make for the best substrates of MTAP, while addition to the *ortho*-position (compounds **1**, **4**, and **7**) should make for the worst. Investigating these functionalities at various positions would test this hypothesis.

Compounds **7–9**, containing primary alcohol groups, were of particular interest, as they were potentially the most cytotoxic. In **Chapter 1**, I discussed the potential toxicity of PTA analogues with alcohols in the C5′ region. MTA analogues have structural similarity to adenosine and could be cytotoxic by competition (in the same manner as antimetabolites), or they could be cytotoxic if they are phosphorylated (on the path to RNA synthesis). Compounds **7–9** have a primary alcohol in the C5′ region, and they are identical to adenosine apart from the phenyl and sulfide groups. This structural similarity could act as a mode of cytotoxicity either by competition or by phosphorylation, so I hypothesized that compounds **7–9** would be most cytotoxic.

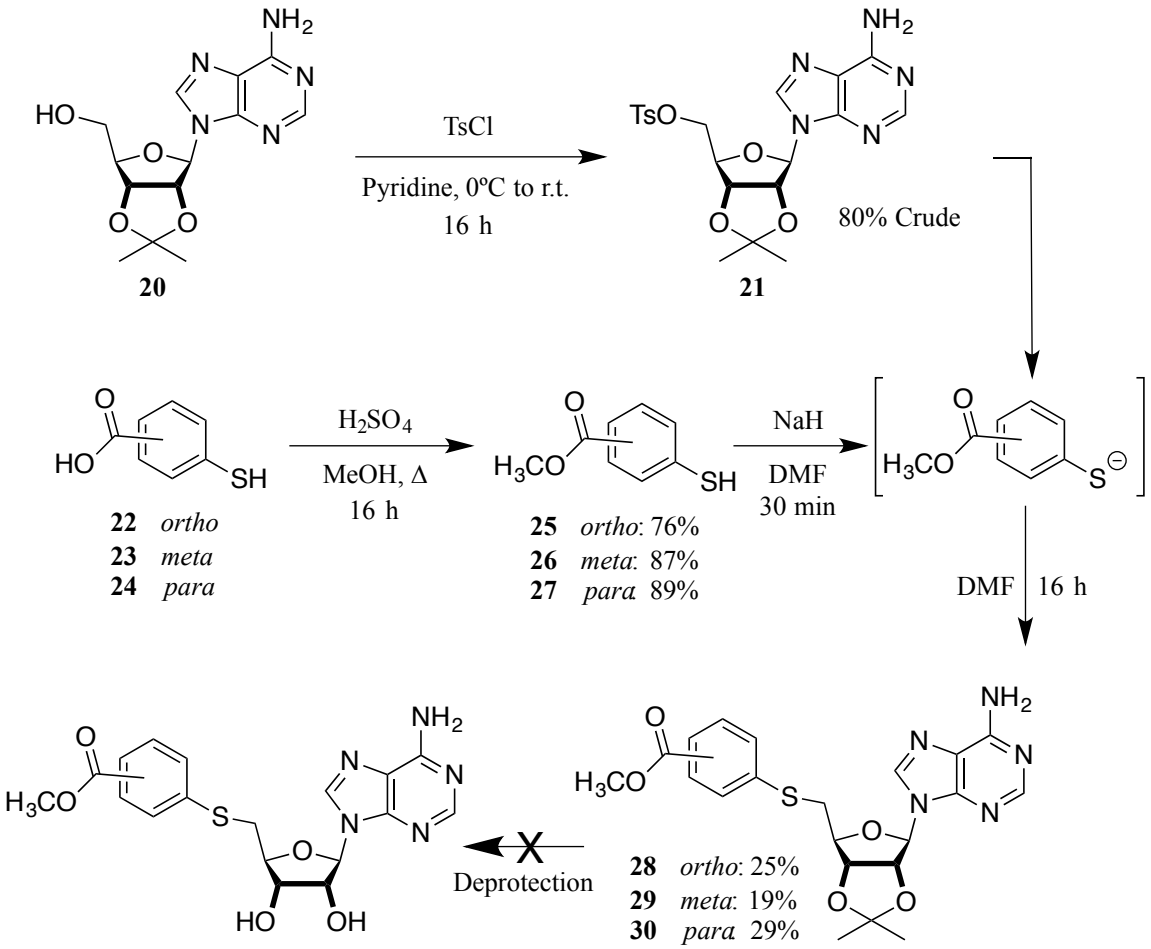
2.3 Synthesis of Compounds 1–3

Figure 2.3 Adenosine alcohol designations.



My first synthetic approach is shown in **Scheme 2.1**. Isopropylideneadenosine (compound **20**) was a commercially available material that provided the basic structure of the PTA derivatives: the adenine and ribose ring. The 2′- and 3′-alcohol groups of the phenyl ring were protected by an isopropylidene group (for alcohol nomenclature of adenosine, see **Figure 2.3**), and so the only available alcohol for tosylation was the 5′-alcohol. Isopropylideneadenosine and tosyl chloride were stirred for 16 hours in pyridine, beginning at 0ºC and warming to room temperature (130–131). After workup, I obtained compound **21** in crude form and good yield. In

Scheme 2.1 First synthetic approach, beginning with isopropylideneadenosine.



parallel, I protected compounds **22–24** by refluxing in methanol and catalytic sulphuric acid (132–133). After purification by flash chromatography, this gave compounds **25–27** in good yields. I then deprotonated the thiol of the mercaptobenzoates with NaH in DMF, and reacted the resulting thiolate with crude compound **21** (134–135). The thiolate attacked the 5-methylene carbon and replaced the tosylate leaving group through nucleophilic substitution. This gave an isopropylidene derivative of the first three target compounds, in low yield.

Deprotection of the isopropylidene group was the last step for compounds **1–3**. I explored a series of conditions for this deprotection, shown in **Table 2.1**, but I did not obtain the desired products. Acidic aqueous conditions were expected to cause deprotection (136). Many conditions have been reported in the literature for the removal of isopropylidene from an isopropylideneadenosine derivative, using hydrochloric acid (137), trifluoroacetic acid (CF_3CO_2H) (138–139), and acetic acid (103, 140, 141). Upon closer inspection, Me_3Si-I, condition 8 in **Table 2.1**, was more likely to deprotect a

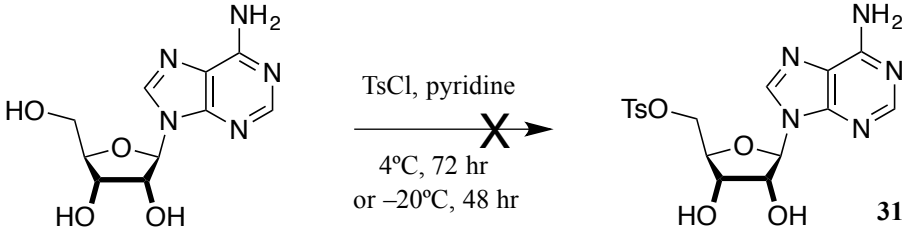
Table 2.1 Deprotection conditions for attempted removal of the isopropylidene group.

Entry	Reaction Conditions	Outcome
1	H_2O	Starting Material
2	Iodine, MeOH	
3	10% aq HCl, CH_2Cl_2	^{1}H NMR spectra showed a complex mixture. This suggested that either the compound fragmented at the hemiaminal ether carbon, or that elimination occurred on the ribose ring.
4	10% aq HCl, THF	
5	CF_3CO_2H, CH_2Cl_2	
6	CF_3CO_2H, THF, H_2O	
7	25% Acetic Acid in H_2O, 110ºC, 4 h	
8	Me_3Si-I, CH_2Cl_2, then H_2O	

methyl ester than an isopropylidene group (142). If the conditions did not return unreacted starting material, they appeared to cause more than a simple deprotection. For conditions 3–7 (**Table 2.1**) the ^{1}H NMR spectra showed a complex mixture of products and this suggested that either the compound fragmented at the hemiaminal ether carbon on the ribose ring (the carbon bonded to adenine), or that the 2′- and 3′-alcohol groups were eliminated from the ribose ring. It is possible that these decomposition steps occurred when the product was concentrated *in vacuo*, while heating. The reaction mixtures were not neutralized before concentrating. Heating a compound with a hemiaminal carbon in aqueous acidic conditions is likely to cause degradation into a free amine, aldehyde, and alcohol (143–144). My failure to neutralize the reaction mixture before concentration could then explain the decomposition. In either case, the yields for **Scheme 2.1** were poor and I decided to avoid the deprotection step in the hope of improving the yields and reducing the number of synthetic steps.

To circumvent the deprotection, I chose to make 5′-tosyladenosine (compound **30**) beginning from adenosine. I had previously used isopropylideneadenosine as my starting material because the protecting group prevented the tosylation of the 2′- and 3′-alcohols, while still allowing for the tosylation of the 5′-alcohol. In principle, adenosine (without the protecting group) could be selectively tosylated at the 5′-alcohol. This alcohol is primary, while the other alcohols are secondary and would suffer greater steric hindrance. Addition of tosyl chloride at cold temperatures might then afford tosylation at only the primary alcohol (145). Indeed, tosylation of adenosine is reported to favour the 5′-alcohol over the aniline nitrogen (146). Furthermore, isolation of the 5′-tosylated product was successful in the past (147). I attempted this reaction using two conditions, shown in

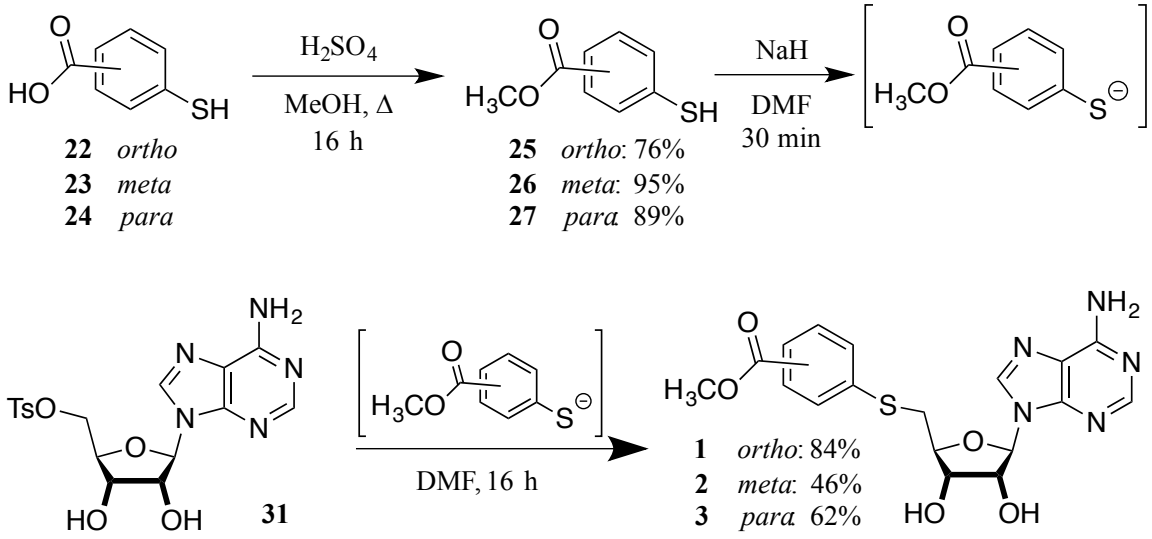
Scheme 2.2 Tosylation of adenosine.



Scheme 2.2. In my first attempt I added adenosine and tosyl chloride to pyridine and stirred them at 4ºC for 72 h (145). In my hands, this was *not* selective for the primary alcohol and gave multiple tosylation products (as analyzed by ^{1}H NMR). Even less reactive conditions (–20ºC, for 48 hours) gave multiple tosylation products. This issue has been reported previously (148). The reaction was possible in principle, but I chose not to spend time optimizing the reaction conditions and the purification. Instead, we discovered that 5′-tosyladenosine was commercially available through ChemImpex, and so I chose to this as my next starting material.

The new synthetic procedure, beginning with 5′-tosyladenosine, is shown in **Scheme 2.3**. Compounds **25–27** were synthesized, deprotonated with NaH, and then

Scheme 2.3 Improved synthetic route to compounds 1–3.

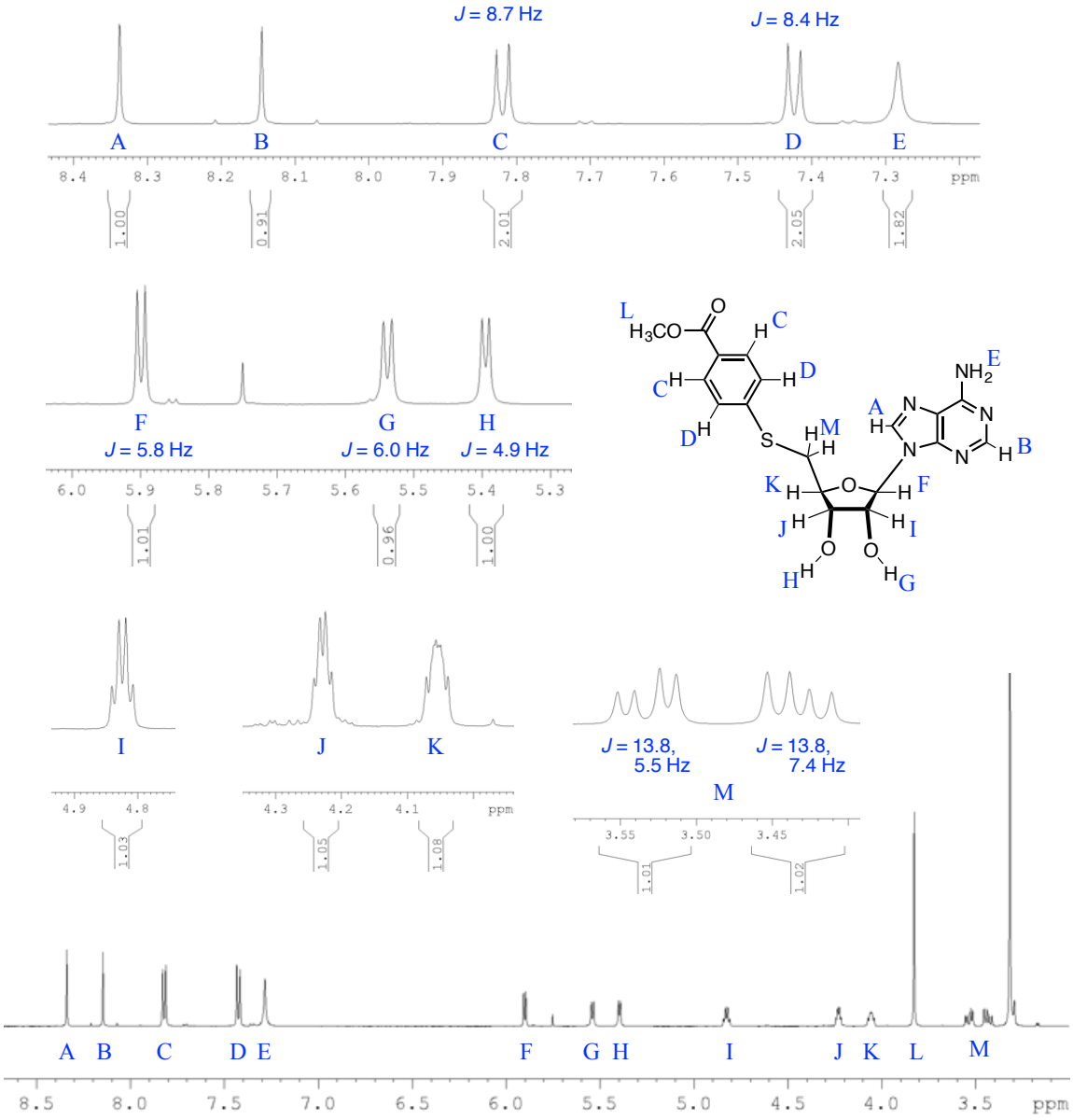


coupled to 5′-tosyladenosine in the same manner as described for **Scheme 2.1**. The products were not sufficiently soluble in solvents for flash chromatography (such as hexanes, CH_2Cl_2, EtOAc, or MeOH) or reverse phase liquid chromatography (such as H_2O or MeCN). Furthermore the patent that previously reported the synthesis of some of these target compounds did not report experimental data for characterization or purification (126). I chose to purify the compounds by recrystallization. Developing this purification required significant optimization, and it was not successful for most solvent systems. Eventually, I settled with a pure MeOH system (at approximately 10 ml of solvent per 100 mg of product). I heated the products to 60ºC, cooled to r.t., recrystallized at 4ºC overnight, and then washed the crystals with cold MeOH and CH_2Cl_2 (which was necessary for removing starting material impurities). This gave the first three target compounds in moderate-to-good yields.

For the complete characterizations of all the final compounds that I synthesized, see **Chapter 4** (Experimental) and **Appendix A** (spectral data). For the synthesis of all my target compounds, I found ^{1}H NMR to be the most revealing. The ^{1}H NMR spectrum for compound **3** is shown in **Figure 2.4** as an exemplar for the characterization of all target compounds. The exact chemical shift and multiplicity of each proton in adenosine is well known (149), and this served as a template for many of the ^{1}H NMR peaks in my experimental spectra. The adenine proton peaks are far from the C5′ region of adenosine, which is where adenosine and compounds **1–9** differ, and so I would not expect much difference between the adenine proton chemical shifts of adenosine and compounds **1–9**. As expected, the broad NH_2 peak (peak E) remained around 7.3 ppm, peak A remained at 8.34 ppm exactly, and peak B remained around 8.13 ppm. The closer the protons were to

the C5′ region, the more the chemical shifts deviated from adenosine. The hemiaminal proton, peak F, a doublet, remained at 5.9 ppm, with a *J* value of 5.8 Hz (rather than 6.2 Hz). Both the 2′- and 3′-alcohols (peaks G and H) shifted downfield, but retained similar coupling constants. The remaining protons on the ribose ring, peaks I, J, and K, were multiplets and each was shifted downfield compared to adenosine. The methylene

Figure 2.4 ^{1}H NMR of compound 3, an exemplar for characterization by NMR.

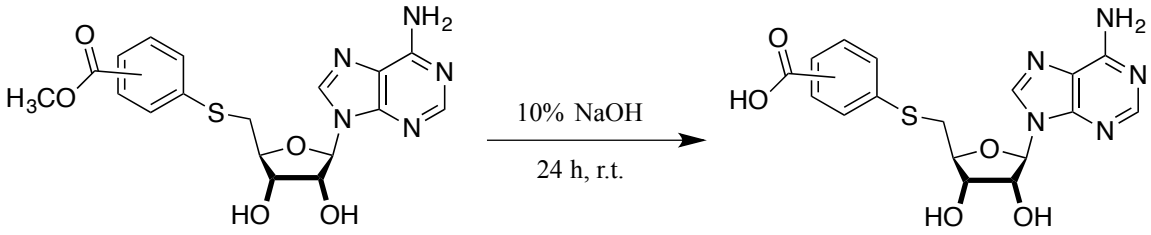


protons, peak M, also shifted downfield but retained the characteristic set of two doublet of doublets (dd), with geminal coupling at J = 13.8 Hz and vicinal coupling to peak K. The remaining peaks included the methyl protons—peak L, the only peak with an integration of 3—and the two sets of aromatic protons, each with an integration of 2. Each of these aromatic peaks (C and D) were approximately doublets, as they mainly coupled to each other. I reasoned that peak C would be further downfield than peak D because of its proximity to the methyl ester functionality (150). This proximity would decrease electron density (and so shielding) around protons C by inductive effects and resonance. There are no resonance structures that remove electron density from the carbons bonded to protons D and these protons are also further from the methyl ester functionality and so would be less affected by induction. I was therefore able to assign all the signals in the ^{1}H NMR spectrum.

2.4 Synthesis of Compounds 4–9

Compounds **4–6** were synthesized by saponification of compounds **1–3** (**Scheme 2.4**) Compounds **1–3** were saponified in water and 10% NaOH for 24 hours, affording the salts of compounds **4–6** in quantitative yields. An acidic workup converted the

Scheme 2.4 Saponification, affording compounds 4–6.



1 *ortho*
2 *meta*
3 *para*

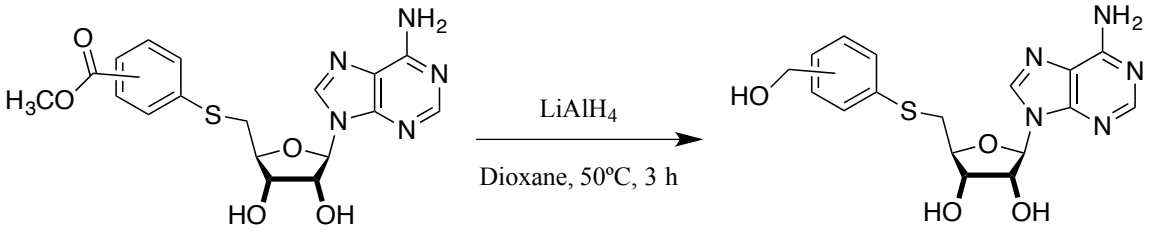
4 *ortho*
5 *meta*
6 *para*

Quantitative
(NaCl present)

carboxylate to the carboxylic acid, and trituration with a small amount of water removed excess salt. The products were identified by the disappearance of the methyl peak in the ^{1}H NMR.

Compounds **7–8** were synthesized by the reduction of the methyl ester of compounds **1** and **2** to a primary alcohol (**Scheme 2.5**). Compounds **1** and **2** were stirred in dioxane and lithium aluminum hydride at 50ºC for 3 hours (140). After neutralization and purification by filtration (rinsing with water), I obtained compounds **7** and **8** in moderate yields. ^{1}H NMR confirmed the synthesis of compounds **7** and **8**, by the disappearance of the methyl peak, and the appearance of a methylene and alcohol peak. Compound **3** did not dissolve in dioxane sufficiently to react with lithium aluminum hydride, and so compound **9** was not obtained by these reaction conditions. I attempted to obtain compound **9** by optimizing reaction conditions, using different solvents, temperatures, and reaction times, but I was unsuccessful (**Table 2.2**) (151, 152).

Scheme 2.5 Reduction, affording compounds 7 and 8.



1 *ortho*
2 *meta*
3 *para*

7 *ortho*: 69%
8 *meta*: 44%
9 *para* trace

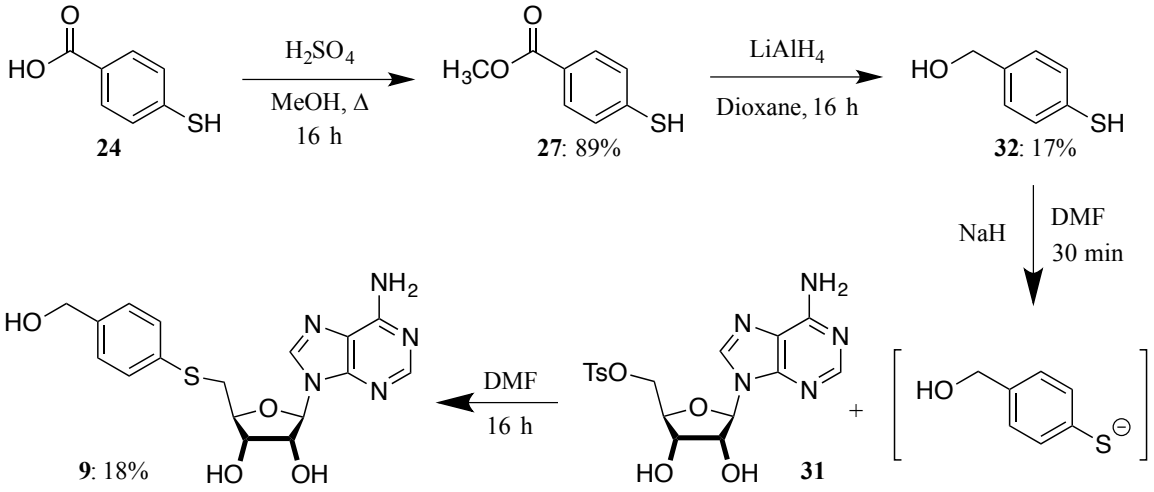
To synthesize compound **9**, I reversed the order of synthetic steps, as shown in **Scheme 2.6**. Instead of reducing the methyl ester *after* coupling compound **27** to 5′-tosyladenosine, I reduced compound **27** to compound **32** (*para*-mercaptobenzyl alcohol). I stirred compound **27** in a slurry of lithium aluminum hydride and dioxane for 16 hours.

Table 2.2 Conditions for the reduction of compound 3.

Entry	Reducing Agent	Solvent	Temperature	Time (h)	Outcome
1	1.5 eq $LiAlH_4$	THF	20ºC	3	S.M.
2	6 eq $LiAlH_4$	Dioxane	50ºC	3	Trace product
3	6 eq $LiAlH_4$	Dioxane	60ºC	4	S.M.
4	10 eq $LiAlH_4$	THF	45ºC	16	S.M.
5	18 eq $LiAlH_4$	Dioxane	80ºC	4	S.M.
6	10 eq $NaBH_4$	1:1:1 THF:MeOH:H_2O	45ºC	3	Trace product

For the filtration, I determined that MeOH dissolved the product and was not suitable for rinsing. I also determined that rinsing with CH_2Cl_2 gave an impure product. Eventually I discovered that rinsing with 5:1 CH_2Cl_2:MeOH gave the most pure compound in low yield (identified by ^{1}H NMR). With only a minimal amount of compound **32**, I did not fully characterize this intermediate, but immediately used it in the next step of **Scheme 2.6**. Purification of compound **9** proved to be very difficult. I explored a series of solvents and found that compound **9** was soluble in a mixture of aqueous HCl and MeCN. I then

Scheme 2.6 Back route to compound 9.

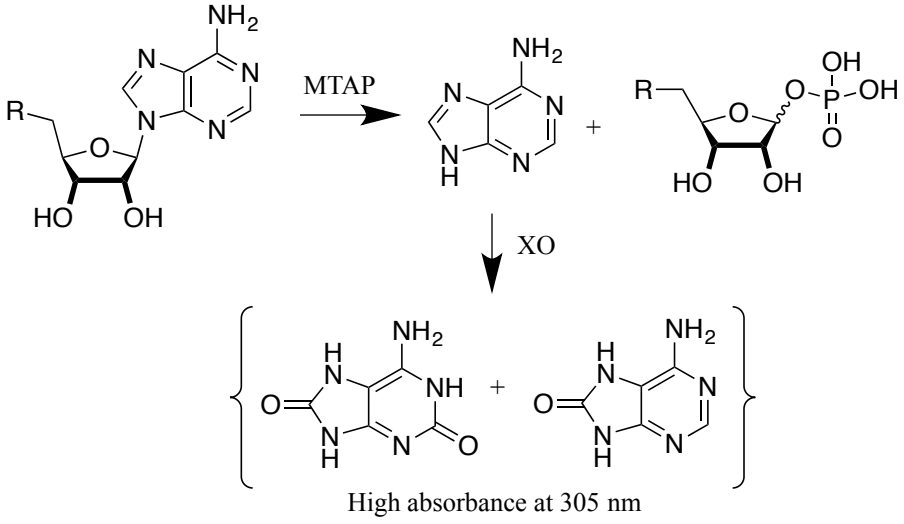


evaporated the solution while heating and recrystallized the product at 4ºC overnight. The crystals still contained impurities and I removed these by trituration with ethanol. Despite the low overall yield of the final compound, sufficient quantities of **9** were available for biological evaluation.

2.5 Suitability as Substrates for MTAP

With compounds **1–9** in hand, I performed an enzyme assay on each to determine if they were good substrates of MTAP. This was a coupled enzyme assay, involving a recombinant MTAP protein and xanthine oxidase (XO) (**Figure 2.5**) (153). The MTAP protein cleaves the substrate into a MTRP analogue and adenine. XO mainly oxidizes adenine into either 8-hydroxyadenine or 2,8-dihydroxyadenine, both of which absorb light at 305 nm (153). If a compound is a substrate for MTAP, adenine and its corresponding oxidized forms will form over time. The rate of increase in absorbance at 305 nm is then a relative indicator of enzyme activity (153). A rapid rate indicates that

Figure 2.5 MTAP-XO coupled enzyme assay.



the compound is a good substrate for MTAP.

The protocol for this assay was based on the protocols described by Kung *et al*. and Savarese *et al.* (103, 153). The absorbance (305 nm) was measured in a 96-well plate that had been warmed to 37ºC. While I tested replicates at both 1 mM and 0.1 mM concentrations of substrate, the data for the 1 mM assay was far noisier than the data from the 0.1 mM assay, and so I chose to only report the latter. Every well also contained XO and was diluted in PBS buffer. Controls were also tested, involving buffer, substrate, and XO, in the absence of the MTAP protein. The absorbance was measured over time for each substrate, in three 5-minute replicates. The slope and slope error was calculated for each replicate, and all three replicates were used to calculate an average slope and the corresponding standard error. These slopes were then set relative to the slope of MTA (and error was calculated for the adjustment). See **Appendix B** for tables of the slopes and standard errors. The average slopes relative to MTA and their standard errors are summarized in **Figure 2.7** below. (Though compound **9** was actually assayed in the second assay, it is included in this summary for sake of simplicity and comparison.) The slope of the change in absorbance was the indicator of how effective each compound was as a substrate for MTAP.

MTA and PTA were tested alongside the compounds I synthesized. MTA is the native substrate and so the rate at which MTAP processes it could be considered ideal. We would not necessarily expect any synthetic substrates to perform better than MTA, and so the rate of cleavage by MTAP was set relative to MTA (100%). PTA was a moderate substrate, at 39%, similar to previous results. The only other compounds as comparable substrates were **8** and **9**, at 35% and 23% (**Figure 2.7**).

Figure 2.6 Summary of substrates.

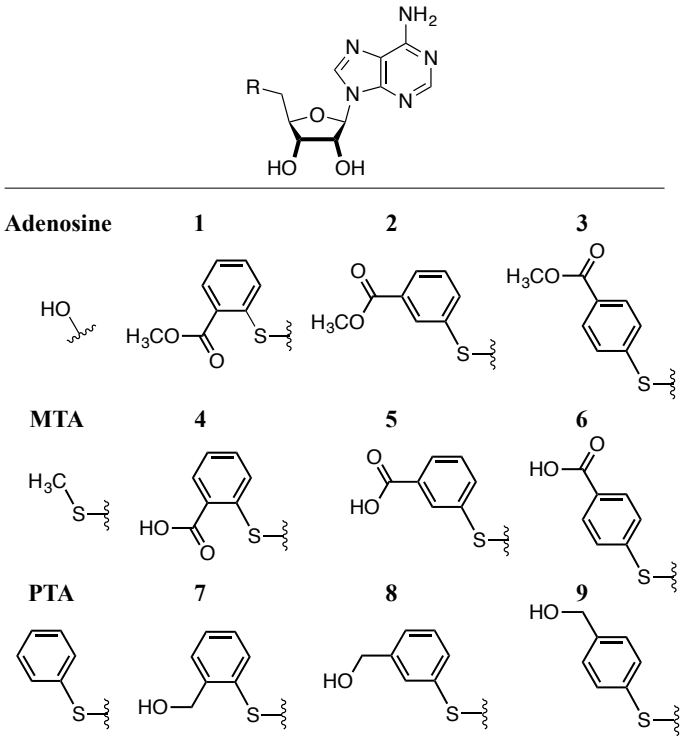
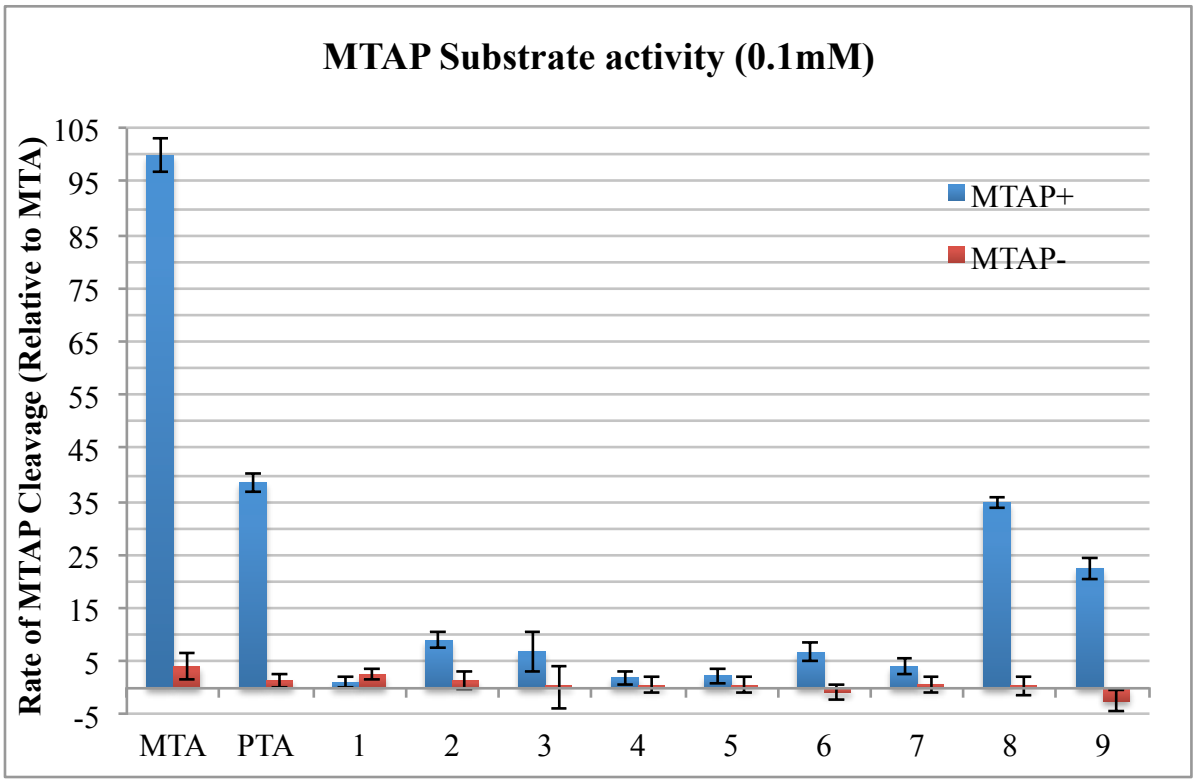


Figure 2.7 MTAP-XO enzyme assay results for compounds 1–9.



The results of this assay reveal significant features of MTAP's substrate preferences around the phenyl ring of PTA. First, neither the methyl ester nor the carboxylic acid functionalities were tolerated at any position on the phenyl ring. It may be that these functional groups are too large or too non-polar. Compounds **8** and **9** contain the primary alcohol functionality, and their activity suggests that the enzyme accommodates the size and polarity of this group. It is only tolerated at certain positions though. Compound **7**, with the same functionality as **8** and **9**, was not an active substrate. The only difference between these three compounds was the position of the primary alcohol on the phenyl ring, at either the *ortho*-, *meta*-, or *para*-position. MTAP does not accommodate the addition of a primary alcohol to the *ortho*-position of the phenyl ring but it tolerates the addition at the *meta*- and *para*-position, retaining some activity. Compound **9**, the *para*-primary alcohol, is a worse substrate than compound **8**, the *meta*-primary alcohol. This is in agreement with the previous work of Kung *et al.*, who explored derivatives of PTA by adding methyl substituents to the phenyl ring; they found that addition to the *ortho*-position made for a bad MTAP substrate, addition to the *para*-position made for a slightly worse substrate than PTA, and addition to the *meta*-position made for a slightly better substrate than PTA (103). Whether the group that is appended to phenyl ring of PTA is a methyl or a primary alcohol, this observation holds. Based on the data from **Figure 2.9** and Kung *et al.*'s previous work, I can form a general rule for the accommodation of substituents on the phenyl ring of PTA. Since the polarity is very distinct between a methyl and primary alcohol this suggests generally that when a functional group is added to the *meta*- or *para*-position and makes for a good substrate of MTAP, addition to the *ortho*-position will give a much worse substrate. As a general rule

for substituents added to the phenyl ring of PTA, the regioisomers have the following relative tolerance by MTAP: *meta* > *para* > *ortho*.

The two important conclusions from the assay for future study are: (1) adding substituents to the *ortho*-position of PTA's phenyl ring can be ignored, as they are not tolerated by MTAP; (2) substituents with a size and polarity similar to the primary alcohol should be well tolerated.

2.6 Cytotoxicity

The compounds were also sent to Dr. Hongwei Cheng at the BC Cancer Agency in Vancouver for cytotoxicity data. Four different cell lines (2 MTAP+ and 2 MTAP–) were tested for each agent by an MTT assay. MTT is a yellow compound, but it is reduced to a purple derivative (formazan) by the mitochondrial reductase enzymes of live cells (and reduced *only* by live cells). Thus the change in absorbance (between 500 and 600 nm) of cell cultures with MTT is indicative of cell viability. The cell lines were incubated for 96 hours and analyzed for cell viability relative to a control. Each cell line was dosed with the compound at three different concentrations (20, 100, and 200 μM). For the graphs of the results, see **Appendix B**.

None of the compounds showed significant cytotoxicity. For a compound to be cytotoxic, we would expect a uniform decrease in cell viability concomitant with an increase in compound concentration. Any data that showed an *increase* in cell viability was seen as a negative result for cytotoxicity. Furthermore, a drop below 50% cell viability was seen as the arbitrary threshold. However, such a drop in cell viability for concentrations of 200 μM would not be considered a significant effect, as this is a

relatively high dose. From these boundaries and the data in **Appendix B**, it is possible that compound **3** showed some minor cytotoxicity against the Du145 cell line.

While I had hypothesized that compounds **7–9** could be cytotoxic, they were not. This negative result was as important as any positive result. This could have been for a number of reasons, including the possibility that the additional phenyl ring (relative to MTA) might obscure the addition of small functional groups like primary alcohols. It was also likely that compounds **7–9** were not phosphorylated due to the presence of the phenyl ring at the 5′-alcohol; if that were true, then compounds **7–9** could not be cytotoxic by inclusion into RNA.

2.7 Summary

Nine derivatives of PTA were synthesized and tested for biological activity. For the synthetic route, 5′-tosyladenosine proved to be the best starting material. Compounds **1–3** were synthesized by coupling the compounds **25–27** to 5′-tosyladenosine. Compounds **4–6** were obtained in quantitative yields by saponification of compounds **1–3**. Compounds **7** and **8** were obtained by reduction of compounds **1** and **2**. Due to a solubility issue, compound **9** could not be synthesized in the same manner as **7** and **8**. Instead, **9** was synthesized by first reducing compound **27** to compound **32**, followed by the coupling of this product to 5′-tosyladenosine. No compounds showed cytotoxicity, but compounds **8** and **9** were moderate substrates of MTAP, albeit slightly reduced (relative to PTA). The results of the MTAP assay indicated something of the shape of MTAP's active site (MTAP tolerates addition of substituents to the *meta*- and *para*-positions of the phenyl ring of PTA, but does not tolerate substituents at the *ortho*-

position) and its affinity to certain functionalities (the primary alcohol). This led to a new series of PTA derivatives discussed in **Chapter 3**.

Chapter 3 Digging Deeper into MTAP's Active Site

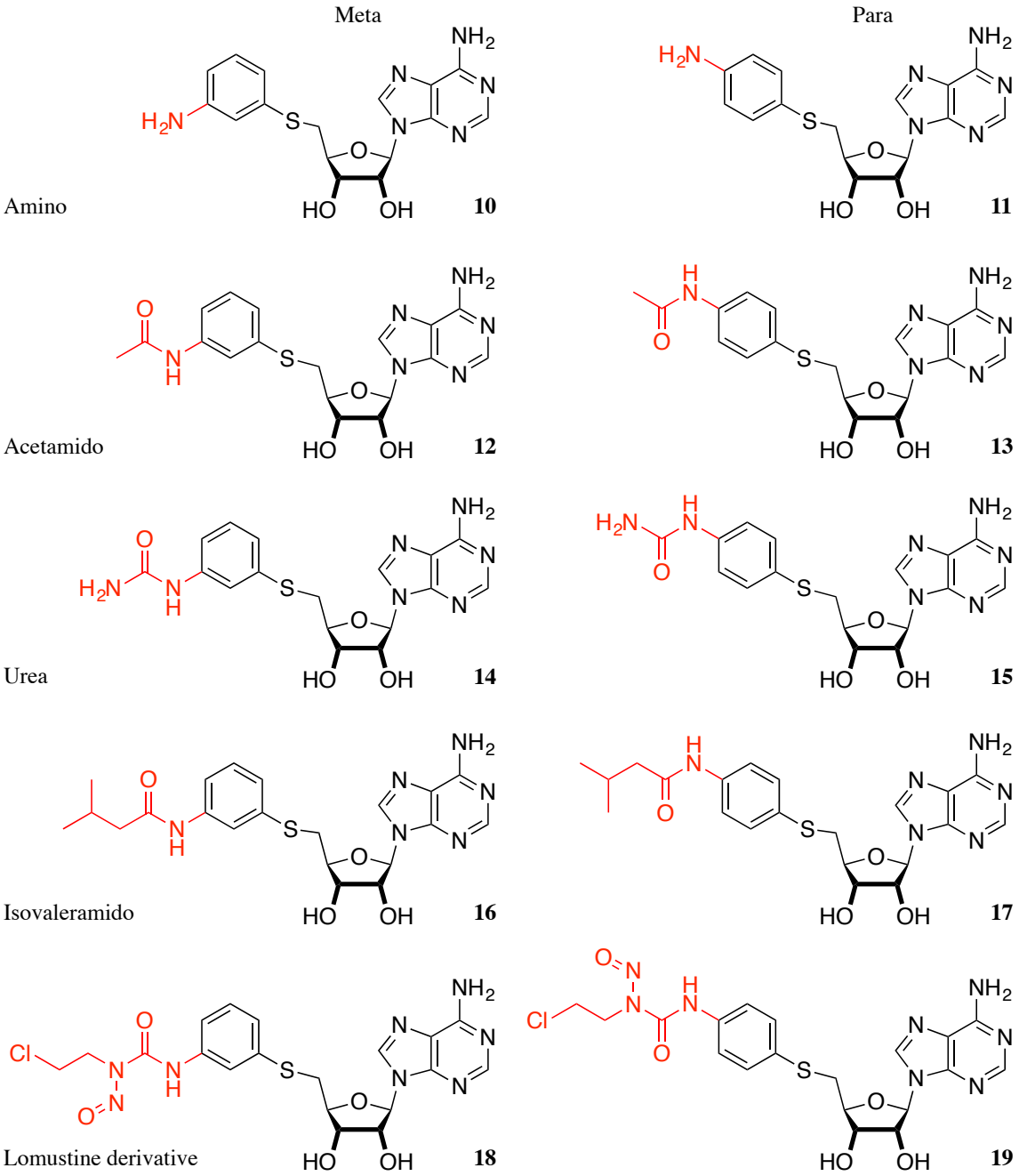
3.1 Introduction

In **Chapter 2** I synthesized nine PTA derivatives and each was tested as substrates for MTAP and for their cytotoxicity. The results from these biological tests revealed that while the primary alcohol was accommodated by MTAP's active site, neither the methyl ester nor the carboxylic acid functionalities were tolerated. They also revealed that any functionality at the *ortho*-position would likely not be tolerated. Furthermore, none of the compounds were cytotoxic. In this chapter I designed 10 more PTA derivatives based on previous results, to further probe the structure-function relationships between MTAP's active site and possible substrates, and with the hope of improving both MTAP's tolerance of the compounds and their cytotoxicity.

3.2 Design of compounds 10–19

The reconnaissance of MTAP's active site revealed that adding substituents to the *ortho*-position of the phenyl ring would not produce effective substrates of MTAP. The results of the MTAP assay also revealed that a primary alcohol substituent on the phenyl ring gave the best substrates, and by extension, other substituents similar to a primary alcohol in size and polarity may also be tolerated. With that in mind, I designed a new set of PTA-derivatives with nitrogen functionalities, focusing on *meta*- and *para*-substitution (**Figure 3.1**). The nitrogen functionalities included a basic amino group (compounds **10** and **11**), acetamide (**12** and **13**), urea (**14** and **15**), isovaleramide groups (**16** and **17**), and lomustine derivatives (**18** and **19**). These were meant to stretch the size of substituents on

Figure 3.1 Compounds 10-19, derivatives of PTA (added functionalities in red).



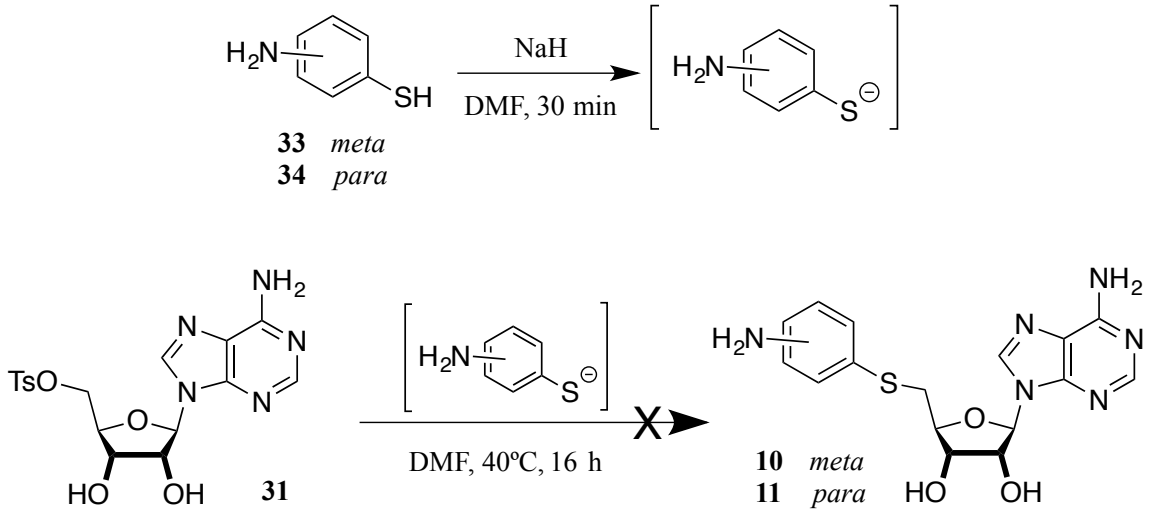
the phenyl ring, while keeping lipophilicity similar to compounds **8** and **9**. Previously the LogP value of compounds **8** and **9** was calculated to be –0.44. The LogP values for this new series of PTA derivatives were –0.63 for **10** and **11**, –0.39 for **12** and **13**, –0.70 for **14** and **15**, 1.07 for **16** and **17**, and 0.71 for **18** and **19** (calculated by ChemDraw). Based

on these values and the size of the new substituents, for this second series of PTA derivatives it was likely that compounds **10** and **11** would be the best substrates for MTAP. **10** and **11** have a smaller substituent than **8** and **9**, but maintain similar LogP values. Compounds **12** and **13** were also possible substrates for MTAP (based on their LogP value), but they had larger substituents than compounds **8** and **9**, which might make for less effective substrates. Compounds **16** and **17** were designed to be a limit for these substrates, since they would likely be less tolerated by MTAP based on their high LogP value and large bulky substituent. While compounds **14** and **15** had a sufficiently low LogP value, it was uncertain whether the size of the urea substituent would be accommodated by MTAP. If they were, then it was possible that compounds **18** and **19**, with a substituted urea from lomustine, might also be tolerated by MTAP. As mentioned in **Chapter 1**, lomustine is an alkylating drug used in chemotherapy. While I was not able to synthesize and test **18** and **19**, I hypothesized that the inclusion of the lomustine functionality to the phenyl ring of PTA could give a very cytotoxic compound, and if it remained as a substrate for MTAP, then it could be selectively toxic against cells that lack MTAP.

3.3 Synthesis of Compounds 10 and 11

I first attempted to synthesize compounds **10** and **11** (**Scheme 3.1**) in the manner of **2** and **3** (**Scheme 2.3**). I began with 5′-tosyladenosine and either 3- or 4-aminothiophenol. The aminothiophenol was deprotonated by NaH in DMF, and then stirred with 5′-tosyladenosine for 16 hours (134–135). It quickly became apparent that the reaction did not proceed smoothly and was not easily purified. In both cases, multiple products were

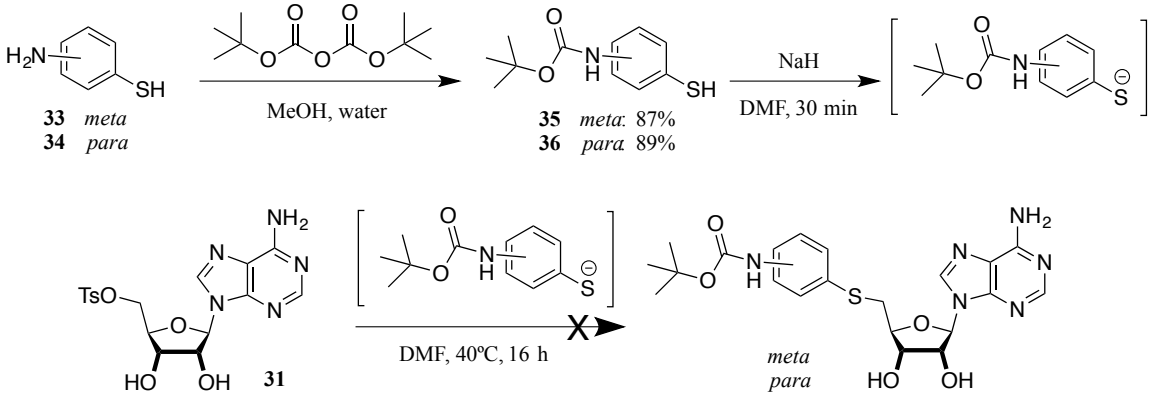
Scheme 3.1 First synthetic approach to 10 and 11.



obtained and purification by recrystallization or LC was not successful (providing only low yields of impure materials). In either purification method, NMR peaks that corresponded to a tosyl species remained with the desired product, which not only frustrated all purification attempts, but raised the question of whether the desired product had been formed at all.

In an attempt to reduce any unwanted products, I protected the amine of 3- and 4-aminothiophenol with a BOC group (**Scheme 3.2**). The hope was also to create a more lipophilic product, allowing for purification by flash chromatography. Stirring BOC

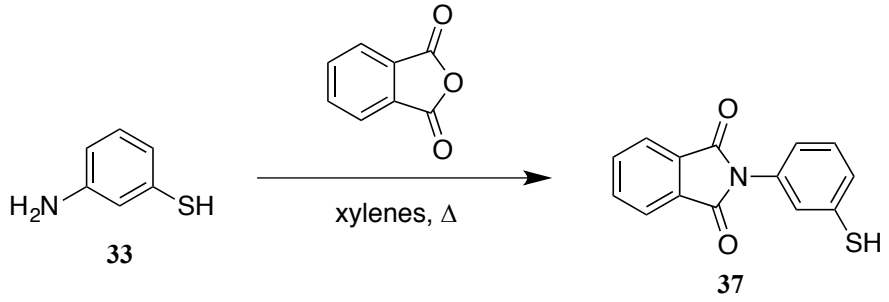
Scheme 3.2 Route to Compounds 10 and 11 through BOC protection.



anhydride with 3- or 4-aminothiophenol in water and methanol, at 35ºC for 16 h, gave the protected amine in high crude yields (154). Attempts to couple the crude product to 5′-tosyladenosine only resulted in a similar complex mixture of impurities; again, recrystallization and LC failed as purification methods. To reduce any unwanted side-reactions, I purified the BOC-protected amines by flash chromatography (pure product obtained in moderate yields) and I used the pure amines in the coupling reaction. Though this reaction was by far the most successful and clean (by ^{1}H NMR), it contained starting material and two different products. These two products had identical ribose and adenine proton peaks, but had different chemical shifts for the aromatic protons of the aminothiophenol as well as the aliphatic protons of the BOC group. So it appeared that the *N*-BOC-3- and *N*-BOC-4-aminothiophenol were coupling to the 5′-tosyladenosine through both the sulfur and the nitrogen, giving the desired product (arising from coupling by the sulfur), and one undesired product (arising from coupling by the nitrogen).

It was possible then that the mono-protected amine, containing an acidic proton, was not sufficiently well protected. To test this, I decided to protect 3-aminothiophenol with a phthalimide group (**Scheme 3.3**). Refluxing the starting material with phthalic anhydride in xylenes for 16 hours, under inert atmosphere (155), followed by flash chromatography, gave two different products. Both had the same number and type of

Scheme 3.3 Protection of 3-aminothiophenol with phthalic anhydride.

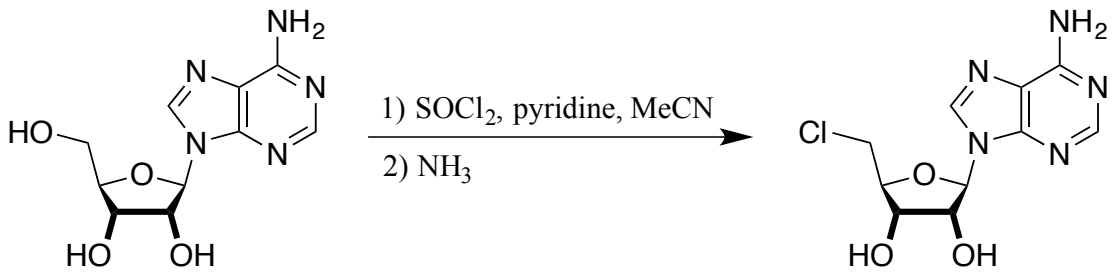


33 **37**

proton peaks by ^{1}H NMR, though they appeared at different chemical shifts (see **Chapter 4** for comparison). It was possible that one was the desired product while the other was the disulfide form, since it could have been oxidized while refluxing. Although the reaction was conducted under inert atmosphere, it is likely that some oxygen was able to enter the flask and react with the product. However, neither of these products were successful in the coupling reaction, returning unreacted starting material in both cases. In my hands, 3-phthalimidothiophenol was not a viable reagent for the synthesis of compounds **10** and **11**.

Due to difficulties with the protected amine coupling reactions, I investigated another route to the synthesis of my targets. I had been using 5′-tosyladenosine because it had worked for the synthesis of compounds **1–9**, but it was possible that there was a better starting material for the synthesis of the next compounds. Kung *et al.* used 5′-chloro-5′-deoxyadenosine as a starting material for the synthesis of similar compounds (103). This seemed reasonable, since it would remove the difficulties associated with the tosylate leaving group. At the same time, compound **10** had been synthesized twice in the past, and both times, it involved 5′-chloro-5′-deoxyadenosine (126, 127). The synthesis of 5′-chloro-5′-deoxyadenosine from adenosine was possible on a gram scale (156, 157) (**Scheme 3.4**).

Scheme 3.4 Chlorination of adenosine.



38: 87%

When I set up the coupling reaction in the same manner as in **Scheme 3.1**, using NaH, stirring in DMF at 40ºC for 16 hours under inert atmosphere (**Scheme 3.5**), I did not obtain the product or a mixture of different products, but unreacted starting material (**Table 3.1**, entry 1). This was a surprising result since the coupling of 3- and 4-aminothiophenol to 5′-chloro-5′-deoxyadenosine had been repeated previously (126, 127). I manipulated the reaction conditions to see if I could push the reaction forward. For a summary of the relevant conditions see **Table 3.1**. Raising the temperature to 100ºC only caused the starting material to degrade (entry 2). Raising it to 80ºC, and

Scheme 3.5 Synthesis of compounds 10 and 11 by 5′-chloro-5′-deoxyadenosine.

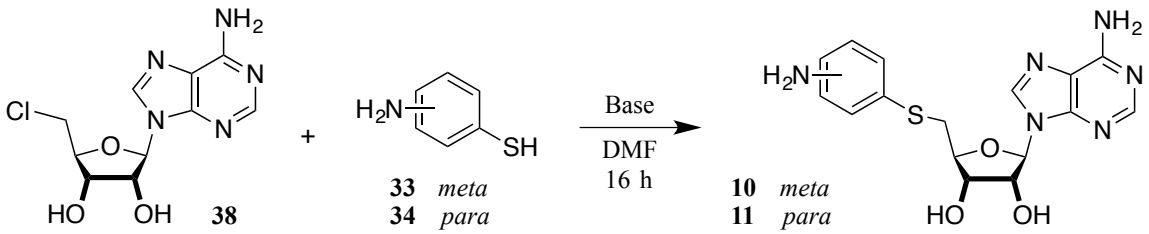


Table 3.1 Conditions for coupling 3- and 4-aminothiophenol to 5′-chloro-5′-deoxyadenosine.

Entry	Isomer	Eq of amino-thiophenol	Base (eq relative to aminothiophenol)	Time and Temperature	Outcome
1	3- and 4-	1	1eq NaH	16 h, 40ºC	S.M.
2	3- and 4-	1	1eq NaH	40 h, 100ºC	S.M. + impurities
3	3- and 4-	1.3	1eq NaH	40 h, 80ºC	S.M.
4	3-	1.3	1.1 eq *t*-BuOK	40 h, 40ºC	1:2.5 of S.M.:product
5	4-	1.3	1.1 eq *t*-BuOK	40 h, 40ºC	1:0.7 of S.M.:product
6	3-	2	1.1 eq *t*-BuOK	16 h, 40ºC	79% yield
7	4-	3	1.1 eq *t*-BuOK	16 h, 40ºC	75% yield
8	3-	1.3	1.7 eq *t*-BuOK	16 h, 40ºC	1:1of S.M.:product
9	4-	1.3	2.5 eq *t*-BuOK	16 h, 40ºC	Mainly S.M.

adding 1.3, instead of 1, equivalents of aminothiophenol, returned starting material with no impurities (entry 3). Finally I saw the product when I switched the base from 1 equivalent of NaH to 1.1 equivalents of a 1M THF solution of potassium *tert*-butoxide (*t*-BuOK) (103). Under these conditions, 3-aminothiophenol was coupled in a ratio of 1:2.5 of starting material to product (entry 4), and 4-aminothiophenol was coupled at a smaller ratio of 1:0.7 (entry 5). Then, I obtained compounds **10** and **11** without any unreacted starting material by adding excess aminothiophenol: 2 equivalents of 3-aminothiophenol and 3 equivalents of 4-aminothiophenol (entries 6 and 7). Since the amount of base was also increased proportionally, it was possible that it was the extra equivalents of *t*-BuOK that caused the reaction to go forward. To check this, I tried the reaction with another set of conditions, maintaining the same total amount of base (the same number of equivalents compared to 5′-chloro-5′-deoxyadenosine), but using less equivalents of aminothiophenol (1.3 eq.) (entries 8 and 9). This gave a mixture of starting material and product, which suggested that an excess of aminothiophenol was required for the reaction to proceed to completion.

The need for a large excess of starting material was odd. The purity of 3- and 4-aminothiophenol was not the 95% claimed by the Aldrich bottles; they were about 80% pure by ^{1}H NMR. Nonetheless, the presence of this amount of impurity did not justify the need for at least 100% excess of starting material. Nor did it explain why the reaction formed *no* product given 1 equivalent of starting material. Although the impurity of starting materials may have been a contributing factor, this could not completely explain the required excess.

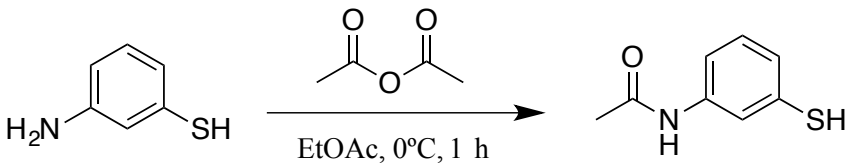
Since compounds **10** and **11** contain a mixture of polar and non-polar

functionalities, purification was difficult; they were not sufficiently soluble in any relevant solvents for either flash chromatography (hexanes, CH_2Cl_2, EtOAc, and MeOH) or LC (water and MeCN). Anglin *et al.* purified compound **10** by recrystallization in MeOH and water (127). After much optimizing of recrystallization conditions I settled with dissolving the impure material in 10 ml of 1:1 water:MeOH, heating to 70ºC, then recrystallizing at 4ºC overnight. I was able to obtain compounds **10** and **11** in 79% and 75% yield.

3.4 Synthesis of Compounds 12–19

The synthesis of compounds **12** and **13** should have been in principle the same as **10** and **11**, beginning instead with 3- and 4-acetamidothiophenol. However, only 4-acetamidothiophenol was commercially available. 3-acetamidothiophenol was synthesized successfully by mixing 3-aminothiophenol with 1 equivalent of acetic anhydride in EtOAc at 0ºC for 1 hour (**Scheme 3.6**) (158). Flash chromatography gave a pure product in 83% yield. In a similar fashion as compounds **10** and **11**, I discovered that the reaction required an excess of acetamidothiophenol starting material. Compound **12** was synthesized with 1.5 equivalents of 3-acetamidothiophenol and 1.5 equivalents of 1M *t*-BuOK. Compound **13** was synthesized with 2 equivalents of 4-acetamidothiophenol and 1.1 equivalents of 1M *t*-BuOK. **12** and **13** were purified by recrystallization in water,

Scheme 3.6 Synthesizing 3-acetamidothiophenol.



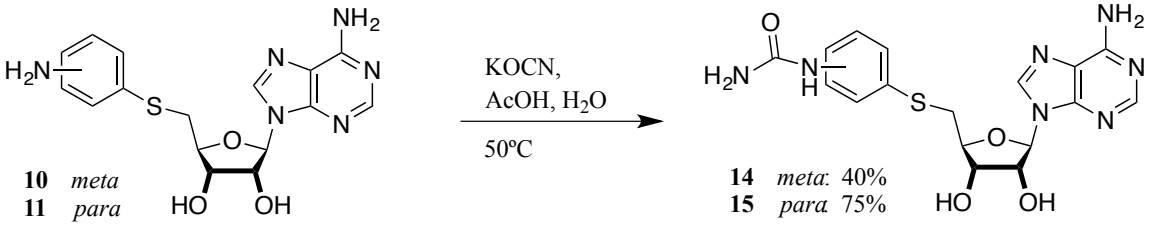
33 **39**: 83%

with yields of 88% and 57%.

Urea derivatives can be synthesized by the reaction of potassium cyanate (KOCN) with substituted amines (159-160). The synthesis of compounds **14** and **15** could have been approached from two different directions then, by either reacting 3- and 4-aminothiophenol with potassium cyanate (and then coupling to 5′-chloro-5′-deoxyadenosine), or by reacting compounds **10** and **11** directly with potassium cyanate. Attempts at the former reaction gave a mixture of products, likely because of the reactivity of the free thiol. So I attempted the latter reaction, shown in **Scheme 3.7**. The first conditions for the formation of compound **14** involved stirring compound **10** in a small amount of water, 0.5 ml glacial acetic acid, and 2 equivalents of KOCN, at 50ºC for 2 hours (159–160). This appeared to give about a 4:1 ratio of product to starting material. Changing the conditions to 2.5 eq of KOCN and for 3 hours, gave no discernible difference to the amount of product. Adding 4 equivalents of KOCN, stirring for 4 hours, forced a complete conversion of compounds **10** and **11** to compounds **14** and **15**. They were purified by recrystallization in water.

The progress of this reaction was monitored by ^{1}H NMR. The formation of **14** from **10** can be seen by a number of peaks: the loss of an NH_2 peak at 5.48 ppm, the rise of a new NH_2 peak at 6.06 ppm, the appearance of an NH peak at 9.09 ppm (the chemical shift of most of the NH_x peaks are pH dependent, though the adenine NH_2 peak appears

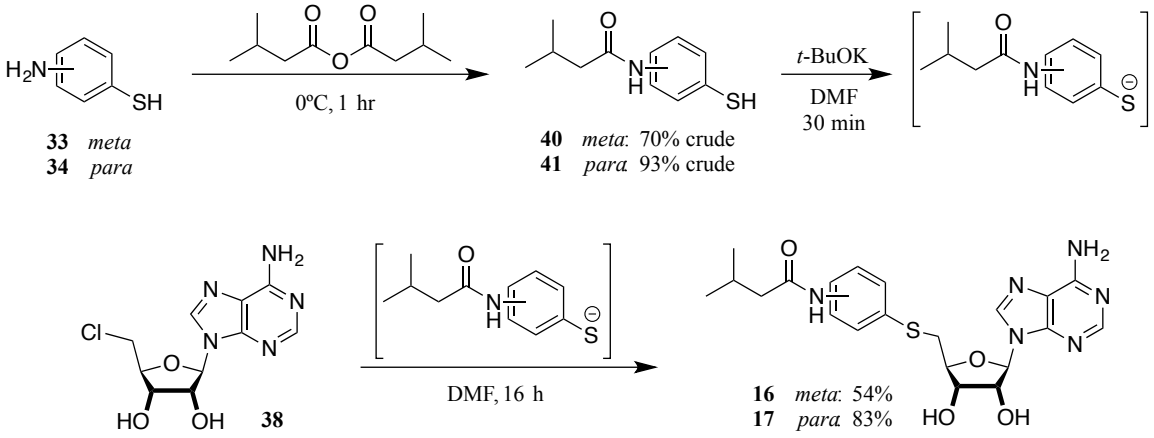
Scheme 3.7 Synthesizing compounds 14 and 15 from 10 and 11.



to remain around 7.3 ppm), and finally the downfield shift of the four aromatic multiplets from 6.93, 6.55, 6.47, and 6.38 ppm, to 7.47, 7.22, 7.12, and 6.86 ppm. This last shift is caused by the change in chemical environment of the aniline to an aromatic urea, which presumably reduces shielding around the aromatic protons.

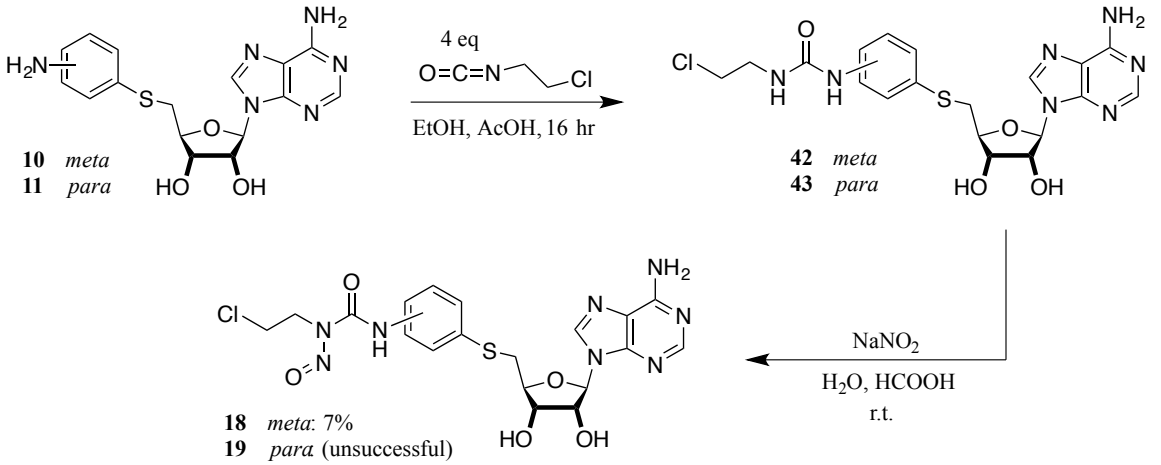
In order to obtain compounds **16** and **17**, I first synthesized compounds **40** and **41** (3- and 4-isovaleramidothiophenol). The procedure was practically identical to the synthesis of 3-acetamidothiophenol. 3- and 4-aminothiophenol were mixed for 3 hours in ethyl acetate with 1 equivalent of isovaleric anhydride (161). Though I tried many different solvent combinations for flash chromatography, I was only able to obtain impure compounds. These were then coupled to 5′-chloro-5′-deoxyadenosine, with 1.5 equivalents of 3-isovaleramidothiophenol and 1.2 equivalents of 4-isovaleramidothiophenol, 1.1 equivalents of *t*-BuOK, stirring for 16 hours at 40ºC. (The excess equivalents were required because of some impurities.) Compounds **16** and **17** were recrystallized in 5 ml of 1:1 water:methanol, heating to 65ºC, then cooling to 4ºC for 16 hours, providing moderate yields of the target compounds.

Scheme 3.8 Synthesis of compounds 16 and 17.



I attempted to synthesize compounds **18** and **19** from compounds **10** and **11** (**Scheme 3.9**). I reasoned that the formation of the substituted urea would be similar to the synthesis of compounds **14** and **15**. I first stirred compounds **10** and **11** with 4 equivalents of 2-chloroethyl isocyanate, forming the substituted urea intermediate (162-163). (I also attempted this reaction with 1.3 equivalents of 2-chloroethyl isocyanate, but as I had expected, the reaction did not go to completion.) Intermediates **42** and **43** were identified by ^{1}H NMR spectroscopy, which showed the addition of two multiplet peaks (each of integration 2) at 3.77 and 3.36 ppm for compound **42** and 3.65 and 3.41 ppm for compound **43**. These correspond to the two pairs of protons on the 2-chloroethyl-substituent. After recrystallization in water, I stirred the intermediate with sodium nitrite in water and formic acid. I used 5 equivalents of sodium nitrite with compound **42**, stirring for 5 hours. This appeared to cause unwanted reactions and afforded only a small amount of compound that I tentatively identified as compound **18**. I then stirred compound **43** with 3 equivalents of sodium nitrite for 2 hours. At first glance, this appeared to give a better transformation than stirring with 5 equivalents of sodium nitrite.

Scheme 3.9 Synthetic route to compounds 18 and 19.



I purified the products by recrystallization in water, and characterized them by ^{1}H NMR and IR spectroscopy. Based on previous reports, when nitrosylation occurs at the correct nitrogen, the chemical shifts of the two 2-chloroethyl multiplets should shift downfield by approximately 0.3 ppm (165). I observed this shift between compounds **42** and **18**, but I did not observe this shift between compounds **43** and **19**. Furthermore the IR spectra were not conclusive. While I might have synthesized compound **18**, I concluded that I had not succeeded in the synthesis of compound **19**. Future work was required for the optimization of reaction conditions for the synthesis of compounds **18** and **19** from **42** and **43**.

3.5 Suitability as Substrates for MTAP

I performed the MTAP-XO assay on compounds **10–17**. The commercial MTAP enzyme was only available in limited quantities and so the assay was carried out at a lower concentration, compared to the first assay. The concentration of MTAP in each well was 0.012 μg/μL, and the concentration of XO was 0.05 U/μL. The enzymes were also older. Both of these factors probably contributed to the far less intense changes in absorbance for the substrates. The change in absorbance over time for MTA in this second assay, was $2x10^{-5}$ AU/s (Absorbance units/second), instead of $8x10^{-5}$ AU/s. With gentler slopes and less intense absorptions, there was more noise in the raw data of the second assay. I performed ten runs instead of three to counter the more noisy data. The slope and slope error was calculated for each replicate, and all ten replicates were used to calculate an average slope and the corresponding standard error. These slopes were then set relative to the slope of MTA (and error was calculated for the adjustment). See

Appendix B for tables of the slopes and standard errors. The average slopes relative to MTA and their standard errors are summarized in **Figure 3.3** below. In this case, MTA and adenosine were used as control substrates.

The first thing to notice in **Figure 3.3** is the rate of MTAP activity for compounds **10** and **11**. At 64% and 46% rates, these compounds are better MTAP substrates than PTA. This was a very promising result. In the previous two chapters I mentioned a trend regarding a single functional group added to PTA at various positions. Compound **10**, the *meta*-isomer, was a better substrate for MTAP than compound **11**, the *para*-isomer. This confirms the expected trend. Though these compounds have been synthesized previously, their ability to be substrates for MTAP was either not measured or not reported (126, 127). To the best of my knowledge, compound **10** was the best substrate for MTAP of all the PTA derivatives synthesized, including others previously reported (103, 126).

Given the success of compounds **10** and **11**, it is surprising that compounds **12** and **13** were not substrates for MTAP. These two sets of compounds have very similar LogP values, and the acetamido group is not too much larger than the amine. For both these reasons I would have expected compounds **12** and **13** to be effective MTAP substrates. However, I noted a large amount of precipitate in the wells containing compound **13**, which indicated that it was not sufficiently soluble in the buffer. The only difference between compounds **12** and **13** is the placement of the acetamido group on the phenyl ring of PTA, and so we would not expect the compounds to have dramatically different solubilities. It is possible that compound **12** was *not* sufficiently insoluble to precipitate out of solution, but also that it *was* sufficiently insoluble to cause aggregation *in* solution; in this case, individual molecules would be unable to act as substrates. The relative

insolubility of compound **12**, then, could account for the results.

Figure 3.2 Summary of compound structures.

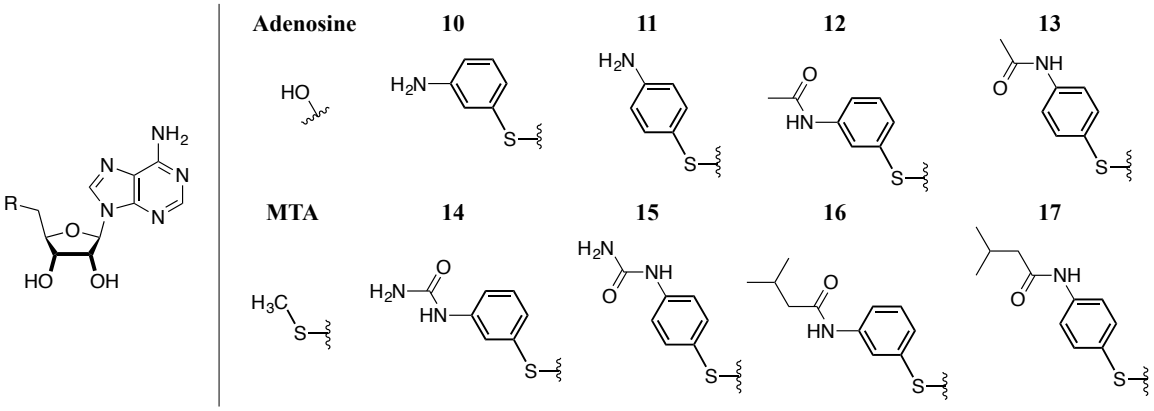
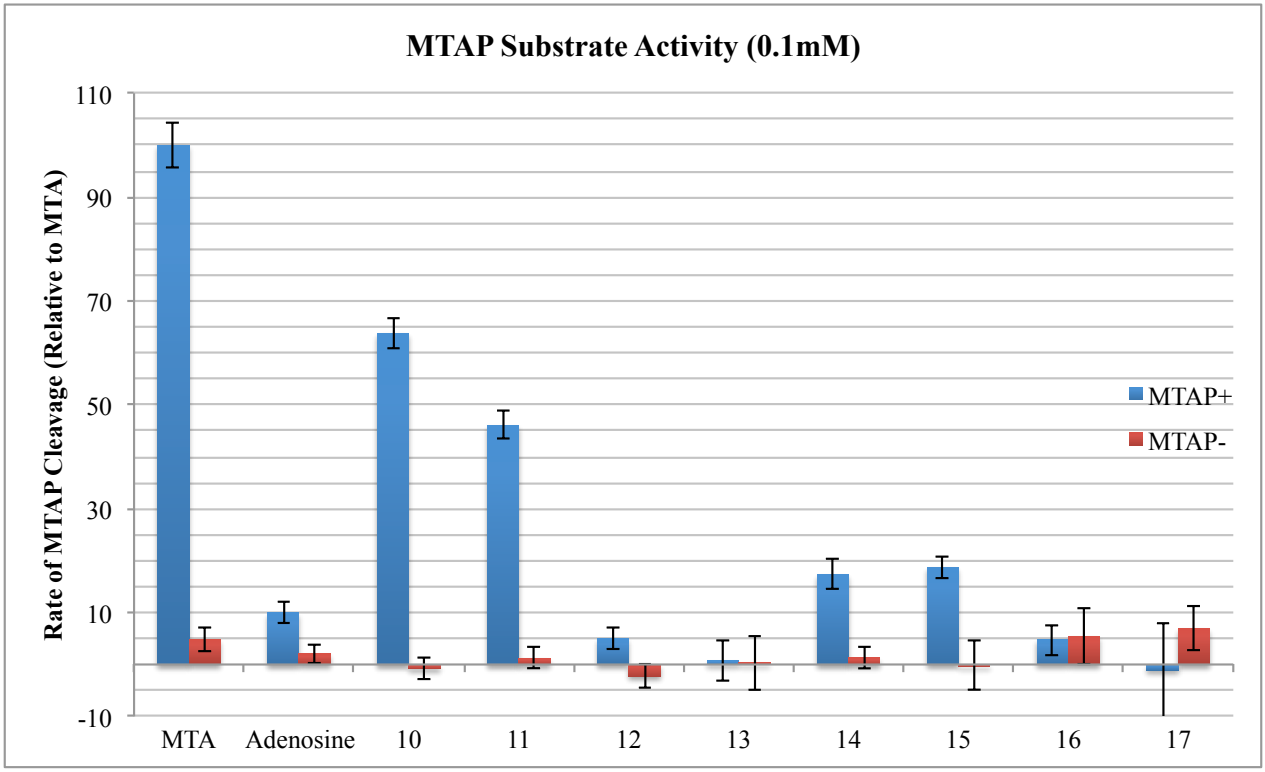


Figure 3.3 MTAP enzyme assay results for compounds 10–17.



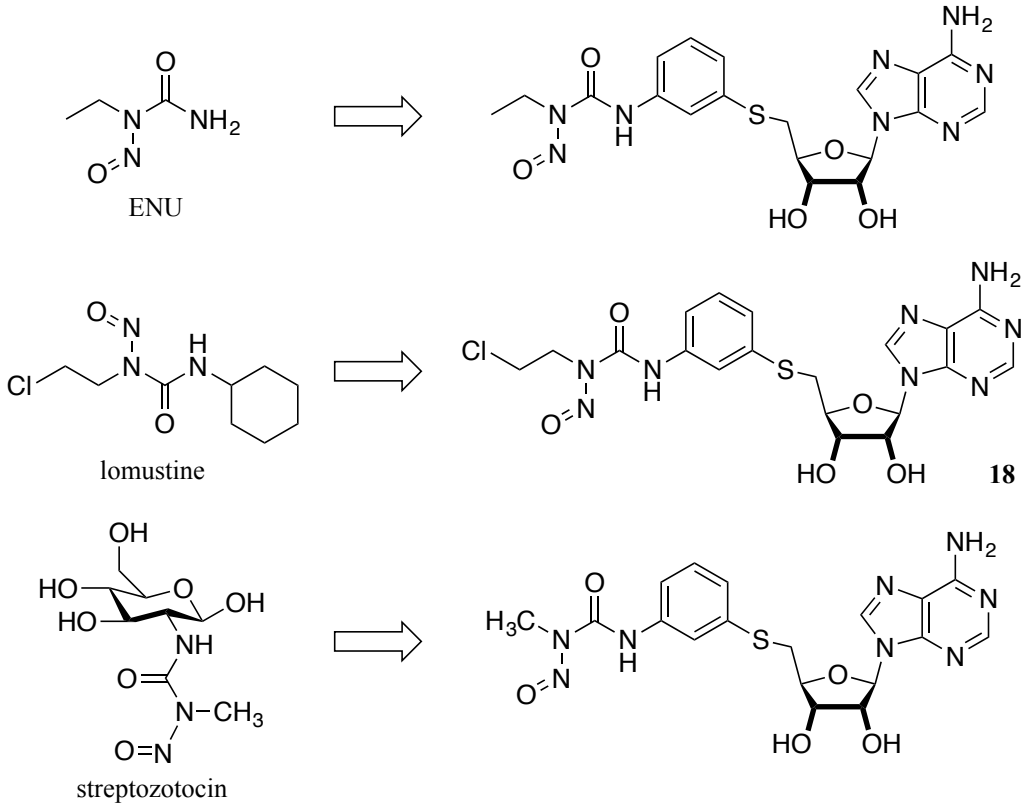
Unlike compound **12**, it is not surprising that compounds **16** and **17** were not substrates of MTAP. They have a high LogP value and a very bulky substituent, and so they were not likely tolerated by MTAP. This sets an important limit for possible PTA derivatives as MTAP substrates.

The substrate activities of compounds **14** and **15**, though fairly reduced, were also promising. Compounds **14** and **15** had not been synthesized previously and they showed activities of 17% and 18%. The addition of an amine substituent to PTA, to make **10** and **11**, made for better substrates of MTAP relative to PTA. The addition of a urea functionality to the amine groups of **10** and **11**, to make **14** and **15**, made for worse substrates. While the tolerance of these compounds by MTAP was much less than **10** and **11**, they still were substrates of MTAP.

The minor success of compounds **14** and **15** has important implications for future work. If a simple urea functionality is tolerated by MTAP, then it is possible that *substituted* ureas are also tolerated. Nitrosoureas are a class of substituted ureas that are cytotoxic, some of which are used in chemotherapy. This of course includes lomustine, which was the inspiration for compounds **18** and **19**. Given the short amount of time available to me, I was not able to successfully synthesize and/or test these molecules. The results of **14** and **15**, however, suggest that such compounds **18** and **19** are an important next step in this project.

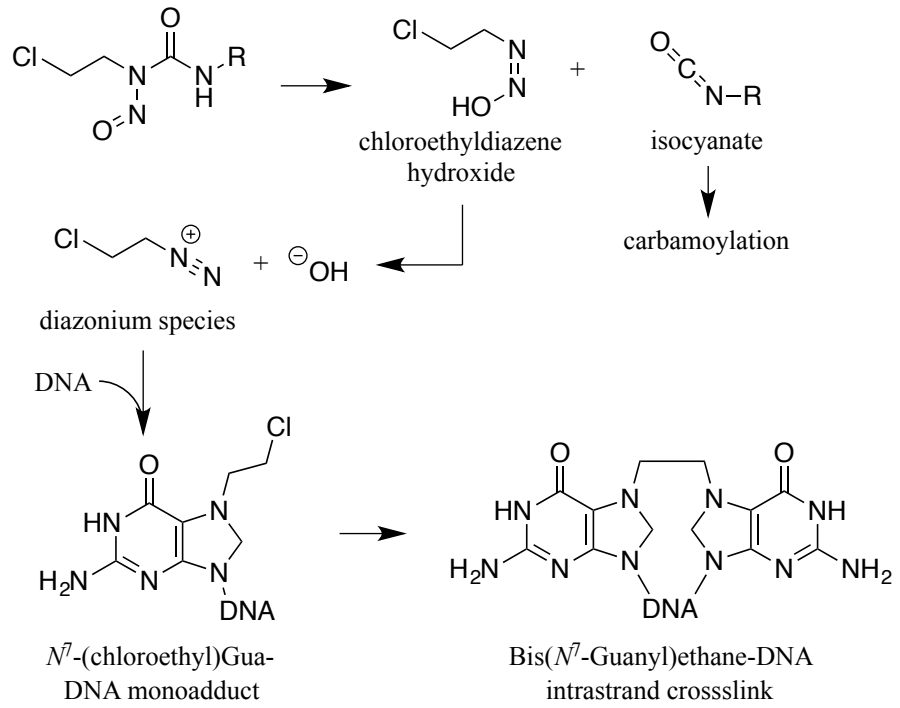
Indeed, there are many nitrosoureas that could be explored, most notably analogues of *N*-ethyl-*N*-nitrosourea (ENU), lomustine, and streptozotocin (**Figure 3.4**). ENU is an alkylating agent commonly used for the mutagenesis of mice (166). Lomustine, which is lipophilic and can cross the blood-brain barrier, is successfully used as a chemotherapeutic for brain tumours (167). Streptozotocin has a glucosamine and the nitrosourea moiety; the sugar acts as a targeting agent, bringing it to the beta cells of the pancreas (168, 169). Consequentially it is successfully used as a chemotherapeutic for treating cancer in the pancreatic islet cells (168, 169). The strategy of streptozotocin is quite similar to compounds **18** and **19**, where the cytotoxic nitrosourea component is appended to PTA, and where PTA could act as a targeting agent, causing the molecule to accumulate in cancer cells that lack MTAP.

Figure 3.4 Nitrosoureas and potential PTA derivatives.



Nitrosoureas are very cytotoxic (165, 171). In aqueous solutions, they decompose to form an alkylating agent and an isocyanate (165, 171). If the nitrosourea is lomustine, it forms chloroethyldiazene hydroxide and isocyanate (165, 171). For a depiction of this breakdown and eventual DNA crosslinking, see **Figure 3.5**. The isocyanate can undergo carbamoylation with proteins (171). The chloroethyldiazene hydroxide then breaks down and forms a diazonium species, which can in turn react with a base on DNA, such as guanidine, forming a monoadduct (171). While this is already quite cytotoxic, the substituent can further react with another guanidine on the same strand of DNA to cause intrastrand crosslinking (171).

Figure 3.5 An example of nitrosourea cytotoxicity by crosslinking.



3.6 Cytotoxicity

The compounds were sent to Dr. Hongwei Cheng at the BC Cancer Agency in Vancouver for cytotoxicity data. The Data is shown in **Appendix B**. The product from

the attempted synthesis of compound **19** showed some cytotoxicity, but since I am not certain of its structure, I will not draw any conclusions from that specific data. Compounds **10** and **16** also showed some cytotoxicity (**Figure 3.6** and **3.7**). Compound **10** was mildly cytotoxic towards the MCF7 cell line, a breast cancer cell line that lacks MTAP; **10** dropped cell viability below 40% at 200µM. I did not expect this compound to be cytotoxic and this result, combined with the fact that **10** was an effective MTAP substrate, makes **10** a very promising compound for future work. Compound **16** showed minor cytotoxicity for three cell lines, dropping the cell viability to about 60% at 200

Figure 3.6 Cytotoxicity data for compound 10.

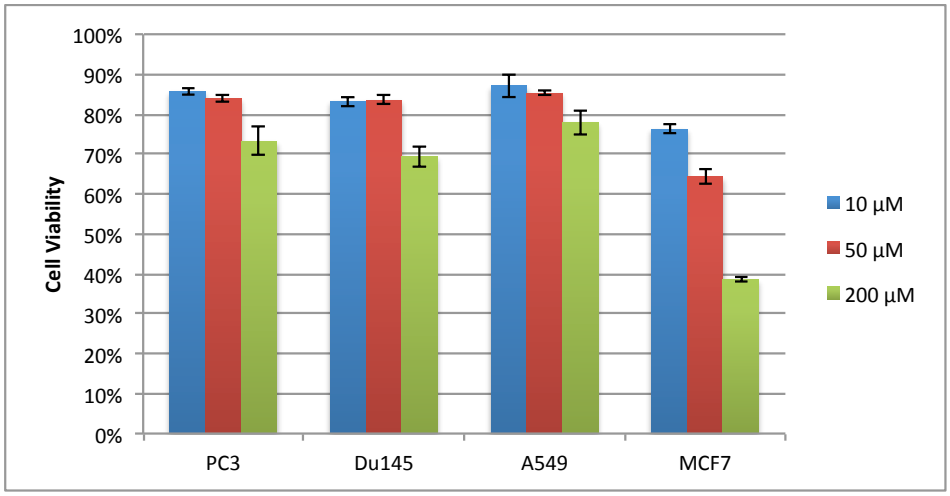
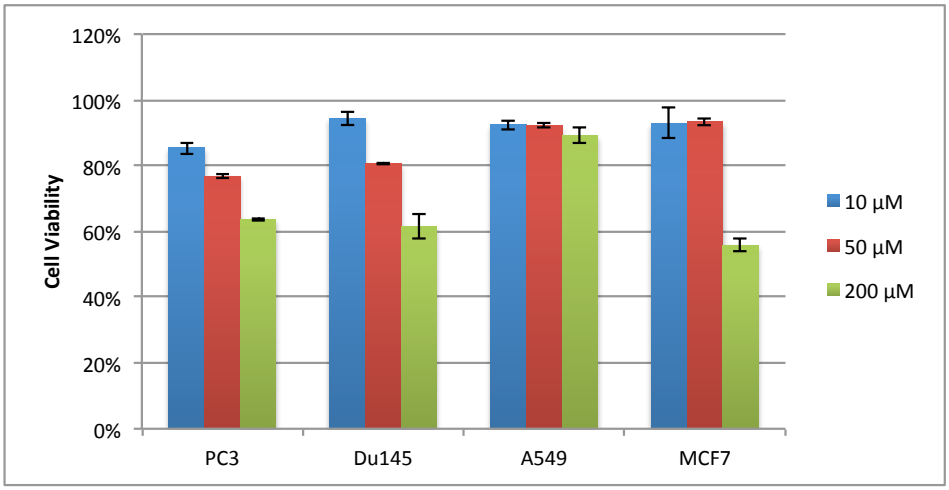


Figure 3.7 Cytotoxicity data for compound 16.



μM. It is promising that these compounds, though not designed for it, showed at least some cytotoxic effects, and it suggests that functional groups that are not explicitly toxic (in contrast to the nitrosoureas), might become cytotoxic once appended to the phenyl ring of PTA.

3.7 Summary and Future Work

Eight more PTA derivatives were synthesized and most were tested for biological activity. I discovered that 5′-chloro-5′-deoxyadenosine was the starting material best suited for these derivatives. The coupling reaction required adjustments, most noticeably the excess of aminothiophenol starting material. Aminothiophenol derivatives were synthesized and then coupled to 5′-chloro-5′-deoxyadenosine, to give compounds **12**, **13**, **16**, and **17**. Reacting compounds **10** and **11** with KOCN gave **14** and **15**. In a similar manner, the intermediates to compounds **18** and **19** (**42** and **43**) were obtained by reacting 2-chloroethyl isocyanate with compounds **10** and **11**. I was unable to synthesize compound **19** from **43**, since the nitrosylation was either not effective or else not selective for the correct nitrogen. I set aside the synthesis, complete characterization, and testing of compounds **18** and **19** for immediate future work.

The results of the MTAP assay revealed that compounds **10** and **11** were good MTAP substrates, better than PTA, and their performance in the MTAP cleavage assay confirmed the previous *meta*/*para* trend. Compounds **16** and **17**, not tolerated by MTAP, set a limit for possible substrates. Compounds **14** and **15** were moderate substrates. Since the urea functionality was tolerated by MTAP, this suggested that substituted ureas might also be accommodated. Nitrosoureas are cytotoxic substituted ureas used in

chemotherapy, and are prime candidates for future work in this project (**figure 3.6**). I did not have time to develop a reliable procedure for the synthesis of compounds **18** and **19**, but my work indirectly suggests these compounds are very promising. Substituted ureas may be tolerated by MTAP, and nitrosoureas are known cytotoxic chemotherapeutics; these are the two ingredients for a cytotoxic MTA analogue.

I did not expect to synthesize the perfect cytotoxic MTA analogue in this thesis; instead I explored the conditions for such a molecule. This involved the design and synthesis of various PTA derivatives to elucidate the structure-function relationships between MTAP and possible substrates. I have succeeded in that task, and this work points towards a new series of PTA derivatives that bring the project closer to its goal of a cytotoxic MTA analogue.

Chapter 4 Experimental

4.1 Materials

The following chemicals were bought from Sigma Aldrich: thiosalicyclic acid, 3-mercaptobenzoic acid, 4-mercaptobenzoic acid, sodium hydride (60% NaH in oil suspension), lithium aluminum hydride, 3-aminothiophenol, 4-aminothiophenol, 4-acetamidothiophenol, thionyl chloride, potassium *tert*-butoxide (1M in THF), acetic anhydride, BOC anhydride, phthalic anhydride, 7N methanolic ammonia, potassium cyanate, ammonium thiocyanate, DMF, hexanes, pyridine, deuterated chloroform, deuterated DMSO, deuterated methanol, 2-chloroethyl isocyanate, and sodium nitrite. The following chemicals were bought from Chem-Impex International: 2′,3′-*O*-isopropylideneadenosine, 5′-tosyladenosine, sodium cyanoborohydride, and adenosine. The following chemicals were bought from Fisher chemicals: methanol, acetone, CH_2Cl_2, ethyl acetate, and xylenes. THF and Dioxane were bought from Caledon. Acetonitrile and glacial acetic acid were bought from Anachemia. Tosyl chloride and isovaleric anhydride were bought from Fluka. Finally, sulfuric acid and ethylene glycol were bought from BDH.

4.2 Instrumentation

Nuclear magnetic resonance (NMR) spectra were recorded using either a Bruker AV 300 MHz NMR or a Bruker Avance 500 MHz NMR spectrometer. Proton (^{1}H) and carbon (^{13}C) spectra are reported in parts per million (ppm) in reference to the deuterated solvent used. The following reference ppm values were used (^{1}H, then ^{13}C): DMSO-d_6, δ

2.50 and δ 39.52; $CDCl_3$, δ 7.26 and δ 77.16; methanol-d_4, δ 3.31 and δ 49.00 (166). The ^1H data are reported as: chemical shift, the multiplicity (where s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad), the coupling constant in Hertz (Hz), and the integration (1H, 2H, etc.). The ^{13}C data are reported as chemical shift and a designation of the carbon as either a quaternary (C), methine (CH), methylene (CH_2), or methyl (CH_3) carbon, as determined by ^{13}C DEPT. Certain ^{13}C spectra shown in **Appendix A** contain an instrumental artifact around 120 ppm; this feature is not characteristic of the compounds in question. Infrared (IR) spectra were recorded with a Perkin Elmer 1000 FT-IR spectrometer and the data are reported as the frequency in cm^{-1}, followed by approximate intensity of absorption (where s = strong, m = medium, w = weak, and br = broad). High resolution mass spectrometry (HRMS) was obtained on an Orbitrap MS, and run in positive mode, except for compounds **25–27**. The liquid chromatography system was a Dionex UltiMate 3000 UHPLC focused, with an analytical column 250 x 4.6 mm, 5 μL particle size. For the MTAP-XO assay, a Spectromax M5 microplate reader was used.

4.3 General Experimental Remarks

Inert atmosphere reaction conditions involved the flame-drying of all round bottom flasks and stir bars under vacuum, followed by the maintenance of a positive pressure of argon for the entire reaction. While DMF was already anhydrous, dioxane, acetonitrile, and pyridine were dried with molecular sieves for inert atmosphere reaction conditions. THF was distilled by sodium benzophenone. Solvents and liquid reagents were transferred by oven-dried stainless steel needles and fresh plastic syringes. After workup,

solvents were dried with sodium sulphate. All solvents were evaporated on rotary evaporators, with a range of temperatures.

Thin layer chromatography (TLC) silica had the following specifications: 0.20 mm, 60 Å pore-size, 230-400 mesh, Macherey-Nagel; TLC plates were visualized by ultraviolet light. The silica gel for flash chromatography had the following specifications: 60, 40–63 μM, from SiliCycle.

4.4 General Procedures

A. Compounds 25–27.

Compounds **22–24** (6.5 mmol) were dissolved in methanol (50 ml) and concentrated sulfuric acid (0.1 ml). The reaction mixture was heated to reflux for 16 h. The mixture was reduced to a volume of 10 ml by rotary evaporation and diluted with saturated aqueous $NaHCO_3$ (10ml). The product was extracted into CH_2Cl_2 (50 ml) 3 times. It was dried, concentrated *in vacuo*, and purified by flash chromatography (100% CH_2Cl_2).

B. Compounds 28–30.

Under inert atmosphere conditions, compounds **25–27** (2.35 mmol) were dissolved in DMF (5 ml). NaH (3.06 mmol) was added and the mixture was stirred for 30 minutes, becoming bright yellow. Compound **21** (1.96 mmol) was dissolved in 5 ml DMF and transferred by cannula to the stirring solution. The resulting mixture was stirred for 16 hours, becoming a brown solution. The product was extracted into ethyl acetate (50 ml),

washed 3 times with distilled water (15 ml), concentrated *in vacuo*, and purified by flash chromatography (solvent gradient from 100% CH_2Cl_2 to 5:1 CH_2Cl_2:MeOH).

C. Compounds 1–3.

Under inert atmosphere conditions, compounds **25–27** (1.50 mmol) were dissolved in DMF (10 ml). NaH (1.94 mmol) was added and the mixture was stirred for 30 minutes, becoming bright yellow. 5′-tosyladenosine (1.25 mmol) was added to the stirring solution. The resulting mixture was stirred for 16 hours, becoming a brown solution. The product was extracted into ethyl acetate (50 ml), washed 3 times with distilled water (15 ml), and concentrated *in vacuo*. The product was purified by recrystallization: the product was dissolved in methanol (10 ml) at 60ºC, cooled to r.t., then recrystallized at 4ºC for 16 h.; the crystals were washed with cold methanol and then cold CH_2Cl_2.

D. Compounds 4-6.

Compounds **1–3** (0.5 mmol) were stirred in NaOH (10%, 3 ml) and water (3 ml) for 24 h. The mixture was neutralized with HCl (10%), concentrated *in vacuo*, and dried to form a white solid. The product was triturated with a small amount of water.

E. Compounds 7 and 8.

Under inert atmosphere conditions, compounds **1** and **2** (0.4 mmol) were stirred in dioxane (4 ml), forming an emulsion. $LiAlH_4$ (2.4 mmol) was added and the mixture was stirred at 50ºC for 3 h. This was neutralized with ethyl acetate (4 ml) and HCl (1M, 2 ml), and then extracted into water (2 ml) 3 times. The grey solid was filtered out of the

aqueous and washed with water. The filtrate was dried *in vacuo*.

F. Compounds 10–13, 16 and 17.

Under inert atmosphere conditions, the aminothiophenol derivatives were dissolved in DMF (10 ml). *t*-BuOK (1M in THF, 1.1 mmol) was added and the reaction mixture was stirred for 30 minutes at room temperature. Compound **38** (1.0 mmol) was added and the resulting mixture was stirred for a further 16 hours at 40ºC. The product was neutralized with HCl (1M) and concentrated *in vacuo*. The resulting crude oil was triturated with CH_2Cl_2 (20 ml) 3 times to form a tan solid. The product was recrystallized from methanol and water.

4.5 Synthetic Procedures

Compound 21.

Under inert atmosphere, at 0ºC, compound **20** (4.88 mmol) was added to dry pyridine (6ml). Tosyl chloride (5.86 mmol) was added, and the resulting mixture was stirred for 16 h, warming to room temperature. The crude product, a hard brown gelatin, was dissolved in ethyl acetate (50 ml) at 50ºC while stirring, and then washed 3 times with aqueous saturated NH_4Cl (15 ml). The organic layer was dried and concentrated *in vacuo*, affording the crude product, a viscous brown oil (80.5% yield).

Compound 25, methyl 2-mercaptobenzoate.

Compound **25** was prepared by general procedure A. Light yellow oil (76% yield);

^{1}H (300MHz $CDCl_3$): δ 7.91 (m, 1H), δ 7.55 (m, 1H), δ 7.41 (m, 1H), δ 7.21 (m, 1H), δ 5.31 (s, 1H), δ 3.83 (s, 3H); ^{13}C (300MHz $CDCl_3$): δ 166.1 (C), 138.1 (C), 132.8 (CH), 131.1 (CH), 131.0 (CH), 125.6 (C), 124.7 (CH), 52.2 (CH3); IR (neat, cm^{-1}) 2998 (w), 2951 (m), 2550 (m), 1706 (s), 1590 (m), 1433 (s), 1252 (s), 1064 (s), 739 (s); HRMS (ESI) calculated for $C_8H_8O_2S - H^+$ 167.0172, found 167.0173.

Compound 26, methyl 3-mercaptobenzoate.

Compound **26** was prepared by general procedure A. Light brown oil (95% yield); ^{1}H (300MHz $CDCl_3$): δ 7.95 (m, 1H), δ 7.82 (m, 1H), δ 7.45 (m, 1H), δ 7.31 (m, 1H), δ 3.91 (s, 3H), δ 3.54 (s, 1H); ^{13}C (300MHz $CDCl_3$): δ 166.4 (C), 133.4 (CH), 131.7(C), 131.0 (C), 130.1 (CH), 129.1(CH), 126.8(CH), 52.3 (CH3); IR (neat, cm^{-1}) 2997 (w), 2951 (m), 2561 (m), 1716 (s), 1574 (m), 1436 (s), 1261 (s), 1126 (s), 737 (s); HRMS (ESI) calculated for $C_8H_8O_2S - H^+$ 167.0172, found 167.0173.

Compound 27, methyl 4-mercaptobenzoate.

Compound **27** was prepared by general procedure A. Light yellow oil (74% yield); ^{1}H (300MHz $CDCl_3$): δ 7.89 (d, J = 8.8 Hz 2H), δ 7.29 (d, J = 8.8 Hz 2H), δ 3.90 (s, 3H), δ 3.61 (s, 1H); ^{13}C (300MHz $CDCl_3$): δ 166.6 (C), 138.3(C), 130.2(CH), 128.1 (CH), 127.1 (C), 52.1 (CH3); IR (neat, cm^{-1}) 2997 (w), 2949 (m), 2846 (w), 2578 (m), 1710 (s), 1591 (m), 1431 (s), 1274 (s), 1108 (s), 757 (s); HRMS (ESI) calculated for $C_8H_8O_2S - H^+$ 167.0172, found 167.0173.

Compound 28.

Compound **28** was prepared by general procedure B. Tan powder (25% yield); ^{1}H (300MHz $CDCl_3$): δ 8.36 (s, 1H), δ 7.94 (m, 1H), δ 7.90 (s, 1H), δ 7.35 (m, 1H), δ 7.30 (m, 1H), δ 7.17 (m, 1H), δ 6.08 (d, *J* = 2.3 Hz, 1H), δ 5.62 (br s, 2H), δ 5.59 (dd, *J* = 6.3, 2.4 Hz, 1H), δ 5.17 (dd, *J* = 6.3, 2.9 Hz, 1H), δ 4.52 (m, 1H), δ 3.92 (s, 3H), δ 3.38 (dd, *J* = 13.5, 7.7 Hz, 1H), δ 3.23 (dd, *J* = 13.6, 6.0 Hz, 1H), δ 1.59 (s, 3H), δ 1.39 (s, 3H).

Compound 29.

Compound **29** was prepared by general procedure B. Tan powder (19% yield); ^{1}H (300MHz $CDCl_3$): δ 8.36 (s, 1H), δ 8.01 (m, 1H), δ 7.85 (s, 1H), δ 7.84 (m, 1H), δ 7.50 (m, 1H), δ 7.30 (t, *J* = 7.8 Hz, 1H), δ 6.05 (d, *J* = 2.0 Hz, 1H), δ 5.55 (br s, 2H), δ 5.54 (dd, *J* = 6.2, 2.0 Hz, 1H), δ 5.12 (dd, *J* = 6.3, 3.1 Hz, 1H), δ 4.40 (m, 1H), δ 3.91 (s, 3H), δ 3.34 (dd, *J* = 13.7, 5.6 Hz, 1H), δ 3.24 (dd, *J* = 13.6, 6.4 Hz, 1H), δ 1.58 (s, 3H), δ 1.39 (s, 3H).

Compound 30.

Compound **30** was prepared by general procedure B. Tan powder (29% yield); ^{1}H (300MHz $CDCl_3$): δ 8.36 (s, 1H), δ 7.88 (m, 1H), δ 7.85 (s, 2H), δ 7.29 (m, 2H), δ 6.06 (d, *J* = 2.0 Hz, 1H), δ 5.56 (br s, 2H), δ 5.55 (dd, *J* = 6.2, 2.1 Hz, 1H), δ 5.14 (dd, *J* = 6.3, 3.2 Hz, 1H), δ 4.42 (m, 1H), , δ 3.90 (s, 3H), δ 3.53 (dd, *J* = 13.6, 7.5 Hz, 1H), δ 3.43 (dd, *J* = 13.8, 6.3 Hz, 1H), δ 1.58 (s, 3H), δ 1.39 (s, 3H).

Compound 31, 5′-tosyladenosine.

Under inert atmosphere conditions, adenosine (7.48 mmol) was dissolved in

pyridine and stirred at 4ºC, 0ºC, or –20ºC (in a 9:1 ethylene glycol:ethanol bath). Tosyl chloride (8.98 mmol, 1.2 eq) was added and the solution immediately turned yellow. The reaction was allowed to warm to room temperature from 0ºC for 16 h; or it was kept at 4ºC for 72 h; or it was kept at –20ºC for 48 h. For each, the result was the same. The mixture was concentrated *in vacuo*, and then extracted into ethyl acetate (30 ml) from saturated aqueous NH_4Cl (5 ml). The solvent was then dried and evaporated to give 38-59% yields of crude mixtures of multiply tosylated products.

Compound 1.

Prepared by general procedure C. White powder (84% yield); ^{1}H (500MHz DMSO): δ 8.36 (s, 1H), δ 8.15 (s, 1H), δ 7.85 (d, *J* = 7.9 Hz, 1H), δ 7.51 (t, *J* = 9.2 Hz, 1H), δ 7.50 (d, *J* = 6.9 Hz, 1H), δ 7.29 (br s, 2H), δ 7.24 (td, *J* = 6.86, 2.2 Hz, 1H), δ 5.91 (d, *J* = 5.7 Hz, 1H), δ 5.54 (d, *J* = 5.9 Hz, 1H), δ 5.40 (d, *J* = 4.9 Hz, 1H), δ 4.82 (m, 1H), δ 4.23 (m, 1H), δ 4.07 (m, 1H), δ 3.82 (s, 3H), δ 3.43 (dd, *J* = 13.9, 5.5 Hz, 1H), δ 3.34 (dd, *J* = 14.1, 7.3 Hz, 1H); ^{13}C (500MHz DMSO): δ 166.1 (C), 156.1 (C), 152.6 (CH), 149.5 (C), 139.9 (CH), 139.9 (C), 132.6 (CH), 130.7 (CH), 127.6 (C), 126.1 (CH), 124.3 (CH), 119.2 (C), 87.5 (CH), 82.4 (CH), 73.0 (CH), 72.6 (CH), 52.1 (CH_3), 34.2 (CH_2); IR (neat, cm^{-1}) 3339 (w), 3172 (m), 2930 (w), 1706 (s), 1671 (s), 1611 (m), 1429 (m), 1303 (m), 1246 (s), 1230 (s), 1115 (m), 1021 (s), 787 (s), 720 (s), 706 (s), 688 (s), 660 (s); HRMS (ESI) calculated for $C_{18}H_{19}N_5O_5S + H^+$ 418.1180, found 418.1180; M.P. 117-119ºC.

Compound 2.

Prepared by general procedure C. White powder (46% yield); ^{1}H (500MHz DMSO): δ 8.32 (s, 1H), δ 8.14 (s, 1H), δ 7.87 (t, *J* = 1.7 Hz, 1H), δ 7.74 (dt, *J* = 7.8, 1.4 Hz, 1H), δ 7.63 (m, 1H), δ 7.43 (t, *J* = 7.9 Hz, 1H), δ 7.28 (br s, 2H), δ 5.89 (d, *J* = 5.9 Hz, 1H), δ 5.52 (d, *J* = 6.2 Hz, 1H), δ 5.38 (d, *J* = 4.9 Hz, 1H), δ 4.82 (m, 1H), δ 4.21 (m, 1H), δ 4.02 (m, 1H), δ 3.82 (s, 3H), δ 3.49 (dd, *J* = 13.6, 5.6 Hz, 1H), δ 3.39 (dd, *J* = 13.6, 7.2 Hz, 1H); ^{13}C (500MHz DMSO): δ 165.7(C), 156.1 (C), 152.6 (CH), 149.4 (C), 139.9 (CH), 137.0 (C), 132.7 (CH), 130.4 (C), 129.4 (CH), 128.2 (CH), 126.4 (CH), 119.2 (C), 87.5 (CH), 82.8 (CH), 72.7 (CH), 72.5 (CH), 52.3 (CH_3), 35.0 (CH_2); IR (neat, cm^{-1}) 3328 (m), 3184 (m), 2925 (m), 1717 (m), 1640 (s), 1599 (m), 1574 (m), 1418 (m), 1287 (s), 1262 (m), 1123 (s), 1029 (s), 747 (s), 646 (s); HRMS (ESI) calculated for $C_{18}H_{19}N_5O_5S + H^+$ 418.1180, found 418.1180; M.P. 94-97ºC.

Compound 3.

Prepared by general procedure C. White powder (69% yield); ^{1}H (500MHz DMSO): δ 8.34 (s, 1H), δ 8.15 (s, 1H), δ 7.82 (d, *J* = 8.7 Hz, 2H), δ 7.43 (d, *J* = 8.4 Hz, 2H), δ 7.28 (br s, 2H), δ 5.90 (d, *J* = 5.8 Hz, 1H), δ 5.54 (d, *J* = 6.0 Hz, 1H), δ 5.40 (d, *J* = 4.9 Hz, 1H), δ 4.82 (m, 1H), δ 4.23 (m, 1H), δ 4.06 (m, 1H), δ 3.82 (s, 3H), δ 3.53 (dd, *J* = 13.8, 5.5 Hz, 1H), δ 3.43 (dd, *J* = 13.8, 7.4 Hz, 1H); ^{13}C (500MHz DMSO): δ 165.8 (C), 156.1 (C), 152.7 (CH), 149.3 (C), 143.4 (C), 139.9 (CH), 129.5(CH), 126.3 (CH), 126.1 (C), 119.2 (C), 87.5 (CH), 82.6 (CH), 72.7 (CH), 72.6 (CH), 52.0 (CH_3), 33.8 (CH_2); IR (neat, cm^{-1}) 3389 (m), 3327 (m), 3114 (m), 2946 (w), 1708 (m), 1686 (m), 1663 (s), 1596 (s), 1571 (m), 1333 (m), 1290 (s), 1119 (s), 1092 (s), 1013 (s), 760 (s); HRMS (ESI) calculated for $C_{18}H_{19}N_5O_5S + H^+$ 418.1180, found 418.1180; M.P. 210-

211ºC.

Compound 4.

Prepared by general procedure D. White powder (118% yield); ^{1}H (500MHz DMSO): δ 8.65 (s, 1H), δ 8.43 (s, 1H), δ 7.86 (m, 1H), δ 7.47 (m, 2H), δ 7.20 (m, 1H), δ 5.96 (d, *J* = 5.7 Hz, 1H), δ 4.76 (m, 1H), δ 4.23 (m, 1H), δ 4.10 (m, 1H), δ 3.43 (dd, *J* = 13.6, 5.6 Hz, 1H), δ 3.29 (dd, *J* = 13.5, 7.5 Hz, 1H); ^{13}C (500MHz DMSO): δ 167.3 (C), 148.7 (C), 141.6 (CH), 140.0 (C), 132.2 (CH), 130.9 (CH), 128.3 (C), 128.0 (CH), 125.6 (CH), 125.5 (CH), 124.0 (CH), 118.8 (C), 87.6 (CH), 82.7 (CH), 73.1 (CH), 72.9 (CH), 33.9 (CH_2); IR (neat, cm^{-1}) 3318 (m), 3147 (m), 2903 (m), 1700 (s), 1416 (m), 1310 (m), 1254 (s), 1084 (s), 1044 (s), 950 (m), 831 (m), 748 (s), 719 (s), 637 (m), 536 (m); HRMS (ESI) calculated for $C_{17}H_{17}N_5O_5S + H^+$ 404.1023, found 404.1023; M.P. 150-153ºC.

Compound 5.

Prepared by general procedure D. White powder (132% yield); ^{1}H (500MHz DMSO): δ 8.36 (s, 1H), δ 8.15 (s, 1H), δ 7.87 (m, 1H), δ 7.69 (m, 1H), δ 7.31 (m, 1H), δ 7.30 (br s, 2H), δ 7.22 (m, 1H), δ 5.89 (d, *J* = 6.0 Hz, 1H), δ 4.80 (m, 1H), δ 4.20 (m, 1H), δ 4.01 (m, 1H), δ 3.41 (dd, *J* = 13.9, 6.0 Hz, 1H), δ 3.28 (dd, *J* = 13.7, 7.2 Hz, 1H); ^{13}C (500MHz DMSO): δ 169.5 (C), 156.0 (C), 152.6 (CH), 149.4 (C), 140.8 (C), 139.9 (CH), 134.3 (C), 129.2 (CH), 128.7 (CH), 128.0 (CH), 126.7 (CH), 125.5 (C), 119.1 (C), 87.3 (CH), 82.7 (CH), 72.7 (CH), 72.6 (CH), 35.5 (CH_2); IR (neat, cm^{-1}) 3304 (w), 3179 (m), 1644 (m), 1595 (s), 1550 (s), 1477 (m), 1379 (s), 1044 (m), 763 (s), 646 (s); HRMS (ESI) calculated for $C_{17}H_{17}N_5O_5S + H^+$ 404.1023, found 404.1023; M.P. 210-215ºC.

Compound 6.

Prepared by general procedure D. White powder (124% yield); ^{1}H (500MHz DMSO): δ 8.36 (s, 1H), δ 8.15 (s, 1H), δ 7.80 (d, *J* = 8.5 Hz, 2H), δ 7.37 (d, *J* = 8.5 Hz, 2H), δ 7.30 (br s, 2H), δ 5.90 (d, *J* = 5.8 Hz, 1H), δ 4.81 (m, 1H), δ 4.22 (m, 1H), δ 4.05 (m, 1H), δ 3.53 (dd, *J* = 13.8, 5.6 Hz, 1H), δ 3.39 (dd, *J* = 13.8, 7.6 Hz, 1H); ^{13}C (500MHz DMSO): δ 167.4 (C), 156.0 (C), 152.6 (CH), 149.4 (C), 141.6 (C), 139.9 (CH), 129.7(CH), 126.3 (CH), 119.1 (C), 87.5 (CH), 82.6 (CH), 72.7 (CH), 72.6 (CH), 34.1 (CH_2); IR (neat, cm^{-1}) 3325 (m), 2968 (w), 1704 (s), 1590 (m), 1488 (m), 1391 (s), 1308 (m), 1183 (s), 1085 (s), 1052 (s), 1009 (m), 950 (m), 772 (s), 725 (s), 627 (s), 515 (s); HRMS (ESI) calculated for $C_{17}H_{17}N_5O_5S + H^+$ 404.1023, found 404.1023; M.P. 193-195ºC.

Compound 7.

Prepared by general procedure E. Clear, colourless oil (69% yield); ^{1}H (500MHz DMSO): δ 8.35 (s, 1H), δ 8.14 (m, 1H), δ 7.44 (m, 1H), δ 7.39 (m, 1H), δ 7.27 (m, 2H), δ 7.20 (m, 2H), δ 5.89 (m, 1H), δ 5.59 (br s, 1H), δ 5.47 (br s, 1H), δ 5.30 (br s, 1H), δ 4.80 (m, 1H), δ 4.53 (s, 2H), δ 4.20 (m, 1H), δ 4.00 (m, 1H). ^{13}C (500MHz DMSO): δ 155.9 (C), 152.6 (CH), 149.4 (C), 141.3 (C), 139.9 (CH), 132.9 (C), 128.1 (CH), 127.2 (CH), 126.7 (CH), 125.7 (CH), 119.0 (C), 87.3 (CH), 82.8 (CH), 72.7 (CH), 72.6 (CH), 60.5 (CH_2), 35.4 (CH_2); IR (neat, cm^{-1}) 3345 (s), 2964 (w), 2924 (w), 2854 (w), 2257 (w), 1646 (s), 1600 (m), 1475 (m), 1420 (m), 1337 (m), 1237 (m), 1090 (m), 1047 (s), 1025 (s), 988 (s), 828 (s), 766 (s), 504 (s); HRMS (ESI) calculated for $C_{17}H_{19}N_5O_4S + H^+$

390.1231, found 390.1232.

Compound 8.

Prepared by general procedure E. Clear, colourless oil (44% yield); ^{1}H (500MHz DMSO): δ 8.36 (s, 1H), δ 8.14 (m, 1H), δ 7.25 (m, 5H), δ 7.12 (m, 1H), δ 5.89 (m, 1H), δ 5.62 (br s, 1H), δ 5.48 (br s, 1H), δ 5.31 (br s, 1H), δ 4.80 (m, 1H), δ 4.44 (s, 2H), δ 4.20 (m, 1H), δ 4.01 (m, 1H). ^{13}C (500MHz DMSO): δ 156.5 (C), 153.1(CH), 149.5 (C), 144.1 (C), 140.4 (CH), 135.9 (C), 129.2 (CH), 126.8 (CH), 126.5 (CH), 124.4 (CH), 119.6 (C), 87.9 (CH), 83.3 (CH), 73.1 (CH), 73.1 (CH), 62.9 (CH_2), 35.7 (CH_2); IR (neat, cm^{-1}) 3350 (s), 2933 (w), 2833 (w), 2254 (w), 2128 (w), 1646 (s), 1599 (m), 1476 (m), 1422 (m), 1334 (m), 1236 (m), 1049 (s), 1023 (s), 996 (s), 825 (s), 764 (s), 572 (s); HRMS (ESI) calculated for $C_{17}H_{19}N_5O_4S + H^+$ 390.1231, found 390.1230.

Compound 32, 4-mercaptobenzyl alcohol.

Under inert atmosphere conditions, compound **27** (1.9 mmol) was dissolved in dioxane (5 ml) and stirred with $LiAlH_4$ (9.5 mmol) for 16 h. The reaction was quenched with ethyl acetate and HCl (15%), until bubbling ceased. The product was concentrated *in vacuo* and filtered, rinsing with 5:1 CH_2Cl_2:methanol. The filtrate was concentrated *in vacuo* and purified by flash chromatography (CH_2Cl_2). The product oil was obtained in 17% yield, and was not fully characterized, but used immediately for the synthesis of compound **9**. Clear, colourless oil (17% yield); ^{1}H (300MHz $CDCl_3$): δ 7.17 (m, 2H), δ 7.12 (m, 2H), δ 4.52 (m, 2H), δ 3.38 (s, 1H), δ 1.99 (br s, 1H).

Compound 9.

Under inert atmosphere conditions, compound **32** (0.314 mmol) was dissolved in DMF (5 ml). NaH (0.408 mmol) was added and the resulting mixture was stirred for 30 minutes, becoming brown. Compound **31** (0.308 mmol) was added and the mixture was stirred for a further 16 hours at 35ºC to give a light yellow-brown solution. The product was concentrated *in vacuo*, dissolved in 1:1 water:MeCN (10 ml) and HCl (1M, 0.5 ml), concentrated *in vacuo* to 1 ml, and recrystallized at 0ºC for 16 hours. The crystals were filtered with cold water, dried, and triturated with ethanol. Clear, colourless oil (18% yield); ^{1}H (500MHz MeOD): δ 8.08 (s, 1H), δ 8.08 (s, 1H), δ 7.26 (m, 2H), δ 7.13 (m, 2H), δ 5.86 (m, 1H), δ 4.44 (s, 2H), δ 4.24 (m, 1H), δ 4.11 (m, 1H), δ 3.22 (m, 1H), δ 3.17(m, 1H); ^{13}C (500MHz MeOD): δ 157.3 (C), 153.9 (CH), 150.7 (C), 141.4 (C), 141.2 (CH), 136.0 (C), 130.7 (CH), 128.7 (CH), 120.6 (C), 90.1 (CH), 85.1 (CH), 74.8 (CH), 74.1 (CH), 64.7 (CH_2), 37.3 (CH_2); IR (neat, cm^{-1}) 3316 (m), 3177 (m), 2899 (m), 1645 (s), 1599 (s), 1576 (s), 1478 (m), 1418 (m), 1334 (m), 1295 (m), 1212 (m), 1125 (s), 1088 (s), 1035 (s), 796 (s), 642 (s); HRMS (ESI) calculated for $C_{17}H_{19}N_5O_4S + H^+$ 390.1231, found 390.1231.

Compound 35, *N*-BOC-3-aminothiophenol.

In 1:1 methanol:water (0.5 ml), compound **33** (0.976 mmol) and *tert*-butyloxycarbonyl anhydride (0.976 mmol) were stirred for 16 h at 35ºC. The product mixture was concentrated *in vacuo* and extracted into CH_2Cl_2 (20 ml) from water (10 ml). Purified by flash chromatography (1:1 hexanes:CH_2Cl_2). Clear, light-brown oil (69% yield); ^{1}H (500MHz $CDCl_3$): δ 7.45 (m, 1H), δ 7.13 (m, 1H), δ 7.02 (m, 1H), δ 6.92 (m, 1H), δ 6.42 (br s, 1H), δ 3.46 (s, 1H), δ 1.53 (s, 9H).

Compound 36, *N*-BOC-4-aminothiophenol.

In 1:1 methanol:water (0.5 ml), compound **34** (0.976 mmol) and *tert*-butyloxycarbonyl anhydride (0.976 mmol) were stirred for 16 h at 35ºC. The product mixture was concentrated *in vacuo* and extracted into CH_2Cl_2 (20 ml) from water (10 ml). Purified by flash chromatography (1:1 hexanes:CH_2Cl_2, then 9:1 CH_2Cl_2:MeOH). White powder (73% yield); ^{1}H (500MHz $CDCl_3$): δ 7.29 (m, 2H), δ 7.25 (m, 2H), δ 7.49 (br s, 1H), δ 3.40 (s, 1H), δ 1.53 (s, 9H).

Compound 37, *N*-(3-thiophenol)phthalimide.

Under inert atmosphere conditions, compound **33** (0.976 mmol) and phthalic anhydride (0.976 mmol) were heated to reflux in xylene (10 ml) for 16 h. The solvent was evaporated and dried at 130ºC for 1 h. Flash chromatography (1:1 hexanes:CH_2Cl_2) afforded two products, both yellow solids. Product 1, ^{1}H (300MHz $CDCl_3$): δ 7.88 (dd, 2H), δ 7.73 (dd, 2H), δ 7.32 (m, 1H), δ 7.29 (m, 1H), δ 7.24 (m, 1H), δ 7.18 (m, 1H), δ 3.49 (s, 1H); Product 2, ^{1}H (300MHz $CDCl_3$): δ 7.82 (dd, 2H), δ 7.70 (dd, 2H), δ 7.60 (m, 1H), δ 7.47 (m, 1H), δ 7.38 (m, 1H), δ 7.26 (m, 1H).

Compound 38, 5′-chloro-5′-deoxyadenosine.

Under inert atmosphere conditions, adenosine (10.0 mmol) was stirred at 0ºC in acetonitrile (30 ml) and pyridine (20 mmol). After stirring for 10 min, thionyl chloride (50.0 mmol) was added drop-wise. The resulting mixture was stirred for 4 h at 0ºC and then warmed to room temperature, stirring for a further 16 h. The yellow emulsion was

filtered and washed with acetonitrile, affording a white precipitate. This intermediate was dissolved in methanol (30 ml) and aqueous NH_4OH (2M, 20 ml), stirring for 1 h. The product was concentrated *in vacuo*, and recrystallized by dissolving in water (40 ml) at 70ºC, cooling to room temperature, and sitting at 4ºC for 16 h. The crystals were washed with cold water and dried. Fluffy white crystals (87% yield); ^{1}H (500MHz DMSO): δ 8.34 (s, 1H), δ 8.15 (s, 1H), δ 7.30 (br s, 2H), δ 5.92 (d, J = 5.7 Hz, 1H), δ 5.58 (d, J = 5.9 Hz, 1H), δ 5.44 (d, J = 5.1 Hz, 1H), δ 4.75 (m, 1H), δ 4.22 (m, 1H), δ 4.09 (m, 1H), δ 3.94 (dd, J = 11.5, 5.1 Hz, 1H), δ 3.84 (dd, J = 11.5, 6.3 Hz, 1H); ^{13}C (500MHz DMSO): δ 156.1 (C), 152.7 (CH), 139.7 (CH), 119.1(C), 87.4 (CH), 83.6 (CH), 72.1 (CH), 71.2 (CH), 44.8 (CH_2); IR (neat, cm^{-1}) 3325 (m), 3136 (m), 2942 (m), 1640 (s), 1598 (s), 1474 (m), 1418 (m), 1333 (m), 1298 (m), 1245 (m), 1208 (m), 1097 (s), 1048 (s), 797 (m), 724 (m), 644 (m), 586 (m); HRMS (ESI) calculated for $C_{10}H_{12}ClN_5O_3 + H^+$ 286.0702, found 286.0702. Product showed characterization identical to a previous report (157).

Compound 10.

Prepared by general procedure F, with the following proportions: compound **33** (3.68 mmol), *t*-BuOK (1M in THF, 4.03 mmol), compound **38** (1.75 mmol). The crude product was recrystallized in 1:1 water:methanol (10 ml), heating to 60ºC and cooling to 4ºC for 16 h; the crystals were rinsed with cold water and CH_2Cl_2. White crystals (79% yield); ^{1}H (500MHz DMSO): δ 8.35 (s, 1H), δ 8.16 (s, 1H), δ 7.30 (br s, 2H), δ 6.93 (m, 1H), δ 6.55 (m, 1H), δ 6.47 (m, 1H), δ 6.38 (m, 1H), δ 5.89 (d, J = 5.7 Hz, 1H), δ 5.48 (br, 2H), δ 5.31 (br s, 2H), δ 4.81 (m, 1H), δ 4.17 (m, 1H), δ 4.00 (m, 1H), δ 3.32 (dd, J = 14.1, 6.4 Hz, 1H), δ 3.19 (dd, J = 13.8, 7.0 Hz, 1H); ^{13}C (500MHz DMSO): δ 156.0 (C),

152.6 (CH),149.5 (C), 149.2 (C), 139.9 (CH), 135.7 (C), 129.4 (CH), 119.2 (C), 115.3 (CH), 113.2 (CH), 111.7 (CH), 87.3 (CH), 82.9 (CH), 72.6 (CH), 72.5 (CH), 35.1 (CH_2), 30.6 (C); IR (neat, cm^{-1}) 3280 (m), 3146 (m), 3132 (m), 2918 (m), 1680 (s), 1652 (s), 1596 (s), 1578 (s), 1480 (s), 1423 (m), 1342 (m), 1297 (s), 1208 (m), 1129 (m), 1089 (s), 1040 (s), 990 (m), 832 (m), 745 (s), 713 (s), 685 (s), 639 (s), 593 (s), 542 (s); HRMS (ESI) calculated for $C_{16}H_{18}N_6O_3S + H^+$ 375.1234, found 375.1230; M.P. 85-88ºC.

Compound 11.

Prepared by general procedure F, with the following proportions: compound **34** (4.20 mmol), *t*-BuOK (1M in THF, 4.62 mmol), compound **38** (1.40 mmol). The crude product was recrystallized in 1:1 water:methanol (10 ml), heating to 60ºC and cooling to 4ºC for 16 h; the crystals were rinsed with cold water and CH_2Cl_2. White crystals (75% yield); ^1H (500MHz DMSO): δ 8.35 (s, 1H), δ 8.15 (s, 1H), δ 7.30 (br s, 2H), δ 7.11 (m, 2H), δ 6.51 (m, 2H), δ 5.87 (d, J = 5.7 Hz, 1H), δ 5.52 (br s, 1H), δ 5.36 (br s, 1H), δ 4.79 (m, 1H), δ 4.13 (m, 1H), δ 3.90 (m, 1H), δ 3.12 (dd, J = 13.6, 6.5 Hz, 1H), δ 2.99 (dd, J = 13.6, 6.9 Hz, 1H); ^{13}C (500MHz DMSO): δ 156.0 (C), 152.6 (CH),149.5 (C), 148.5 (C), 139.9 (CH), 134.3 (CH), 133.7 (CH), 119.2 (C), 118.3 (C), 114.4 (CH), 114.2 (CH), 87.1 (CH), 83.0 (CH), 72.5 (CH), 72.5 (C), 38.8 (CH_2); IR (neat, cm^{-1}) 3276 (m), 3132 (m), 2897 (m), 1674 (m), 1651 (s), 1600 (s), 1575 (s), 1496 (s), 1421 (m), 1341 (m), 1294 (s), 1127 (s), 1098 (s), 1036 (s), 817 (s), 717 (s), 630 (s), 513 (s); HRMS (ESI) calculated for $C_{16}H_{18}N_6O_3S + H^+$ 375.1234, found 375.1231; M.P. 96-99ºC.

Compound 39, 3-acetamidothiophenol.

Compound **33** (2.40 mmol) was dissolved in ethyl acetate (10 ml) and stirred at 0ºC. Acetic anhydride (2.40 mmol) was added and the resulting mixture was stirred for 1 h. The reaction mixture was diluted in ethyl acetate (10 ml) and washed 3 times with brine (10 ml). The product from the organic layer was concentrated *in vacuo* and purified by flash chromatography (1:1 hexanes:ethyl acetate). Clear, colourless oil (83% yield); ^{1}H (500MHz DMSO): δ 10.1 (s, 1H), δ 7.87 (m, 1H), δ 7.76 (m, 1H), δ 7.28 (m, 1H), δ 7.16 (m, 1H), δ 2.03 (s, 3H); ^{13}C (500MHz DMSO): δ 168.6 (C), 140.2 (C), 136.1 (C), 129.6 (CH), 121.4 (CH), 118.0 (CH), 116.9 (CH), 23.9 (CH_3); IR (neat, cm^{-1}) 3301 (m), 3170 (w), 3111 (w), 3063 (w), 1662 (s), 1585 (s), 1539 (s), 1480 (s), 1419 (s), 1369 (m), 1307 (m), 1289 (m), 1257 (m), 1015 (m), 866(m), 777 (s), 684 (s), 537 (s); HRMS (ESI) calculated for $C_8H_9NOS + H^+$ 168.0478, found 168.0478.

Compound 12.

Prepared by general procedure F, with the following proportions: compound **39** (0.788 mmol), *t*-BuOK (1M in THF, 0.866 mmol), compound **38** (0.525 mmol). The crude product was recrystallized in water (2 ml), heating to 60ºC and then 4ºC for 16 h; the crystals were rinsed with cold water and CH_2Cl_2. White crystals (88% yield); ^{1}H (500MHz DMSO): δ 9.95 (s, 1H), δ 8.34 (s, 1H), δ 8.15 (s, 1H), δ 7.62 (m, 1H), δ 7.37 (m, 1H), δ 7.27 (br s, 2H), δ 7.21 (m, 1H), δ 7.01 (m, 1H), δ 5.87 (d, J = 6.0 Hz, 1H), δ 5.58 (br s, 1H), δ 5.44 (br s, 1H), δ 4.81 (m, 1H), δ 4.19 (m, 1H), δ 4.00 (m, 1H), δ 3.38 (dd, 1H), δ 3.26 (dd, J = 13.8, 7.0 Hz, 1H); ^{13}C (500MHz DMSO): δ 168.5 (C), 156.0 (C), 152.6 (CH), 149.4 (C), 139.9 (CH), 139.8 (C), 136.1 (C), 129.3 (CH), 122.5 (CH), 119.1 (C), 118.2 (CH), 116.5 (CH), 87.4 (CH), 82.7 (CH), 72.6 (CH), 72.5 (CH), 35.1

(CH_2), 24.0 (CH_3); IR (neat, cm^{-1}) 3267 (m), 3127 (m), 2886 (w), 1652 (s), 1605 (s), 1587 (s), 1544 (s), 1477 (s), 1420 (s), 1295 (s), 1206 (m), 1090 (s), 1044 (s), 776 (m), 684 (s), 638 (s), 537 (s); HRMS (ESI) calculated for $C_{18}H_{20}N_6O_4S + H^+$ 417.1340, found 417.1340; M.P. 83-86ºC.

Compound 13.

Compound **13** was prepared by general procedure F, with the following proportions: 4-acetamidothiophenol (1.05 mmol), *t*-BuOK (1M in THF, 1.16 mmol), compound **38** (0.525 mmol). The crude product was recrystallized in 7 ml 5:2 water:methanol, heating to 70ºC and then 4ºC for 16 h; the crystals were rinsed with cold water and CH_2Cl_2. The product was triturated with CH_2Cl_2 and methanol. White crystals (57% yield); ^1H (500MHz DMSO, TFA salt): δ 9.96 (s, 1H), δ 8.78 (s, 1H), δ 8.47 (s, 1H), δ 7.53 (m, 2H), δ 7.31 (m, 2H), δ 7.28 (br s, 2H), δ 5.88 (d, J = 6.0 Hz, 1H), δ 5.52 (br d, 1H), δ 5.36 (br d, 1H), δ 4.79 (m, 1H), δ 4.16 (m, 1H), δ 3.69 (m, 1H), δ 3.34 (dd, J = 13.9, 6.2 Hz, 1H), δ 3.21 (dd, J = 13.8, 7.1 Hz, 1H) δ 2.02 (s, 3H); ^{13}C (500MHz DMSO, TFA salt): δ 168.2 (C), 156.0 (C), 152.6 (CH), 149.4 (C), 139.9 (CH), 137.9 (C), 130.1 (CH), 128.7 (C), 119.6 (CH), 119.2 (C), 87.3 (CH), 82.8 (CH), 72.6 (CH), 72.5 (CH), 36.4 (CH_2), 23.9 (CH_3); IR (neat, cm^{-1}) 3266 (m), 3111 (m), 2879 (w), 1646 (s), 1593 (s), 1533 (s), 1495 (s), 1396 (s), 1295 (s), 1088 (s), 1042 (s), 815 (s), 716 (s), 648 (s), 503 (s); HRMS (ESI) calculated for $C_{18}H_{20}N_6O_4S + Na^+$ 439.1159, found 439.1157; M.P. 94-96ºC.

Compound 14.

Compound **10** (0.400 mmol) was ground into methanol (8 ml) and glacial acetic acid (0.5 ml), stirring at 0ºC. KOCN (1.60 mmol) was dissolved in water (1 ml) and transferred to stirring solution. The reaction was warmed to 20ºC, stirring for 30 min, then heated to 50ºC, stirring for 4 h. The product was neutralized with NaOH (10%, 4.5 ml), and concentrated *in vacuo* to give a cloudy aqueous mixture. The product was recrystallized at 4ºC for 16 h. White solid (40% yield); ^{1}H (500MHz DMSO): δ 9.09 (s, 1H), δ 8.34 (s, 1H), δ 8.15 (s, 1H), δ 7.47 (m, 1H), δ 7.26 (br s, 2H), δ 7.22 (m, 1H), δ 7.12 (m, 1H), δ 6.86 (m, 1H), δ 6.06 (br s, 2H), δ 5.88 (d, J = 6.0 Hz, 1H), δ 4.78 (m, 1H), δ 4.18 (m, 1H), δ 4.01 (m, 1H), δ 3.37 (dd, 1H), δ 3.23 (dd, J = 13.8, 7.1 Hz, 1H); ^{13}C (500MHz DMSO): δ 156.1 (C), 156.0 (C), 153.1 (C), 152.6 (CH), 141.4 (C), 139.9 (CH), 135.8 (C), 129.1 (CH), 120.4 (CH), 119.1 (C), 117.0 (CH),115.3 (CH), 87.4 (CH), 82.8 (CH), 72.6 (CH), 72.6 (CH), 35.2 (CH_2); IR (neat, cm^{-1}) 3304 (m), 3177 (m), 2893 (w), 1652 (s), 1576 (s), 1544 (s), 1476 (s), 1333 (s), 1300 (s), 1245 (s), 1207 (s), 1090 (s), 1045 (s), 686 (s), 646 (s); HRMS (ESI) calculated for $C_{17}H_{19}N_7O_4S + Na^+$ 440.1111, found 440.1111; M.P. 156-157ºC.

Compound 15.

Compound **11** (0.400 mmol) was ground into methanol (8 ml) and glacial acetic acid (0.5 ml), stirring at 0ºC. KOCN (1.60 mmol) was dissolved in water (1 ml) and transferred to the stirring mixture. The reaction was warmed to 20ºC, stirring for 30 min, then heated to 50ºC, stirring for 4 h. The product was neutralized with NaOH (10%, 4.7 ml), and concentrated *in vacuo* to give a cloudy aqueous mixture. The product was recrystallized at 4ºC for 16 h. White solid (75% yield); ^{1}H (500MHz DMSO, TFA salt): δ

8.64 (s, 1H), δ 8.62 (s, 1H), δ 8.43 (s, 1H), δ 7.36 (m, 2H), δ 7.26 (m, 2H), δ 5.92 (d, *J* = 6.1 Hz, 1H), δ 4.70 (m, 1H), δ 4.14 (m, 1H), δ 3.98 (m, 1H), δ 3.28 (dd, *J* = 13.9, 6.3 Hz, 1H), δ 3.15 (dd, *J* = 13.9, 6.9 Hz, 1H); ^{13}C (500MHz DMSO, TFA salt): δ 155.9 (C), 155.8 (C), 151.6 (CH), 148.7 (C), 147.0 (C), 141.9 (C), 139.6 (CH), 131.1 (CH), 125.8 (C), 118.4 (CHn), 87.5 (CH), 83.4 (CH), 73.1 (CH), 72.4 (CH), 37.0 (CH_2); IR (neat, cm^{-1}) 3311 (m), 3113 (m), 2893 (w), 1674 (s), 1576 (s), 1534 (s), 1496 (s), 1402 (s), 1342 (s),1297 (s), 1211 (s), 1130 (s), 1087 (s), 1045 (s), 817 (s), 719 (s), 639 (s); HRMS (ESI) calculated for $C_{17}H_{19}N_7O_4S + Na^+$ 440.1111, found 440.1110; M.P. 141-144ºC.

Compound 40, 3-isovaleramidothiophenol.

Compound **33** (2.40 mmol) was dissolved in ethyl acetate (10 ml) and stirred at 0ºC. Isovaleric anhydride (2.40 mmol) was added and the resulting mixture was stirred for 3 h, heating to 50ºC for the last hour. The mixture was diluted in ethyl acetate (10 ml) and washed 3 times with brine (10 ml). The product from the organic layer was concentrated *in vacuo* and purified by flash chromatography (CH_2Cl_2). Clear, colourless oil (70% yield, crude); ^{1}H (500MHz DMSO): δ 9.82 (s, 1H), δ 7.64 (m, 1H), δ 7.25 (m, 1H), δ 7.14 (m, 1H), δ 6.94 (m, 1H), δ 5.35 (br s, 1H), δ 2.16 (d, 2H), δ 2.06 (m, 1H), δ 0.92 (d, 6H). ^{13}C (500MHz DMSO): δ 176.1 (C), 145.4 (C), 141.3 (C), 134.9 (CH), 126.6 (CH), 123.3 (CH), 122.4 (CH), 50.8 (CH_2), 30.7 (CH), 27.5 (CH_3).

Compound 41, 4-isovaleramidothiophenol.

Compound **34** (2.40 mmol) was dissolved in ethyl acetate (10 ml) and stirred at 0ºC. Isovaleric anhydride (2.40 mmol) was added and the resulting mixture was stirred

for 3 h, heating to 50ºC for the last hour. The mixture was diluted in ethyl acetate (10 ml) and washed 3 times with brine (10 ml). The product from the organic layer was concentrated *in vacuo* and purified by flash chromatography (9:1 hexanes:ethyl acetate). Clear, yellow oil (93% yield, crude); ^{1}H (300MHz DMSO): δ 9.80 (s, 1H), δ 7.47 (m, 2H), δ 7.20 (m, 2H), δ 5.35 (br s, 1H), δ 2.15 (d, 2H), δ 1.95 (m, 1H), δ 0.91 (d, 6H); ^{13}C (300MHz DMSO): δ 176.0 (C), 144.7 (C), 135.3 (C), 134.6 (CH), 125.0 (CH), 50.8 (CH_2), 30.8 (CH), 27.5 (CH_3).

Compound 16.

Prepared by general procedure F, with the following proportions: compound **40** (0.788 mmol), *t*-BuOK (1M in THF, 0.866 mmol), and compound **38** (0.525 mmol). The crude product was recrystallized in 5 ml 1:1 water:methanol, heating to 65ºC and then 4ºC for 16 h; the crystals were rinsed with cold water and CH_2Cl_2. White crystals (54% yield); ^{1}H (500MHz DMSO): δ 9.87 (s, 1H), δ 8.35 (s, 1H), δ 8.16 (s, 1H), δ 7.65 (m, 1H), δ 7.40 (m, 1H), δ 7.29 (br s, 2H), δ 7.21 (m, 1H), δ 7.00 (m, 1H), δ 5.89 (d, J = 6.0 Hz, 1H), δ 5.51 (d, J = 6.1 Hz, 1H), δ 5.37 (d, J = 4.9 Hz, 1H), δ 4.82 (m, 1H), δ 4.19 (m, 1H), δ 4.01 (m, 1H), δ 3.39 (dd, J = 13.7, 6.5 Hz, 1H), δ 3.28 (dd, J = 13.8, 7.0 Hz, 1H), δ 2.17 (d, J = 7.1 Hz, 1H), δ 2.06 (m, 1H), δ 0.92 (d, J = 6.6 Hz, 1H); ^{13}C (500MHz DMSO): δ 170.8 (C), 156.1 (C), 152.6 (CH), 149.4 (C), 139.9 (CH), 139.8 (C), 136.1 (C), 129.3 (CH), 122.4 (CH), 119.2 (C), 118.3 (CH),116.5 (CH), 87.4 (CH), 82.7 (CH), 72.7 (CH), 72.5 (CH), 45.6 (CH_2), 35.1 (CH_2), 25.5 (CH), 22.2 (CH_3); IR (neat, cm^{-1}) 3252 (m), 3135 (m), 2957 (m), 1654 (s), 1592 (s), 1532 (m), 1478 (s), 1406 (m), 1298 (s), 1211 (m), 1123 (s), 1042 (s), 755 (s), 691 (s), 641 (s), 593 (s), 546 (s); HRMS (ESI)

calculated for $C_{21}H_{26}N_6O_4S + H^+$ 459.1809, found 459.1810; M.P. 178-180ºC.

Compound 17.

Prepared by general procedure F, with the following proportions: compound **41** (0.573 mmol), *t*-BuOK in THF (1M in THF, 0.631 mmol), and compound **38** (0.478 mmol). The crude product was recrystallized in 5 ml 1:1 water:methanol, heating to 55ºC and then 4ºC for 16 h; the crystals were rinsed with cold water and CH_2Cl_2. White crystals (83% yield); ^{1}H (500MHz DMSO): δ 9.88 (s, 1H), δ 8.35 (s, 1H), δ 8.15 (s, 1H), δ 7.55 (m, 2H), δ 7.31 (m, 2H), δ 7.28 (br s, 2H), δ 5.88 (d, *J* = 5.7 Hz, 1H), δ 5.50 (d, , *J* = 5.9 Hz, 1H), δ 5.36 (d, , *J* = 4.9 Hz, 1H), δ 4.79 (m, 1H), δ 4.15 (m, 1H), δ 3.95 (m, 1H), δ 3.34 (dd, 1H), δ 3.21 (dd, , *J* = 14.0, 7.0 Hz, 1H) , δ 2.16 (d, *J* = 6.7 Hz, 1H), δ 2.06 (m, 1H), δ 0.91 (d, *J* = 6.2 Hz, 1H); ^{13}C (500MHz DMSO): δ 170.6 (C), 156.1 (C), 152.6 (CH), 149.5 (C), 139.8 (CH), 137.9 (C), 130.1 (CH), 128.7 (C), 119.7 (CH), 119.2 (C), 87.3 (CH), 82.8 (CH), 72.6 (CH), 72.5 (CH), 45.6 (CH_2), 36.4 (CH_2), 25.5 (CH), 22.3 (CH_3); IR (neat, cm^{-1}) 3286 (s), 3145 (s), 2959 (m), 1652 (s), 1606 (s), 1531 (m), 1495 (m), 1398 (m), 1340 (m), 1296 (m), 1207 (m), 1087 (s), 1043 (s), 826 (m), 746 (s), 637 (m); HRMS (ESI) calculated for $C_{21}H_{26}N_6O_4S + H^+$ 459.1809, found 459.1811; M.P. 188-189ºC.

Compound 18.

Under inert atmosphere conditions, compound **10** (0.366 mmol), 2-chloroethyl isocyanate (1.46 mmol), and glacial acetic acid (5 drops), were stirred in anhydrous ethanol (10 ml) for 16 hours at r.t.. The resulting clear gel was neutralized with NaOH

(10%, 20 drops) and concentrated *in vacuo*. The addition of EtOAc (20 ml) and water (20 ml) precipitated a tan solid. The solid was collected and recrystallized in methanol and water, heating to 65ºC, and then cooling to 4ºC overnight. The intermediate compound **42** was checked by NMR; ^{1}H (300MHz DMSO): δ 8.34 (s, 1H), δ 8.15 (s, 1H), δ 7.55 (m, 1H), δ 7.37 (m, 1H), δ 7.28 (br s, 2H), δ 7.22 (m, 1H), δ 6.98 (br s, 1H), δ 6.96 (m, 1H), δ 5.89 (d, *J* = 6.0 Hz, 1H), δ 5.51 (br s, 1H), δ 5.36 (br s, 1H), δ 4.80 (m, 1H), δ 4.20 (m, 1H), δ 4.01 (m, 1H), δ 3.77 (m, 2H), δ 3.41 (dd, *J* = 13.9, 6.0 Hz, 1H), δ 3.36 (m, 2H), δ 3.28 (dd, *J* = 13.9, 7.3 Hz, 1H). Compound **42** (0.0917 mmol) was dissolved in water (2 ml) and formic acid (1 ml) at 0ºC. Sodium nitrite (0.458 mmol) was added and the resulting mixture was stirred, warming to r.t. over 5 hours. The orange precipitate was filtered and rinsed with water. Recrystallization was performed in methanol, heating to 65ºC, then cooling to 4ºC overnight. Orange solid (7% yield over two steps); ^{1}H (500MHz DMSO): δ 8.34 (s, 1H), δ 8.15 (s, 1H), δ 7.70 (m, 1H), δ 7.55 (m, 1H), δ 7.38 (m, 1H), δ 7.29 (br s, 2H), δ 7.22 (m, 1H), δ 5.89 (d, *J* = 5.7 Hz, 1H), δ 5.54 (d, *J* = 6.3 Hz, 1H), δ 5.39 (d, *J* = 5.2 Hz, 1H), δ 4.82 (m, 1H), δ 4.22 (m, 1H), δ 4.04 (m, 1H), δ 3.95 (m, 2H), δ 3.83 (m, 2H), δ 3.47 (dd, *J* = 13.9, 5.8 Hz, 1H), δ 3.36 (dd, *J* = 14.0, 7.2 Hz, 1H); ^{13}C (500MHz DMSO): 157. 0 (C), 156.5 (C), 153.2 (CH), 149.9 (C), 140.4 (CH), 139.1 (C), 137.8 (C), 130.2 (CH), 124.8 (CH), 119.8 (C), 118.8 (CH), 117. 2 (CH), 88. 3 (CH), 83.3 (CH), 73.5 (CH), 73.1 (CH), 42.6 (CH_2), 38. 4 (CH_2), 35.4 (CH_2); IR (neat, cm^{-1}) 3334 (m), 3124 (m), 2908 (w), 1708 (m), 1646 (s), 1592 (s), 1483 (s), 1409 (s), 1215 (s), 1086 (s); M.P. 161-163ºC.

Attempted synthesis of compound 19.

Under inert atmosphere conditions, compound **11** (0.535 mmol), 2-chloroethyl isocyanate (2.14 mmol), and glacial acetic acid (25 drops), were stirred in anhydrous ethanol (10 ml) for 16 hours at r.t. (slurry). The resulting clear gel was neutralized with NaOH (10%) and concentrated *in vacuo*. The addition of EtOAc (20 ml), CH_2Cl_2 (10 ml) and water (20 ml) precipitated a tan solid. The solid was collected and recrystallized in methanol and water, heating to 65ºC, and then cooling to r.t. for 1 hour. The intermediate compound **43** was checked by NMR; ^{1}H (300MHz DMSO): δ 8.72 (s, 1H), δ 8.35 (s, 1H), δ 8.15 (s, 1H), δ 7.36 (m, 2H), δ 7.29 (br s, 2H), δ 7.28 (m, 2H), δ 6.41 (m, 1H), δ 5.88 (d, J = 6.0 Hz, 1H), δ 5.49 (d, J = 6.1 Hz, 1H), δ 5.33 (d, J = 4.8 Hz, 1H), δ 4.79 (m, 1H), δ 4.15 (m, 1H), δ 3.94 (m, 1H), δ 3.65 (m, 2H), δ 3.41 (m, 2H), δ 3.29 (dd, J = 14.1, 6.5 Hz, 1H), δ 3.17 (dd, J = 13.1, 7.5 Hz, 1H). Compound **43** (0.198 mmol) was stirred in water (3 ml) and formic acid (0.5 ml) at 0ºC (slurry). Sodium nitrite (0.569 mmol) was added and the resulting mixture was stirred at r.t. for 2 hours. The yellow slurry was heated to 70ºC and then cooled to 4ºC overnight. Tan solid (36% yield over two steps); ^{1}H (500MHz DMSO): δ 8.48 (s, 1H), δ 8.35 (s, 1H), δ 8.15 (s, 1H), δ 7.40 (m, 2H), δ 7.28 (br s, 2H), δ 7.26 (m, 2H), δ 5.88 (d, J = 6.0 Hz, 1H), δ 4.78 (m, 1H), δ 4.15 (m, 1H), δ 3.94 (m, 1H), δ 3.62 (m, 2H), δ 3.38 (m, 2H), δ 3.28 (dd, J = 13.8, 6.3 Hz, 1H), δ 3.15 (dd, J = 13.6, 6.7 Hz, 1H); ^{13}C (500MHz DMSO): δ 156.0 (C), 155.2 (C), 152.6 (CH), 149.5 (C), 139.9 (CH), 131.0 (CH), 130.9 (CH), 125.8 (C), 119.1 (C), 118.2 (CH), 87.2 (CH), 82.9 (CH), 72.6 (CH), 72.5 (CH), 44.1 (CH_2), 41.2 (CH_2), 37.1 (CH_2); IR (neat, cm^{-1}) 3315 (m), 3127 (m), 2940 (w), 2832 (m), 164 (s), 1596 (s), 1553 (s), 1499 (s), 1359 (s), 1237 (s), 1091 (s), 989 (m), 812 (m), 774 (s), 634 (s), 509 (s); M.P. 166-167ºC.

4.7 MTAP-XO Coupled Assay Procedure

The protocol for this assay was based on the protocols described by Kung *et al.* and Savarese *et al.* (103, 153). The MTAP enzyme was purchased from Origene, and the xanthine oxidase (XO) enzyme was purchased from Sigma-Aldrich. The enzymes were thawed on ice. All solutions were diluted in PBS buffer (1xPBS, pH 7.4, 0.1M) and the microplate reader was kept at 37ºC. The final volume of each well in the 96 well plate was 100 µL. The substrates (MTA, PTA, adenosine compounds 1-17) were diluted from 100 mM DMSO stock solutions to working solutions of 5 mM and 0.5 mM. 20 µL of the 5 mM solution gave wells with a substrate final concentration of 1 mM, while 20 µL of the 0.5 mM solutions gave wells with a final substrate concentration of 0.1 mM. Each substrate was tested with the MTAP enzyme present (MTAP+), using 20 µL of a 0.02µg/µL (or 0.012µg/µL) MTAP working solution, and with MTAP absent (MTAP–). Each well also contained 0.5 units of XO, from 10 µL of a 0.05 U/µL XO working solution. In summary, each well contained: (1) 10µL of 0.05 U/µL XO working solution, (2) 20 µL of substrate working solution (either 5 mM or 0.5 mM), (3) 20 µL of 0.02µg/µL (or 0.012µg/µL) MTAP working solution (MTAP+), or 0 µL of MTAP working solution (MTAP–), and (4) diluted to 100 µL with 50 µL PBS buffer (MTAP+) or 70 µL PBS buffer (MTAP–). The absorbance of the wells was measured at 305 nm, monitoring for 5 minute intervals. The slope of the change in absorbance of MTAP+ wells gave a value analogous to the rate of cleavage by MTAP.

The first assay (for compounds **1**–**8**) involved a 0.02µg/µL MTAP working solution and three 5-minute runs. MTA and PTA were used as controls. The slope and

slope error (using the LINEST array function on Excel) was measured for each replicate, and all three replicates were used to calculate an *average* slope and the corresponding standard error (calculated as the square root of the sum of the squares of the standard error of each replicate). These slopes were then set relative to the slope of MTA and error was calculated for the adjustment: each substrate slope was divided by the slope of MTA and multiplied by 100; and the following equation was used to calculate the standard error: $\frac{Dz}{z} = \sqrt[2]{\left(\frac{Da}{a}\right)^2 + \left(\frac{Db}{b}\right)^2}$, where Dz is the standard error of the value z, etc. See **Appendix B** for tables of the slopes and standard errors.

The second assay (for compounds **9**–**17**) involved a 0.012µg/µL MTAP working solution and *ten* 5-minute runs. MTA and adenosine were used as controls. The slopes and standard errors were calculated in the same manner as the first assay. See **Appendix B** for the data.

4.8 MTT Assay (Cytotoxicity)

Both MTT assays were performed by Dr. Hongwei Cheng at the BC Cancer Agency, Vancouver. The protocol was constructed according to those described by Slater *et al.*, Van de Loosdrecht *et al.*, and Alley *et al.* (172–174). The assay was performed in a 96 well assay, and each condition was in triplicate. Before the first day, PC3 (prostrate cancer cells, high metastatic potential, MTAP+), Du145 (prostrate cancer cells, moderate metastatic potential, MTAP+), A549 (adenocarcinomic human alveolar basal epithelial cells, MTAP–), MCF7 (breast cancer cells, MTAP–) cell lines were grown on 10 cm petri dishes in complete media (RPMI1640 medium with 10% FBS) overnight at 37ºC. On the first day: trypsin (3 ml, 0.25% trypsin-EDTA solution, phenol red) was added to each

petri dishes to detach cells. 5 ml of complete media was added to the trypsinized cells. The suspension of cells was centrifuged for 5 minutes in a sterile 15 ml falcon tube at 500 rpm in a swinging bucket rotor (approximately 400 x g). The media was drained and the cells were resuspended with complete media, for a total volume of 1.0 ml. The cells were counted using a cell counter machine. The cells were diluted to 75 000 cells/ml using complete media. 100 µL of the cells were added to each well and incubated overnight (37ºC). On day two: each cell was treated with the agonist (compounds **1-18**), for final concentrations of 20, 100, and 200µM in the first assay, or 10, 50, and 200 µM in the second assay; and the volume in each well was adjusted to 100 µL. The cells were incubated overnight (37ºC). On day three: 20 µL of 5 mg/mL MTT solution (in PBS, filtered sterilized) was added to each well. The suspensions were incubated for 3.5 hours at 37ºC. The media was removed (carefully, without disturbing cells or rinsing with PBS). 150 µL MTT solvent (4 mM HCl, 0.1% Nondet P-40 in isopropanol) was added to each well. The 96-well plate was covered with tinfoil and agitated on an orbital shaker for 15 minutes. The absorbance was read at 590 nm with a reference filter of 620 nm. Each concentration of agonist with each cell line was performed in triplicate, and the standard error was calculated for these replicates (represented in **Appendix B** as error bars). The average absorbances of the triplicate wells was set relative to the average absorbance of the triplicate wells containing the control cell line (no agonist). In the second assay, the control was not run in triplicate and is not shown in the bar graphs (**Appendix B**).

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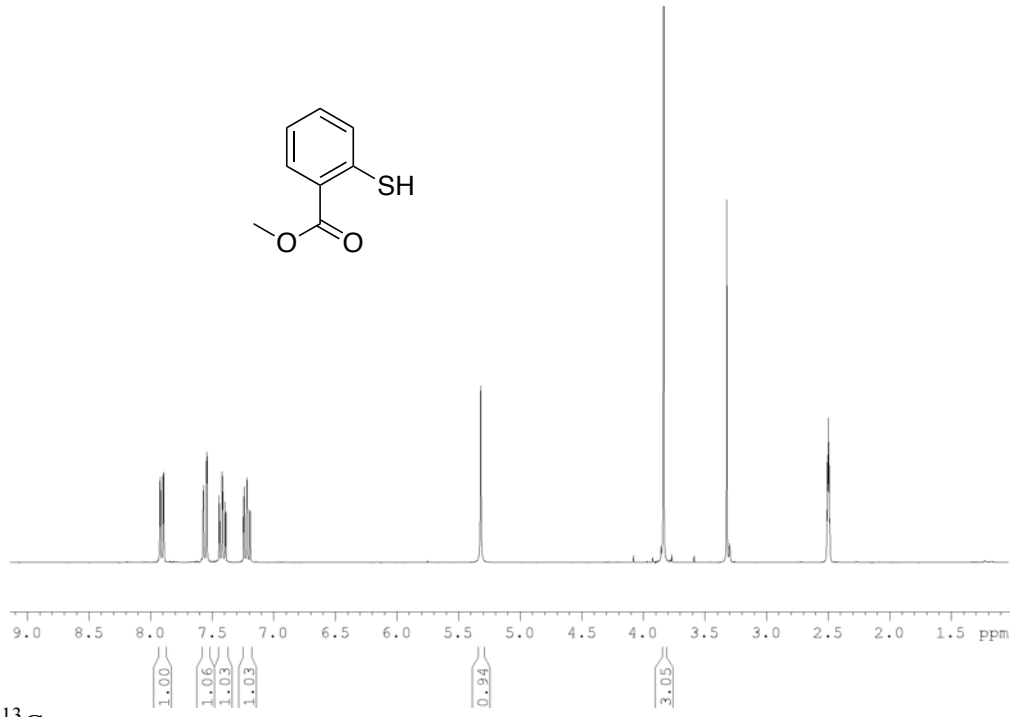
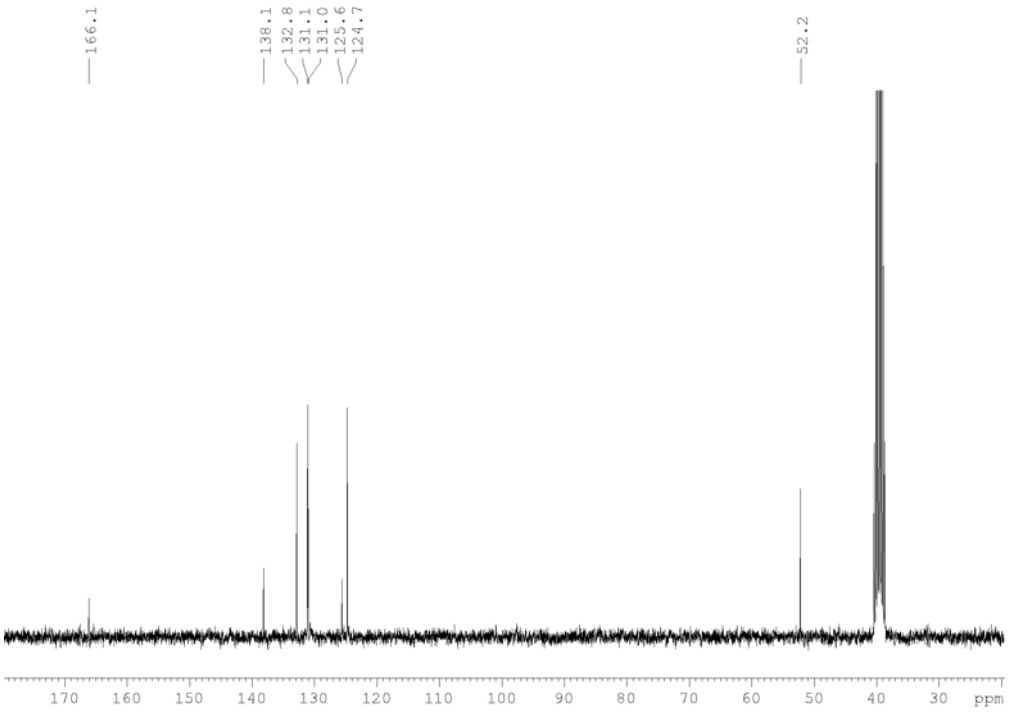
Appendix A Spectral Data (NMR)

Compound 25 ($CDCl_3$)

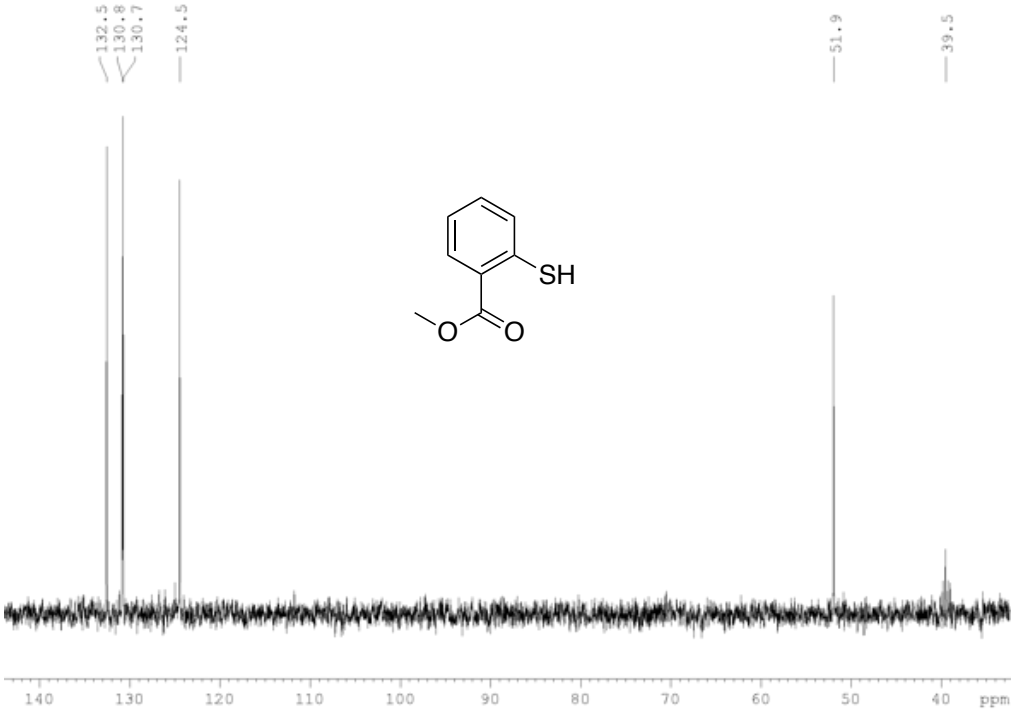
^1H



^{13}C

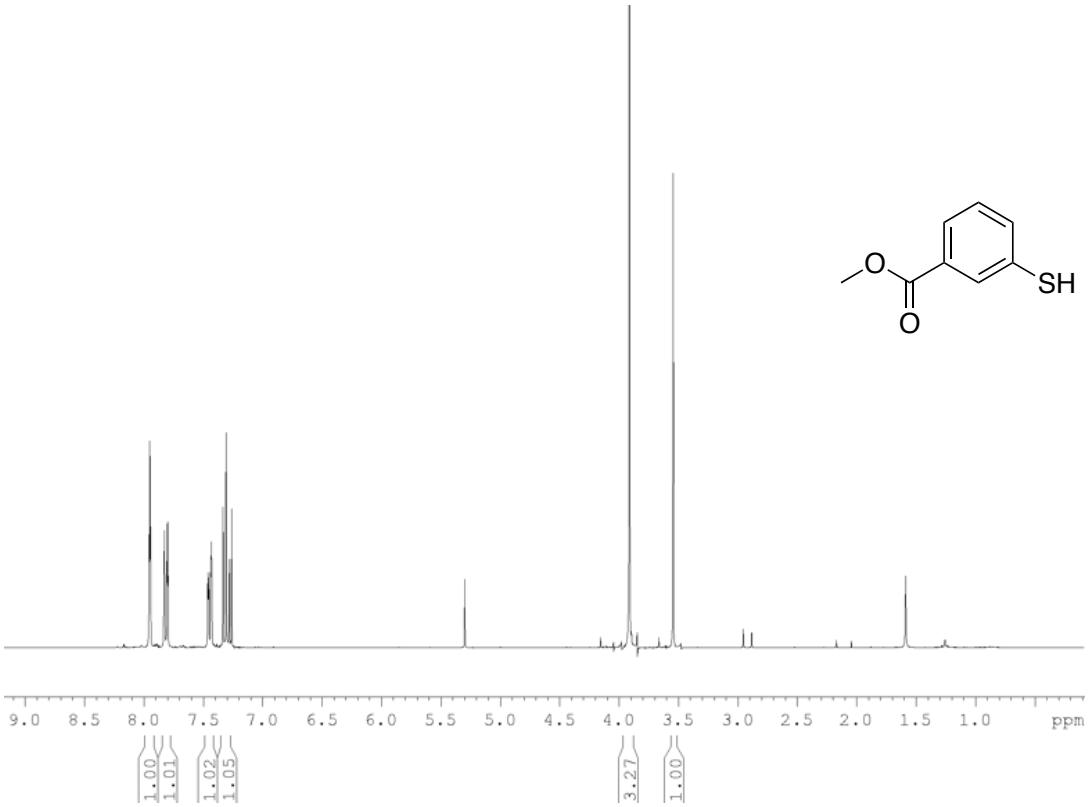


DEPT

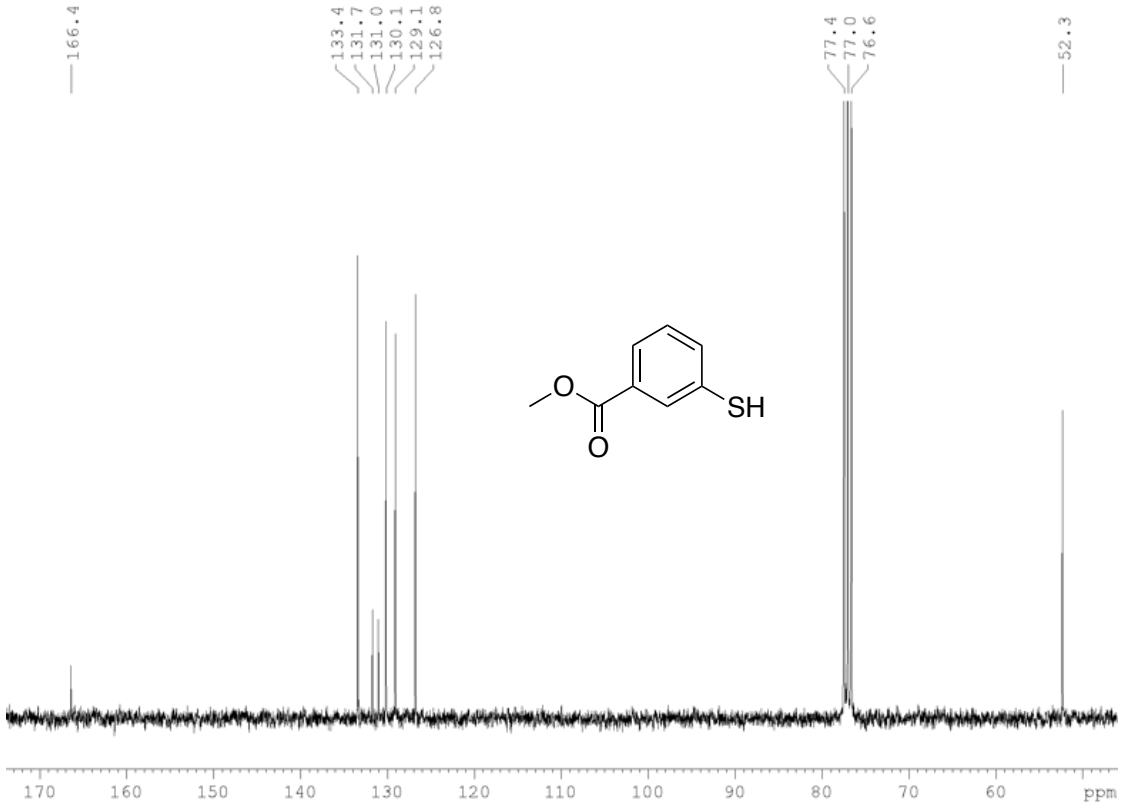


Compound 26 ($CDCl_3$)

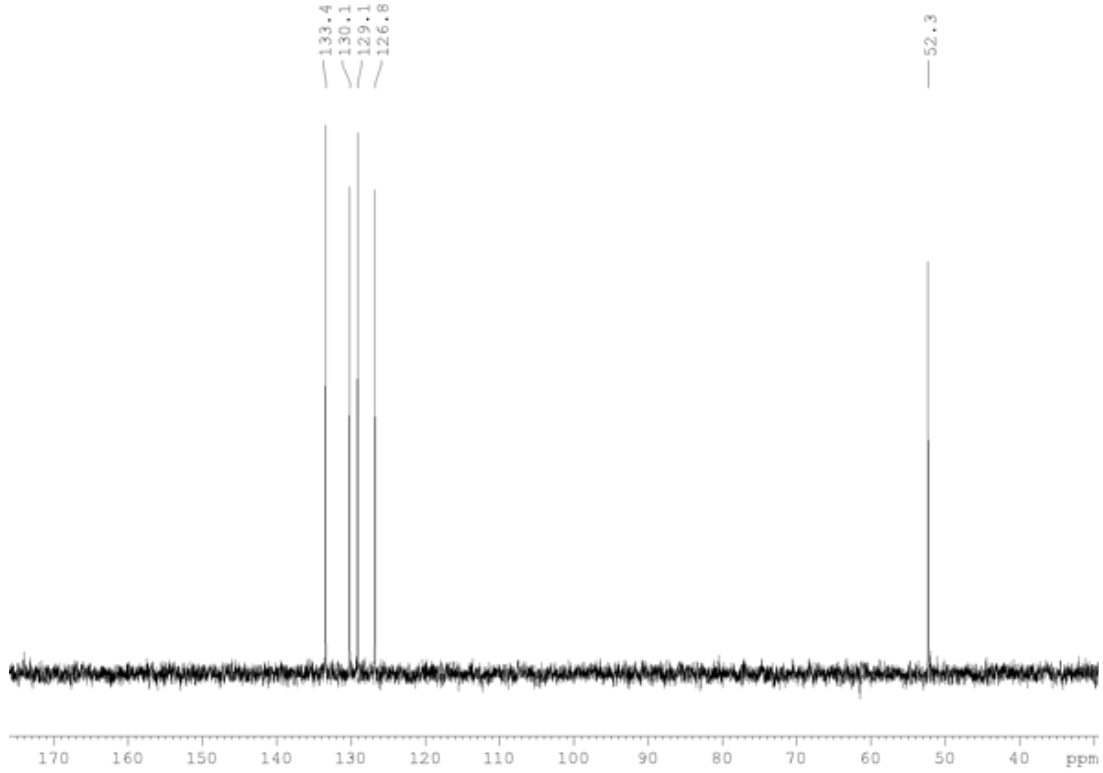
^1H



^{13}C


166.4
133.4
131.7
131.0
130.1
129.1
126.8
77.4
77.0
76.6
52.3
O
O
SH
170
160
150
140
130
120
110
100
90
80
70
60
ppm

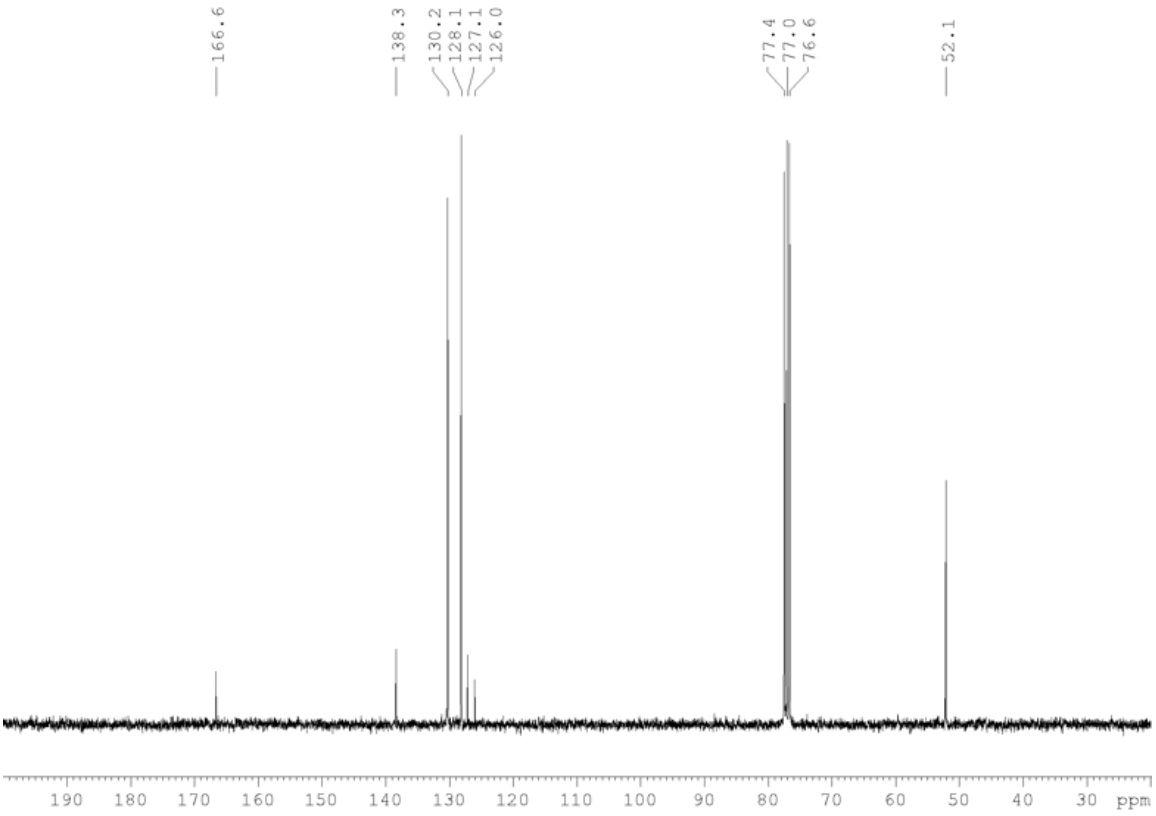
DEPT


133.4
130.1
129.1
126.8
52.3
170
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm

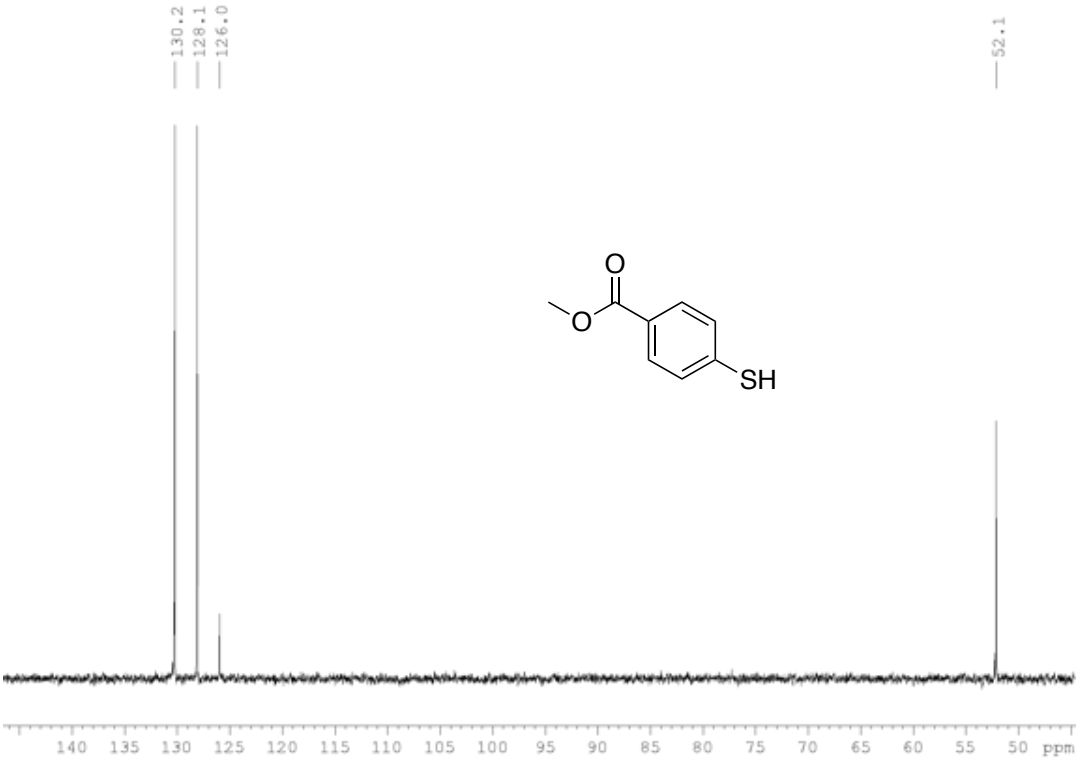
Compound 27 ($CDCl_3$)

^1H

^{13}C

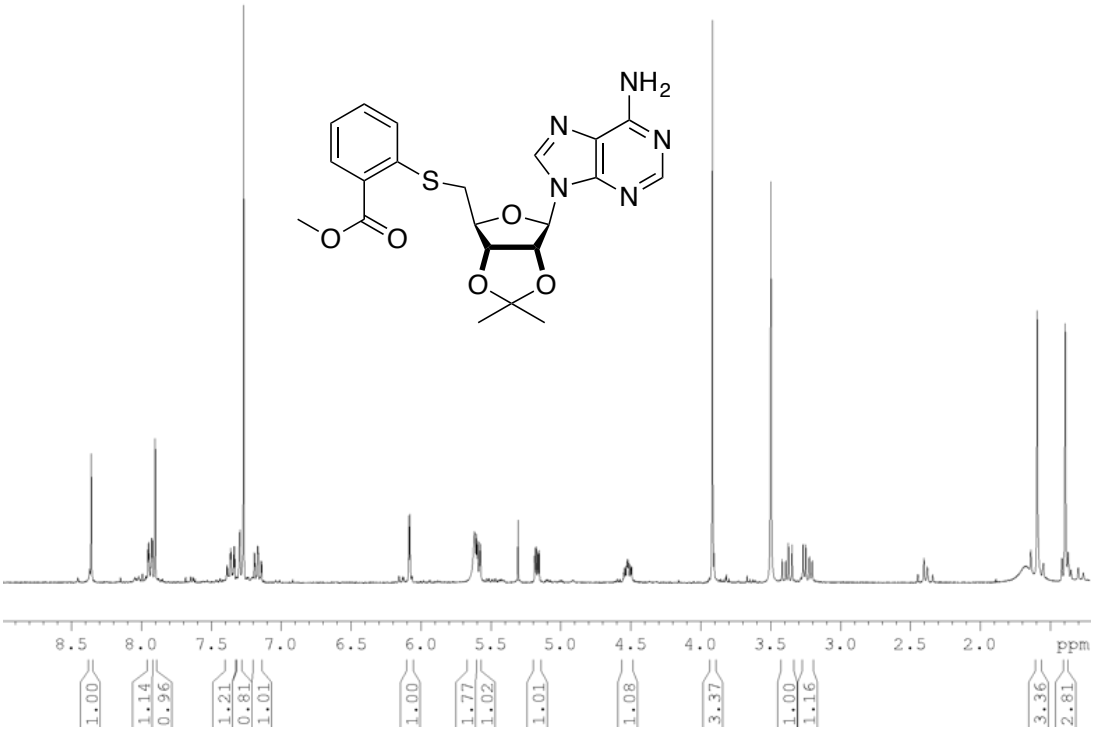


DEPT



Compound 28 ($CDCl_3$)

^1H



Compound 29 ($CDCl_3$)

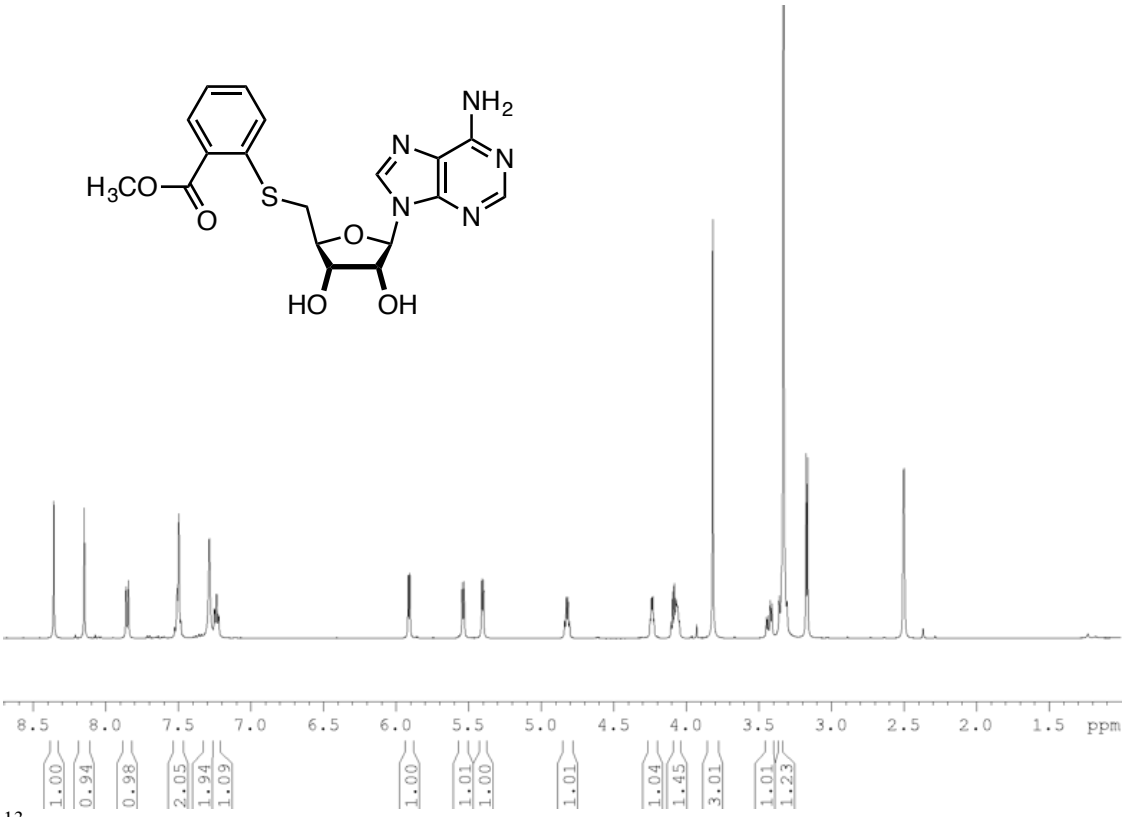
^1H

Compound 30 ($CDCl_3$)

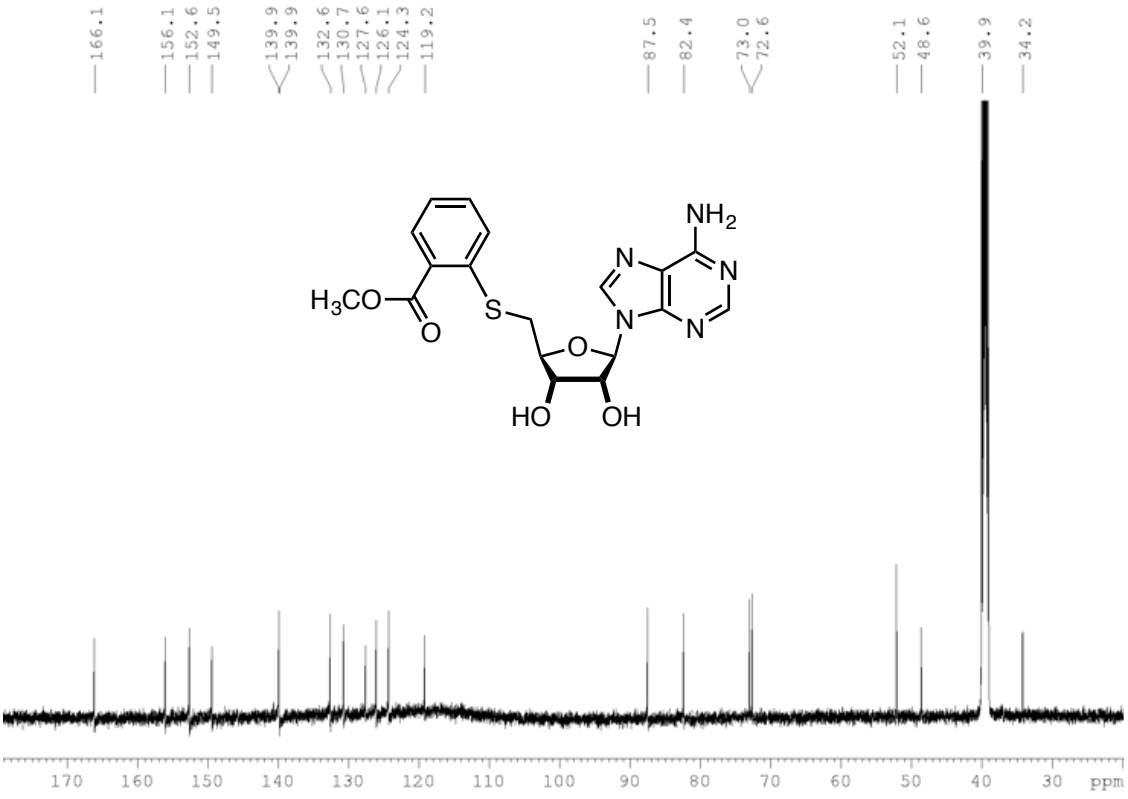
^1H

Compound 1 (DMSO)

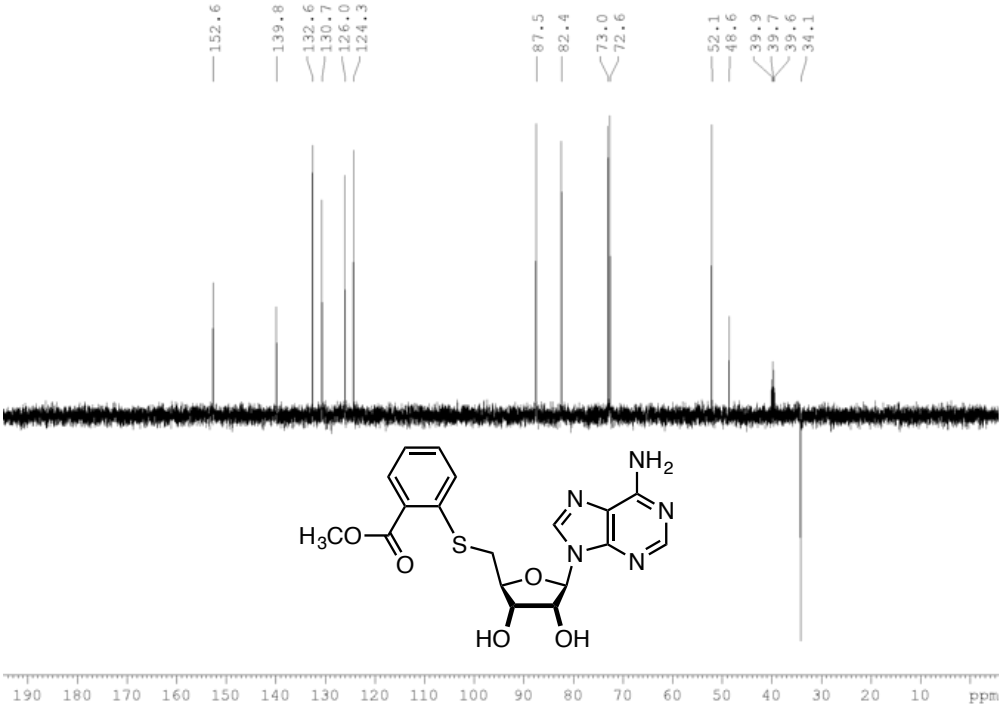
^{1}H



^{13}C

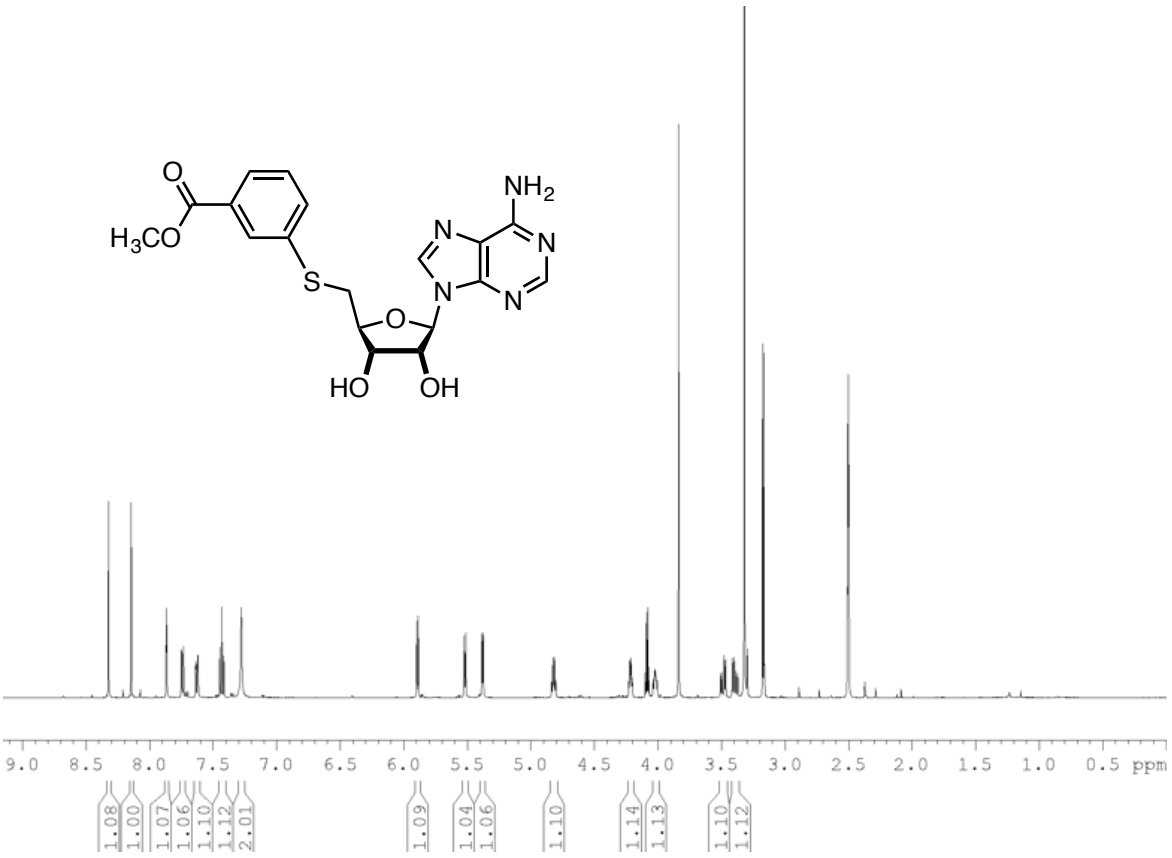


DEPT

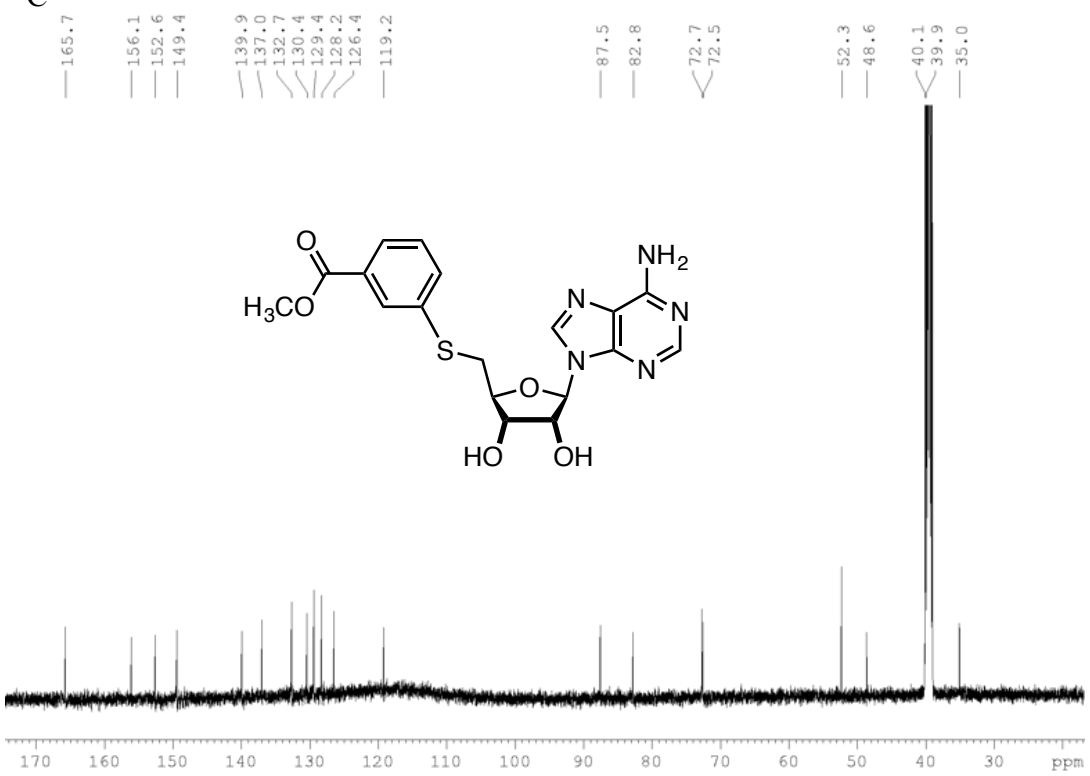


Compound 2 (DMSO)

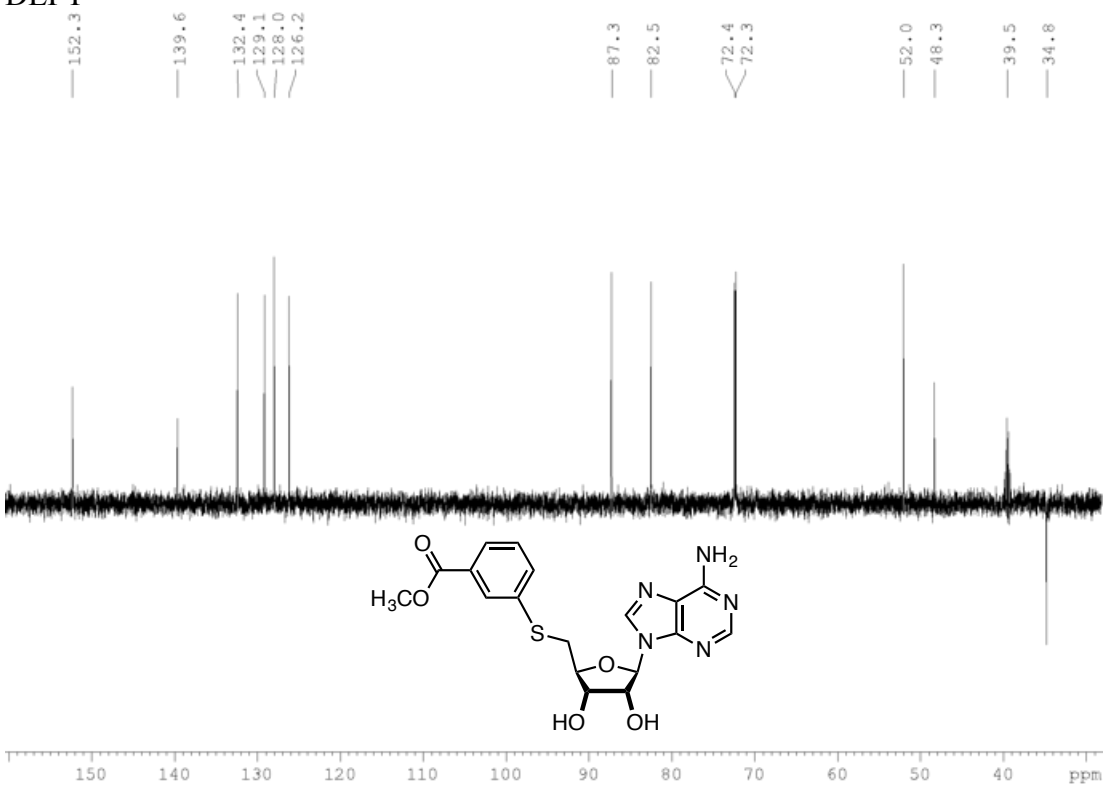
^{1}H



^{13}C

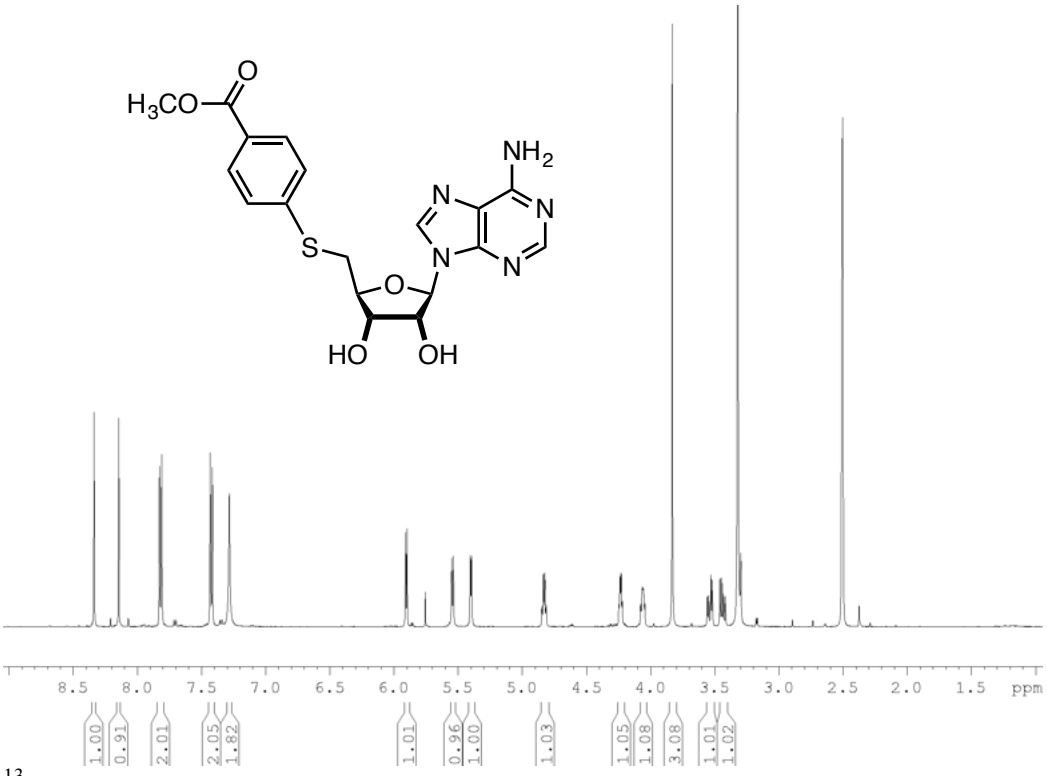

165.7
156.1
152.6
149.4
139.9
137.0
132.7
130.4
129.4
128.2
126.4
119.2
87.5
82.8
72.7
72.5
52.3
48.6
40.1
39.9
35.0
O
H3CO
NH2
N
N
S
N
N
O
HO
OH
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm

DEPT

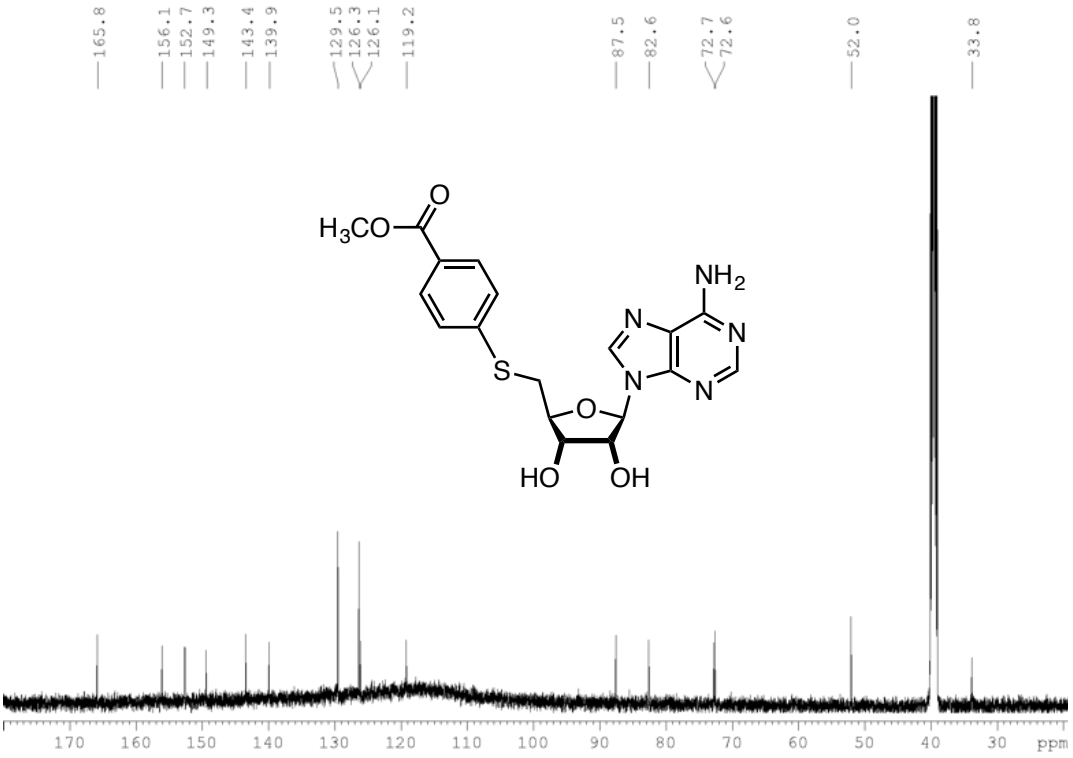

152.3
139.6
132.4
129.1
128.0
126.2
87.3
82.5
72.4
72.3
52.0
48.3
39.5
34.8
O
H3CO
NH2
N
N
S
N
N
O
HO
OH
150
140
130
120
110
100
90
80
70
60
50
40
ppm

Compound 3 (DMSO)

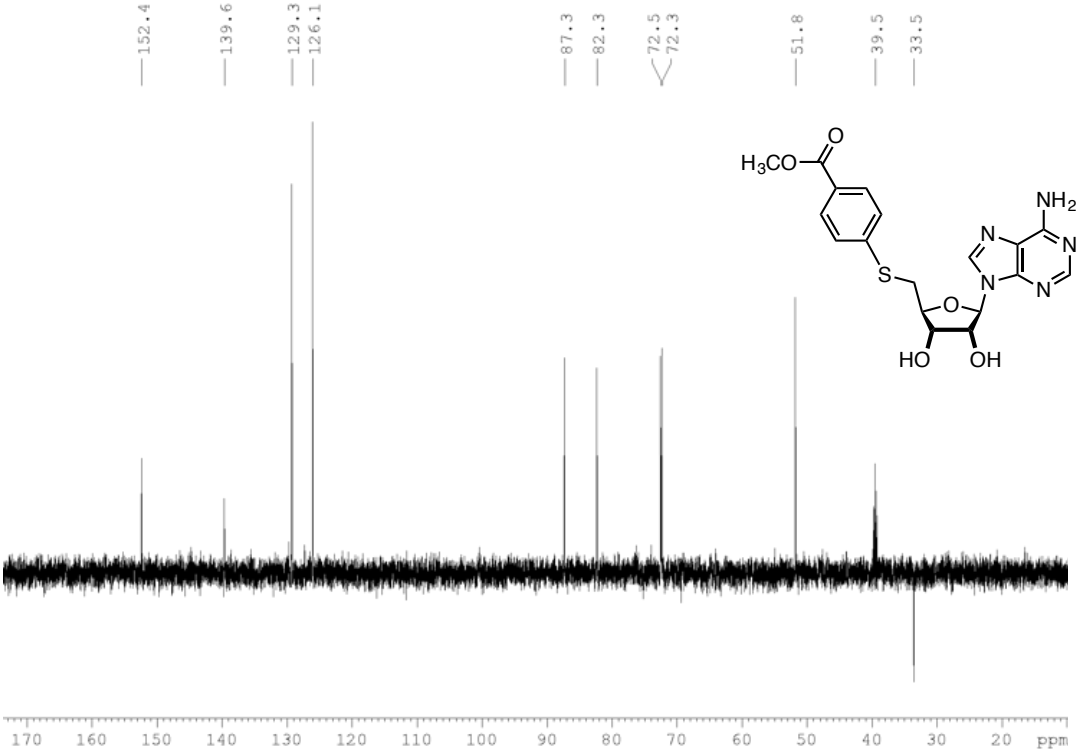
^{1}H



^{13}C

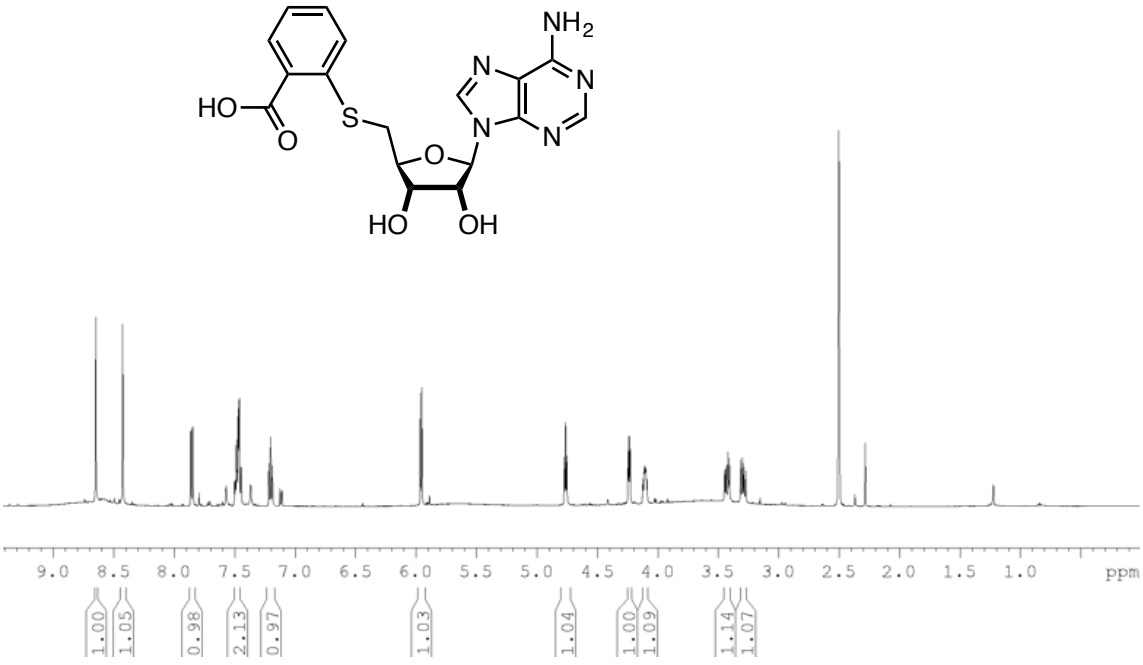


DEPT

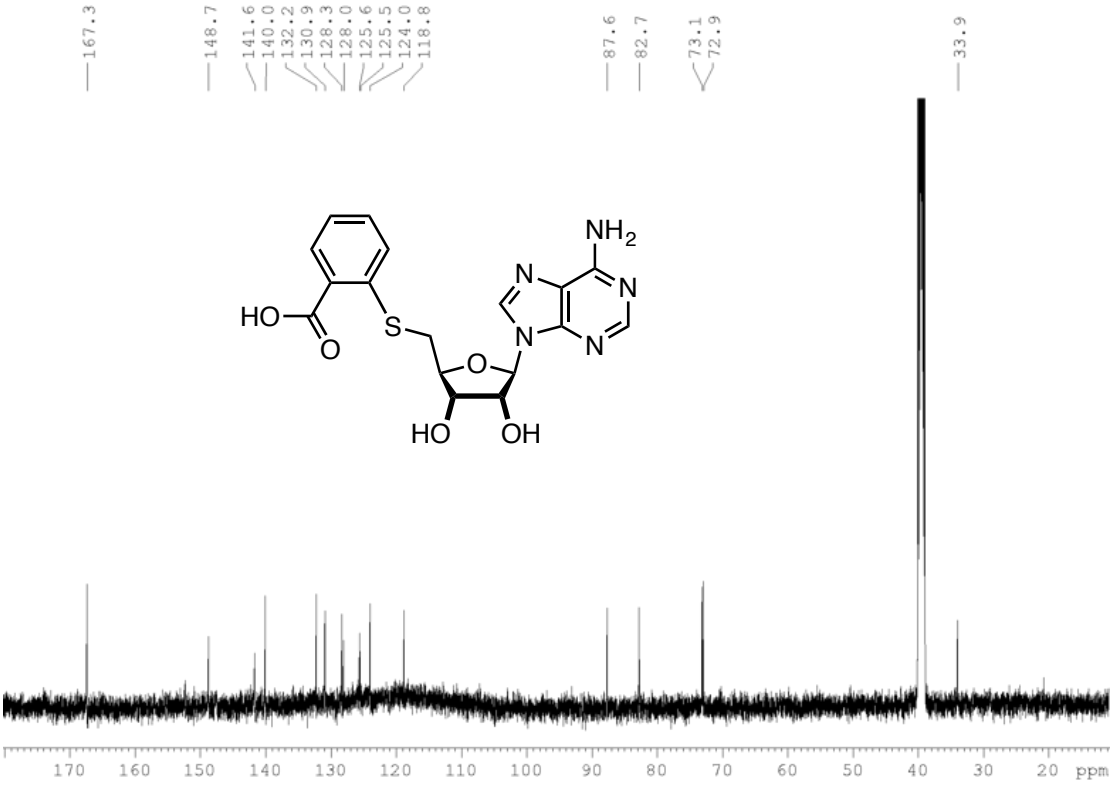


Compound 4 (DMSO)

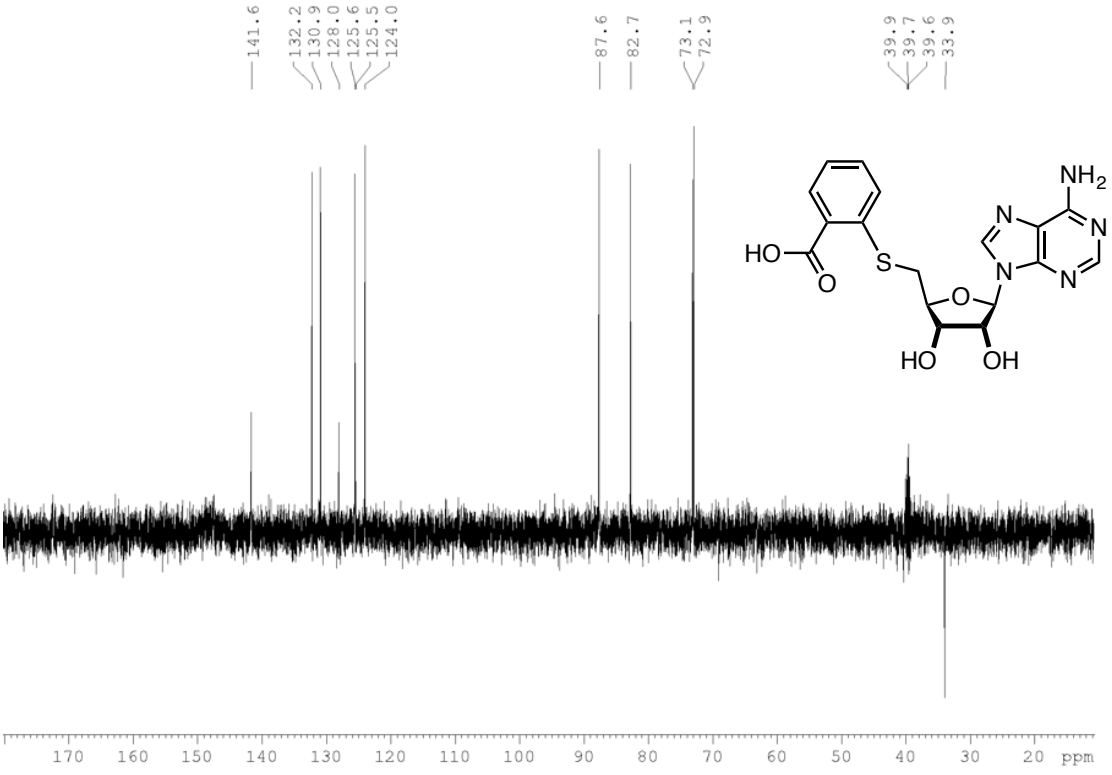
^{1}H



^{13}C

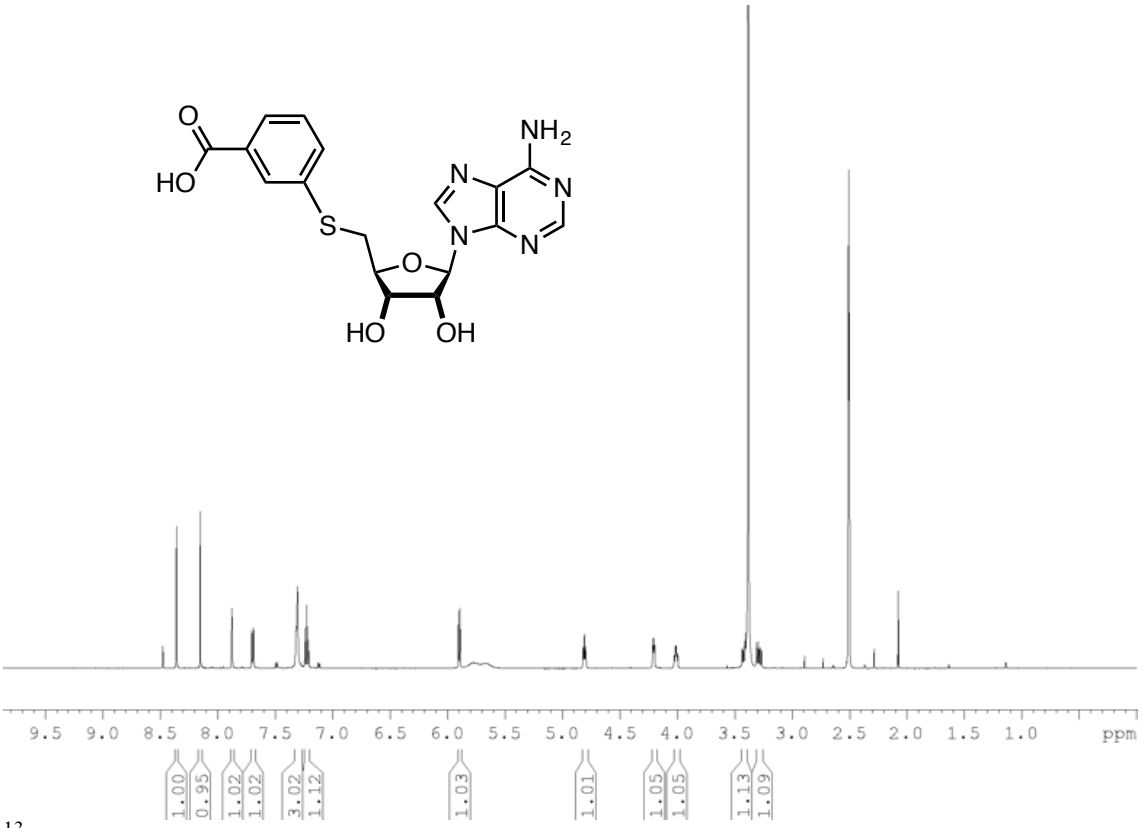

167.3
148.7
141.6
140.0
132.2
130.9
128.3
128.0
125.6
125.5
124.0
118.8
87.6
82.7
73.1
72.9
33.9
NH2
N
N
N
N
HO
O
S
O
HO
OH
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

DEPT

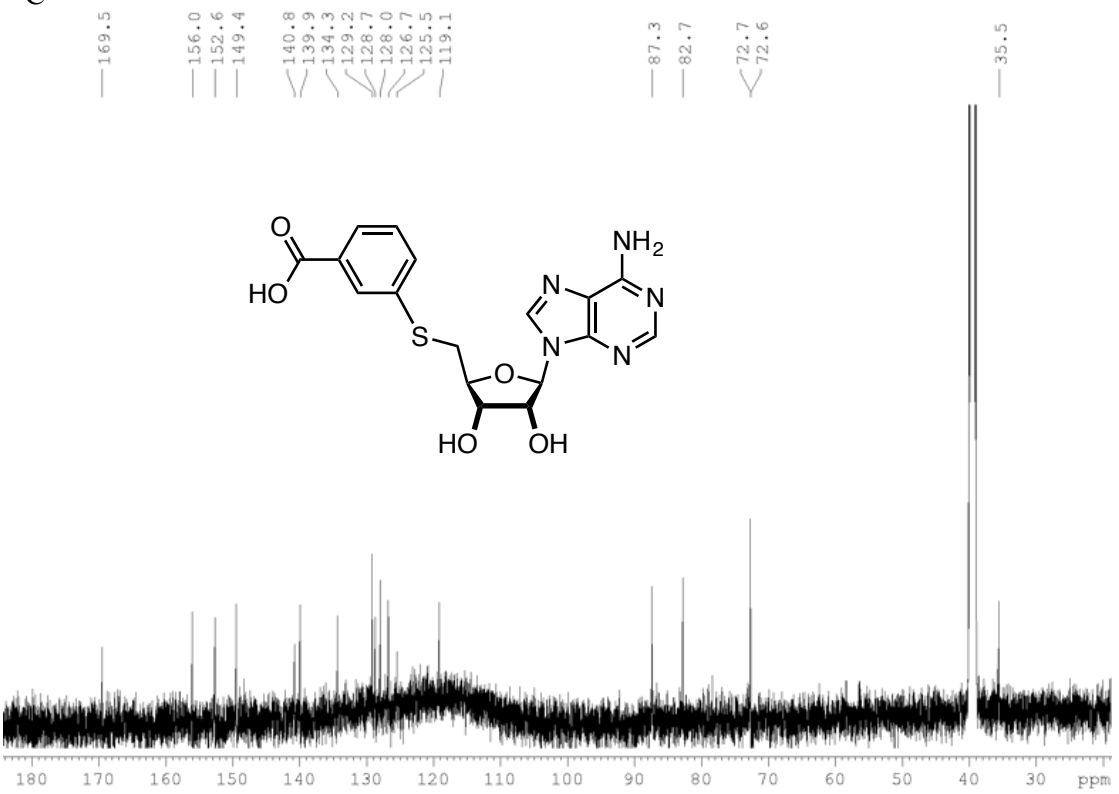

141.6
132.2
130.9
128.0
125.6
125.5
124.0
87.6
82.7
73.1
72.9
39.9
39.7
39.6
33.9
NH2
N
N
N
N
HO
O
S
O
HO
OH
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

Compound 5 (DMSO)

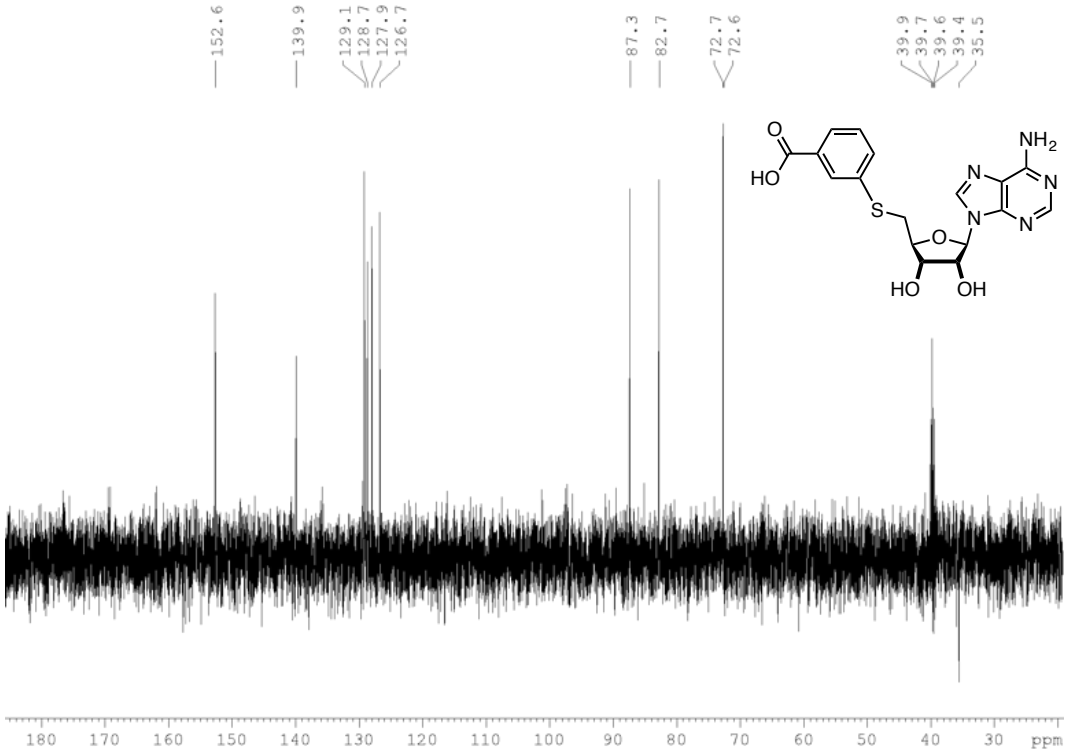
^{1}H



^{13}C

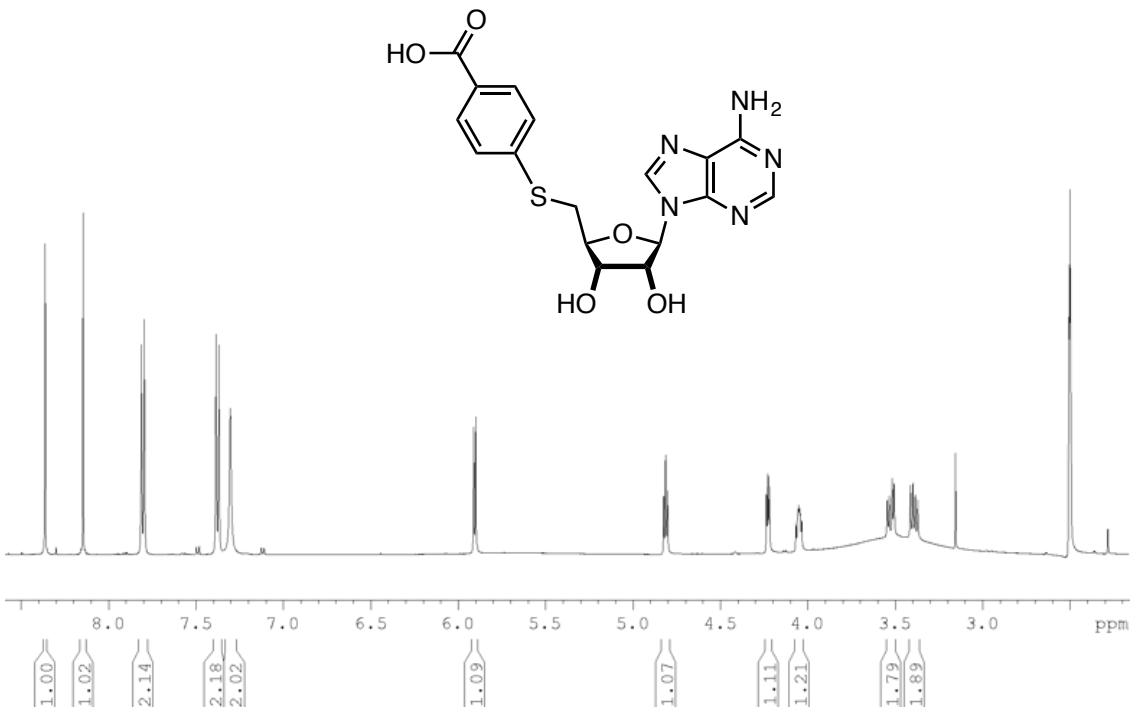


DEPT

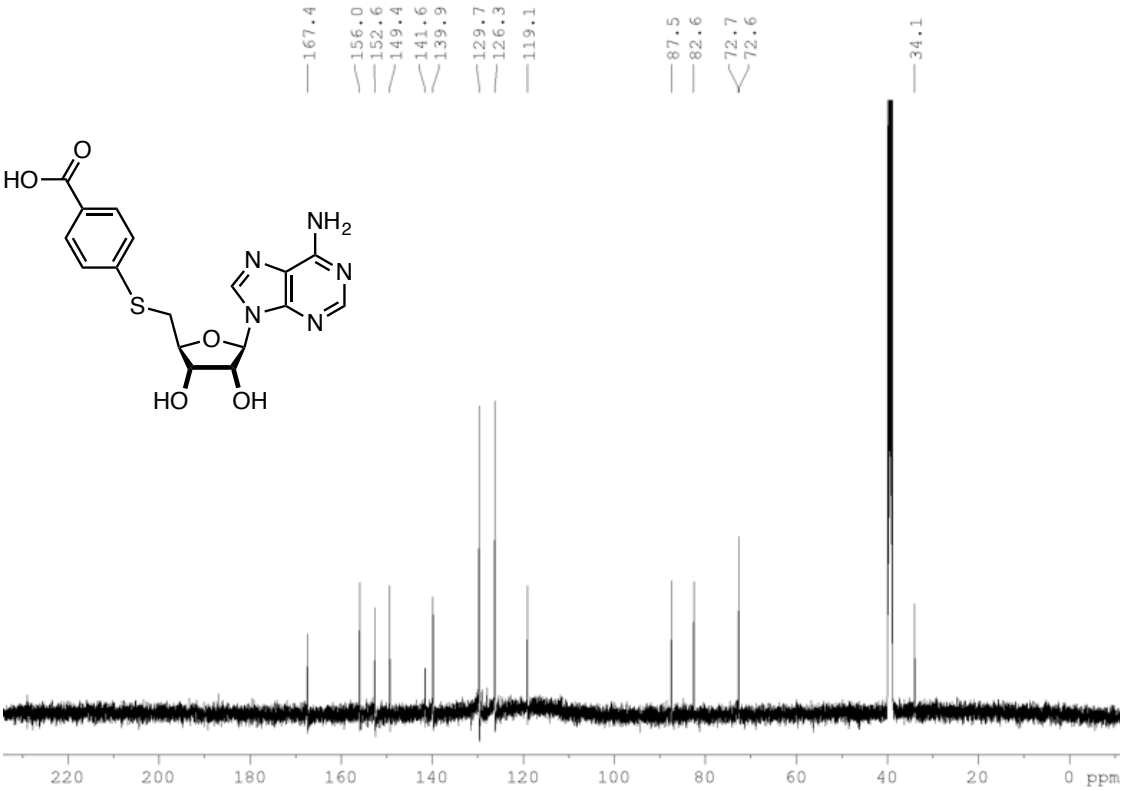


Compound 6 (DMSO)

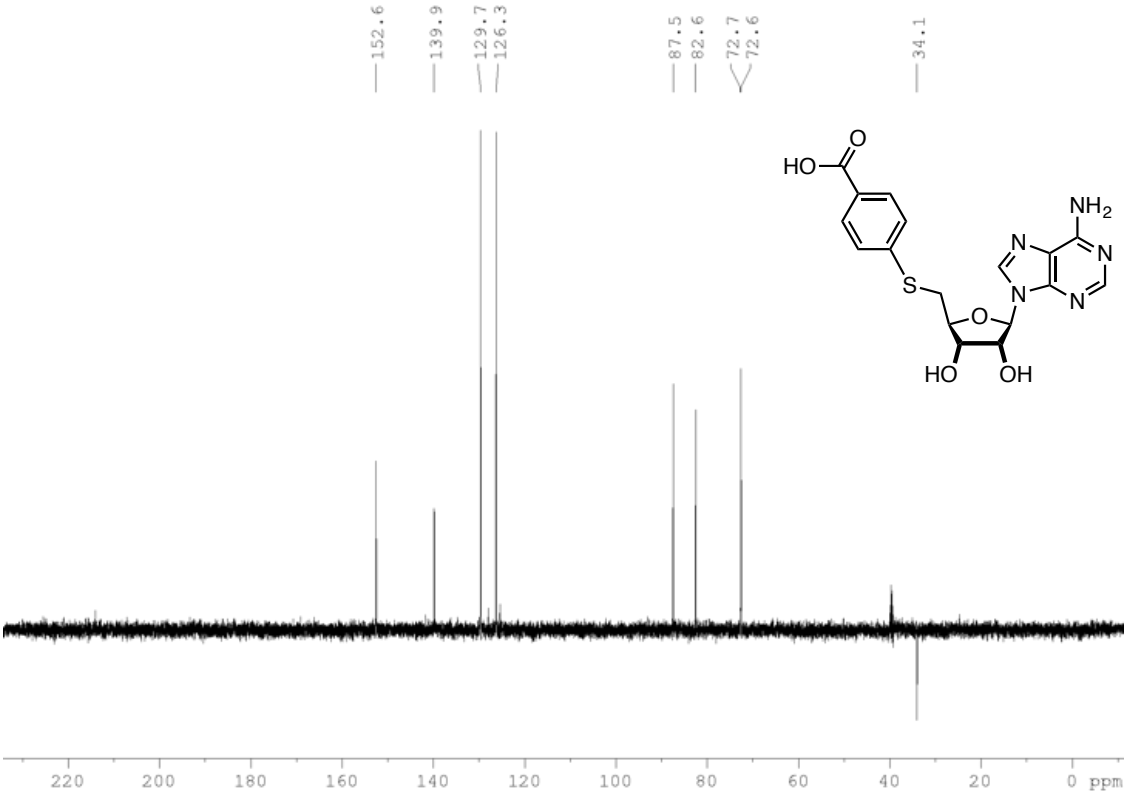
^{1}H



^{13}C

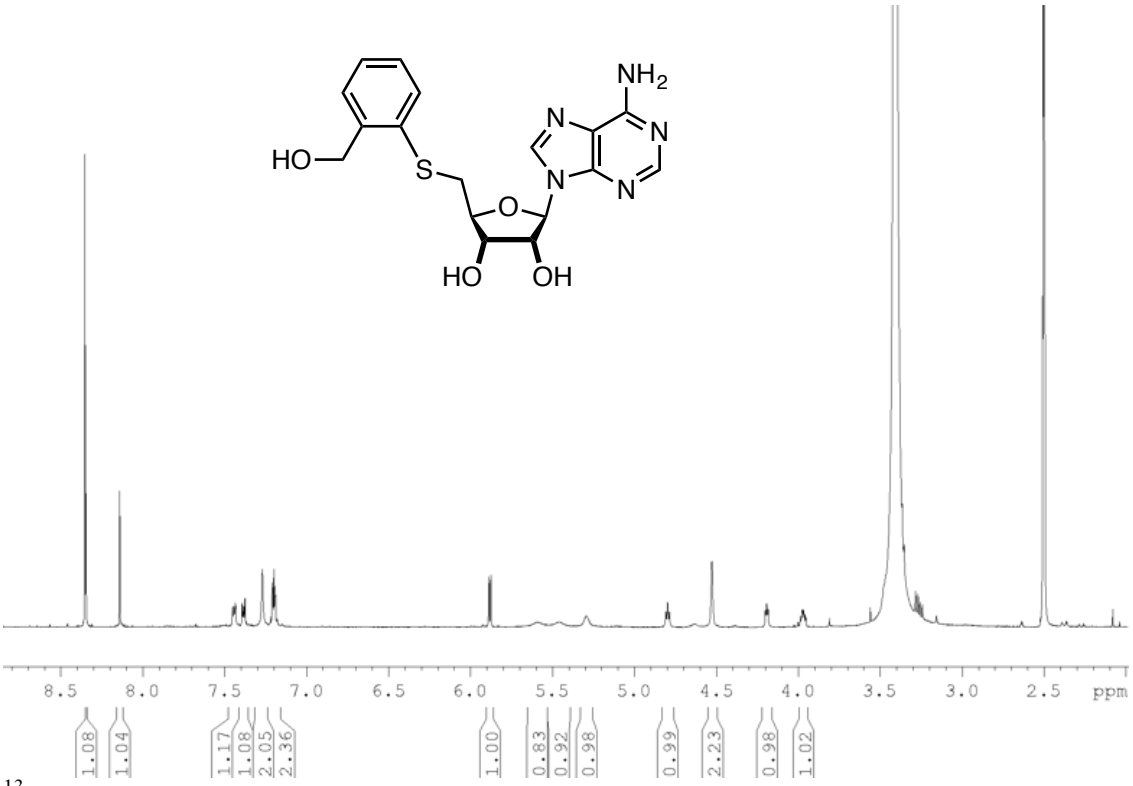

167.4
156.0
152.6
149.4
141.6
139.9
129.7
126.3
119.1
87.5
82.6
72.7
72.6
34.1
HO
O
NH2
N
N
N
N
S
O
HO
OH
220
200
180
160
140
120
100
80
60
40
20
0 ppm

DEPT

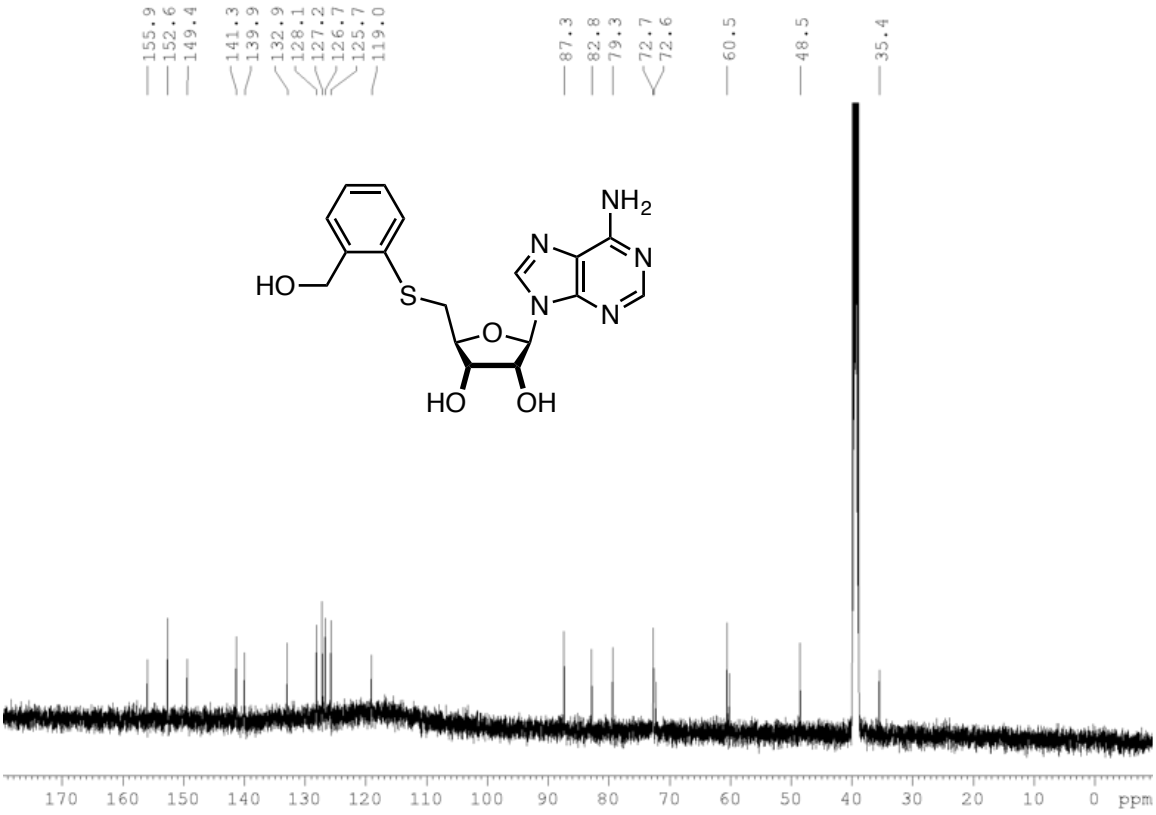

152.6
139.9
129.7
126.3
87.5
82.6
72.7
72.6
34.1
HO
O
NH2
N
N
N
N
S
O
HO
OH
220
200
180
160
140
120
100
80
60
40
20
0 ppm

Compound 7 (DMSO)

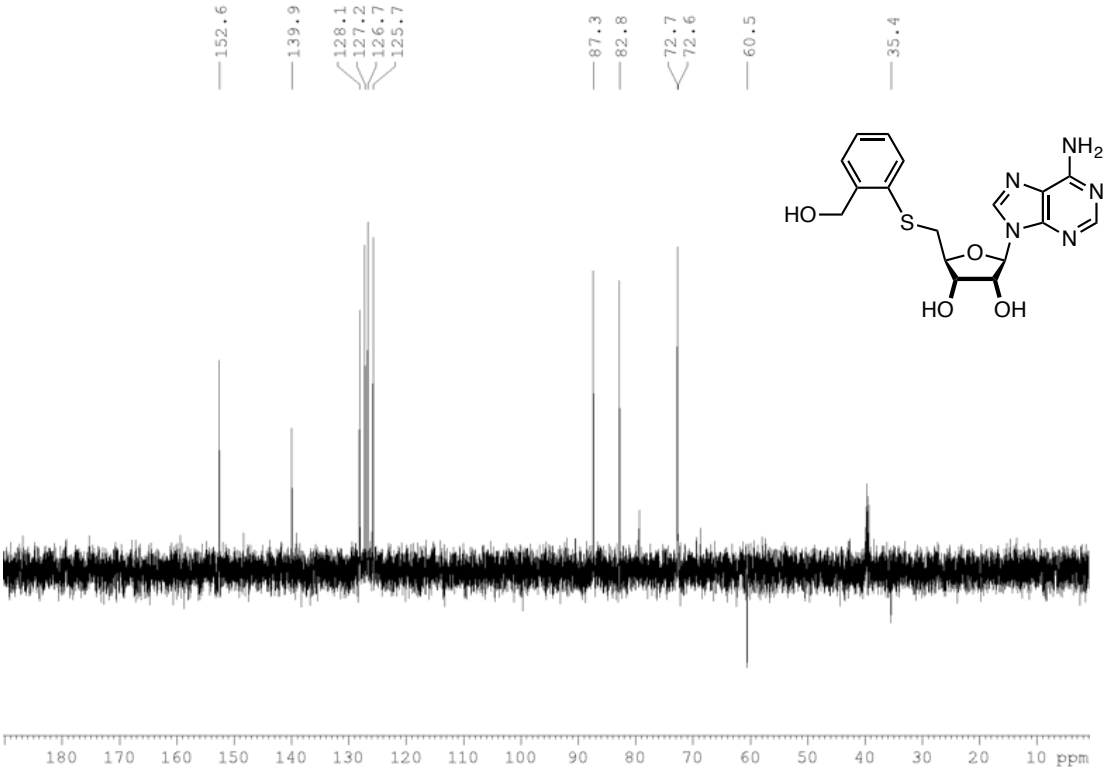
^{1}H



^{13}C

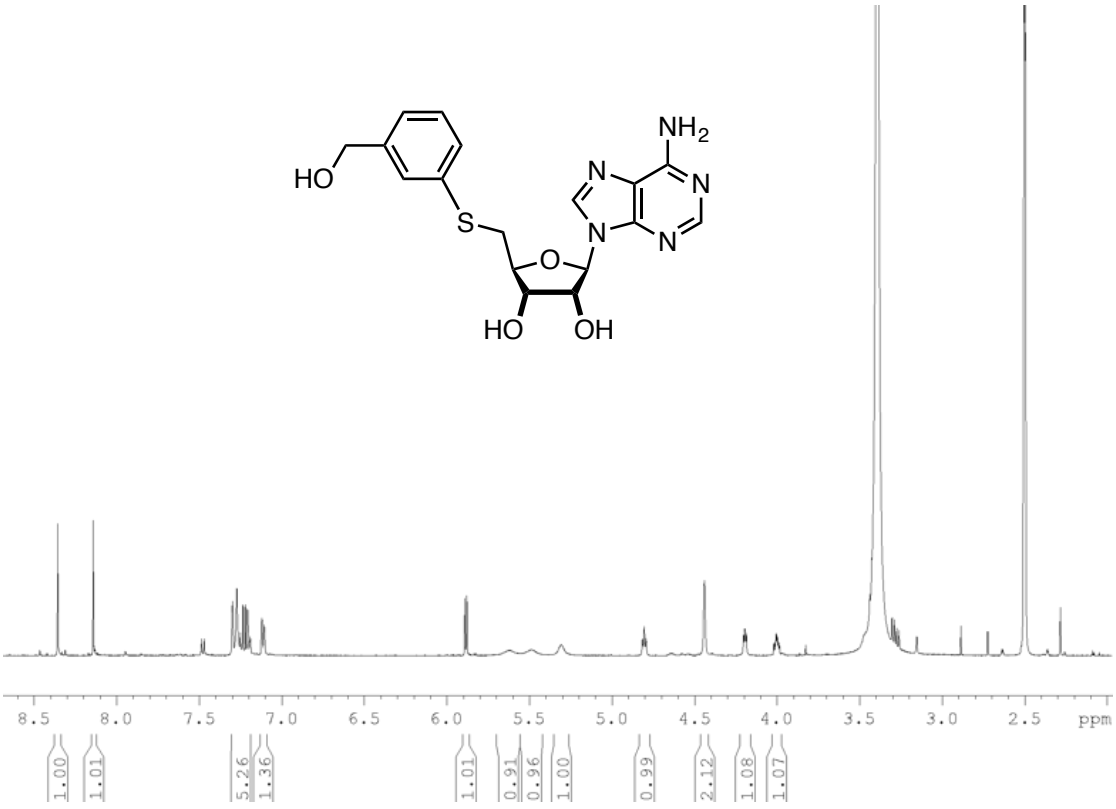


DEPT

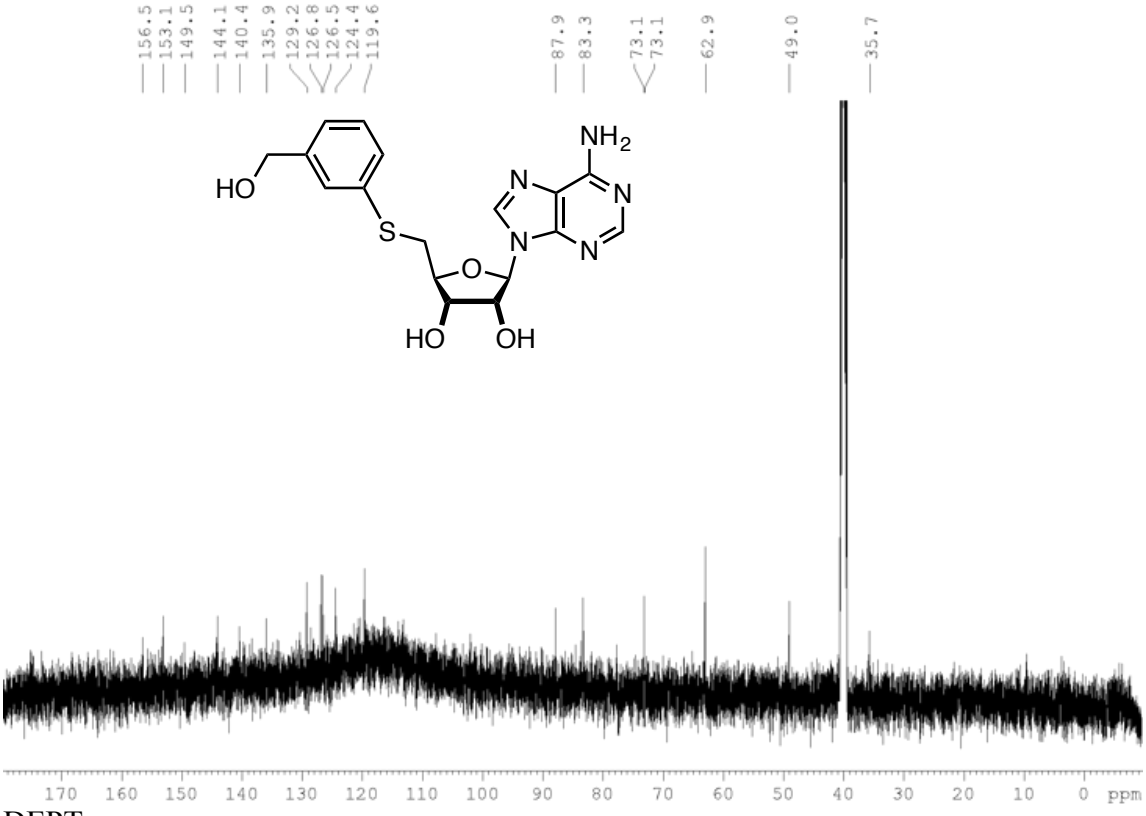


Compound 8 (DMSO)

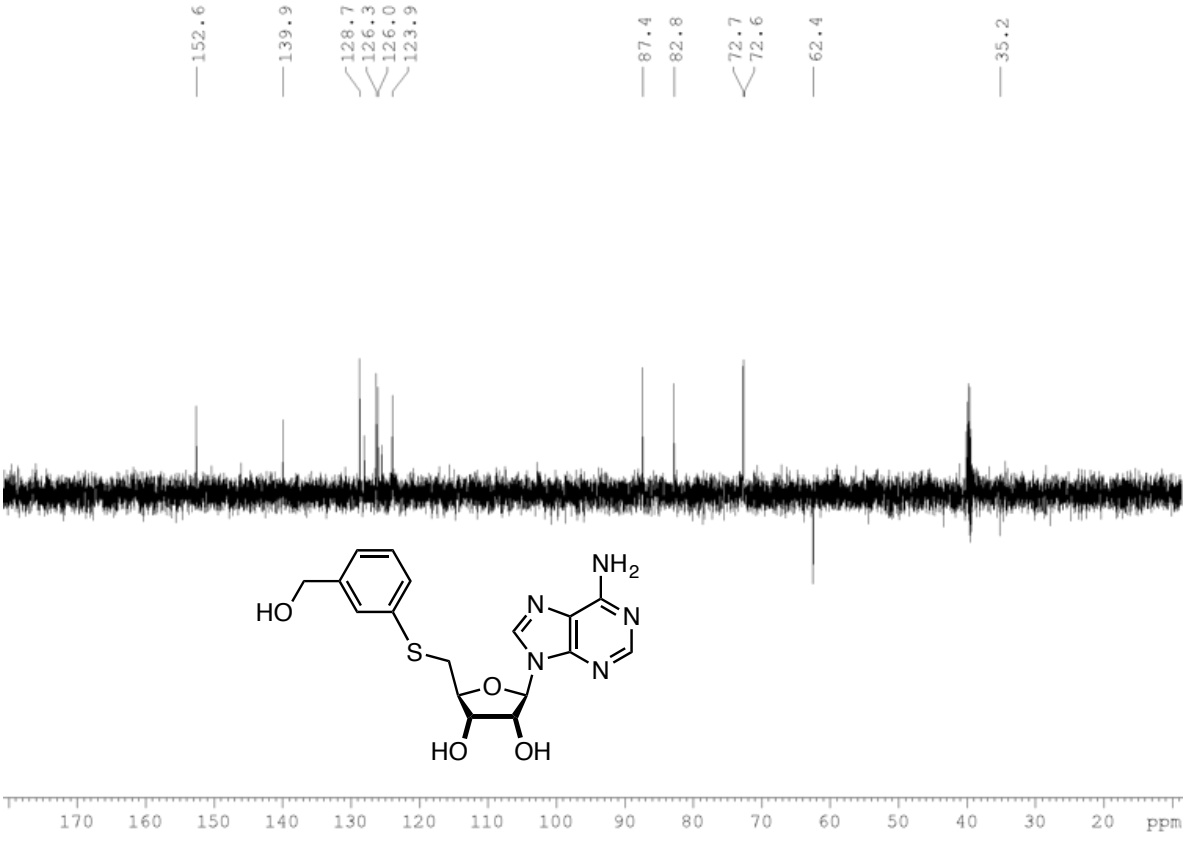
^{1}H



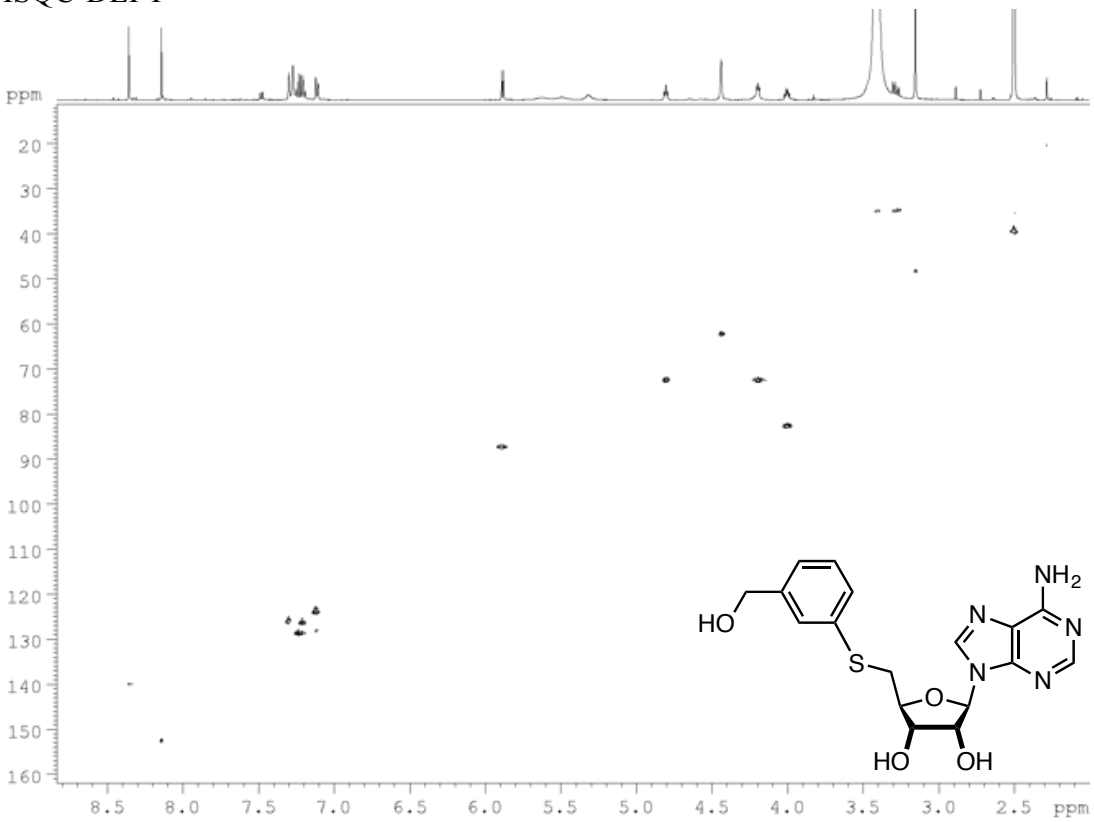
^{13}C


156.5
153.1
149.5
144.1
140.4
135.9
129.2
126.8
126.5
124.4
119.6
87.9
83.3
73.1
73.1
62.9
49.0
35.7
NH2
HO
N
N
N
N
S
O
HO
OH
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

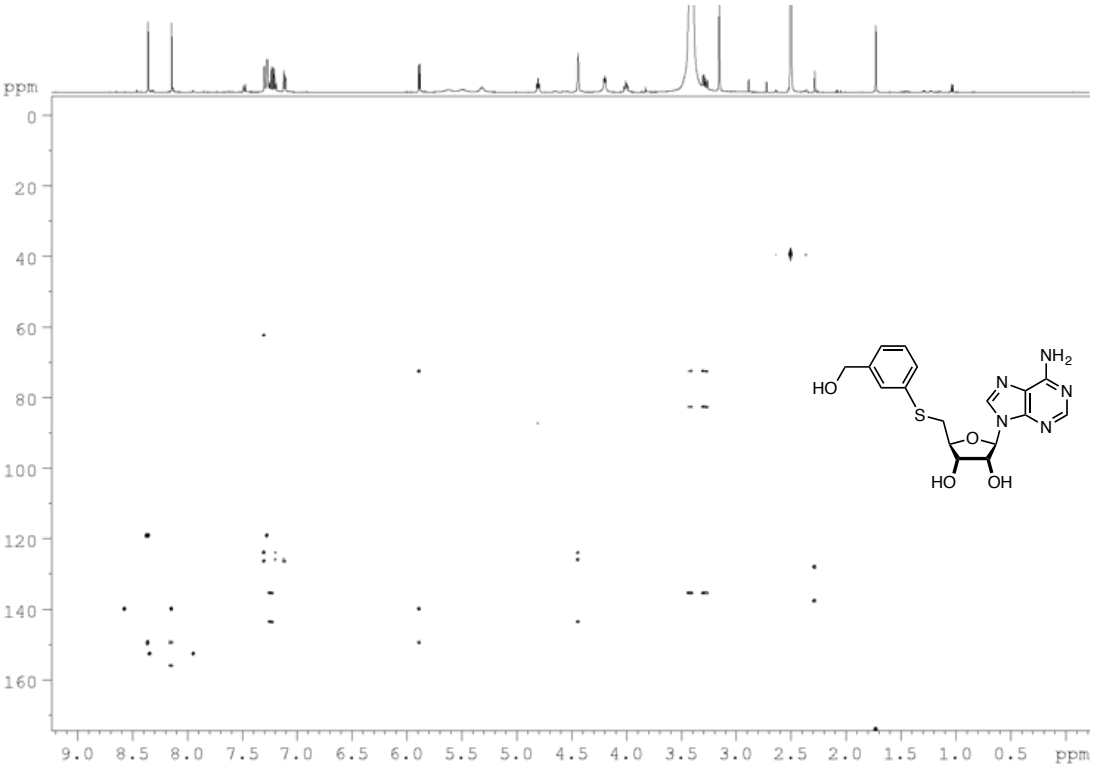
DEPT


152.6
139.9
128.7
126.3
126.0
123.9
87.4
82.8
72.7
72.6
62.4
35.2
NH2
HO
N
N
N
N
S
O
HO
OH
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

HSQC-DEPT

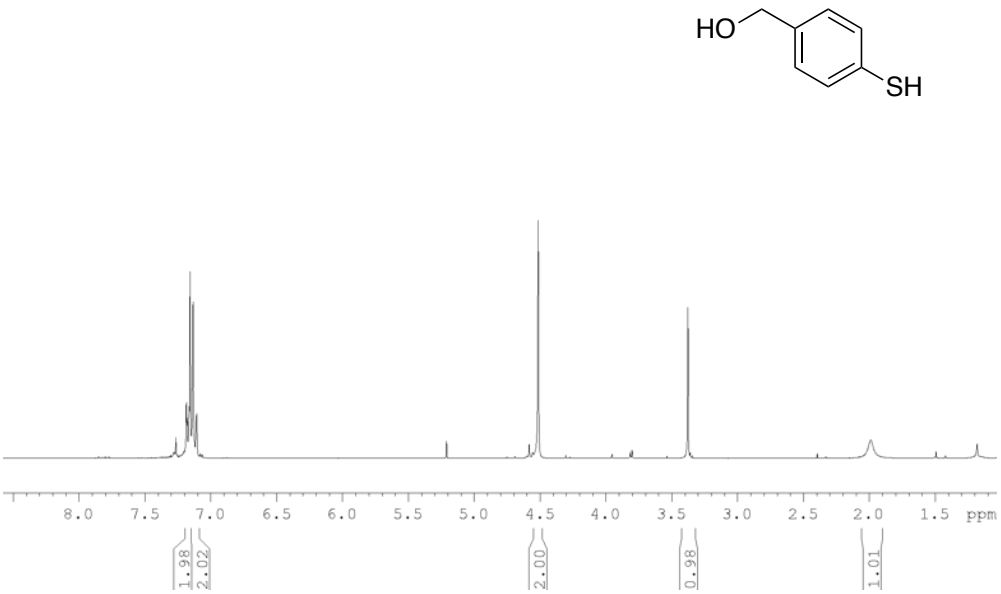


HMBC



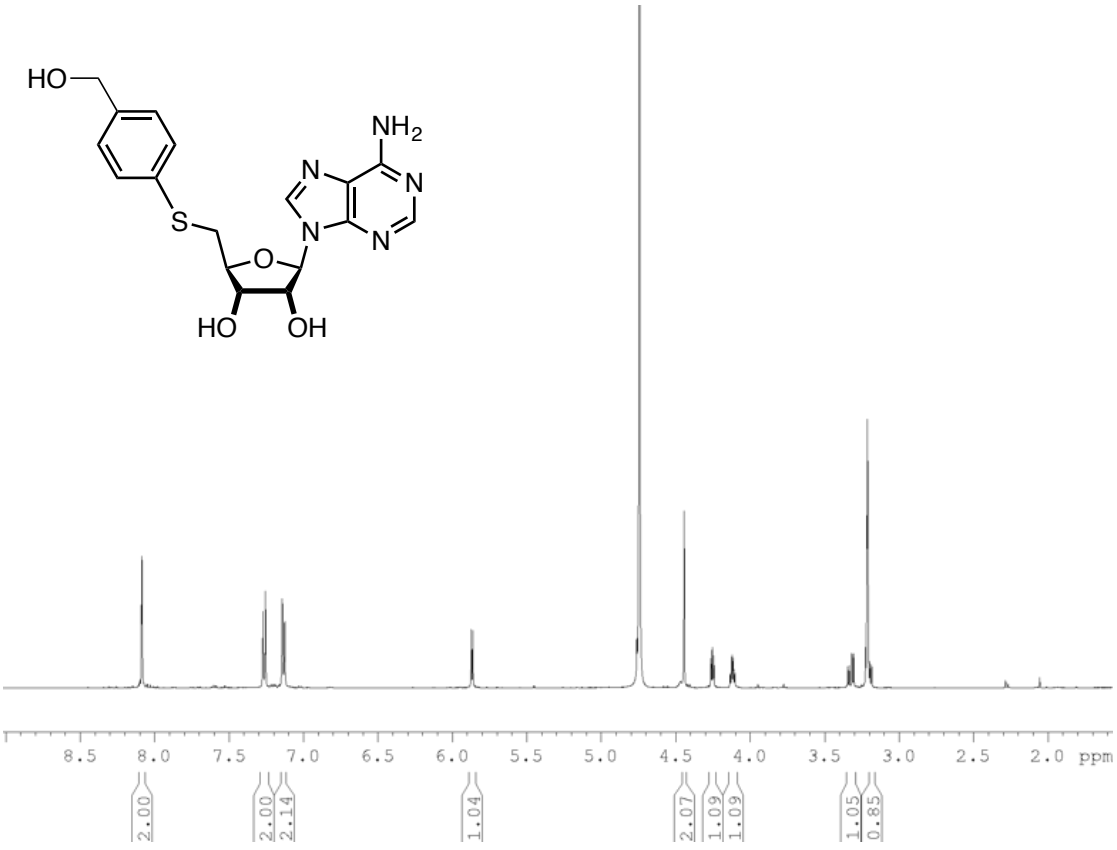
Compound 32 ($CDCl_3$)

^1H

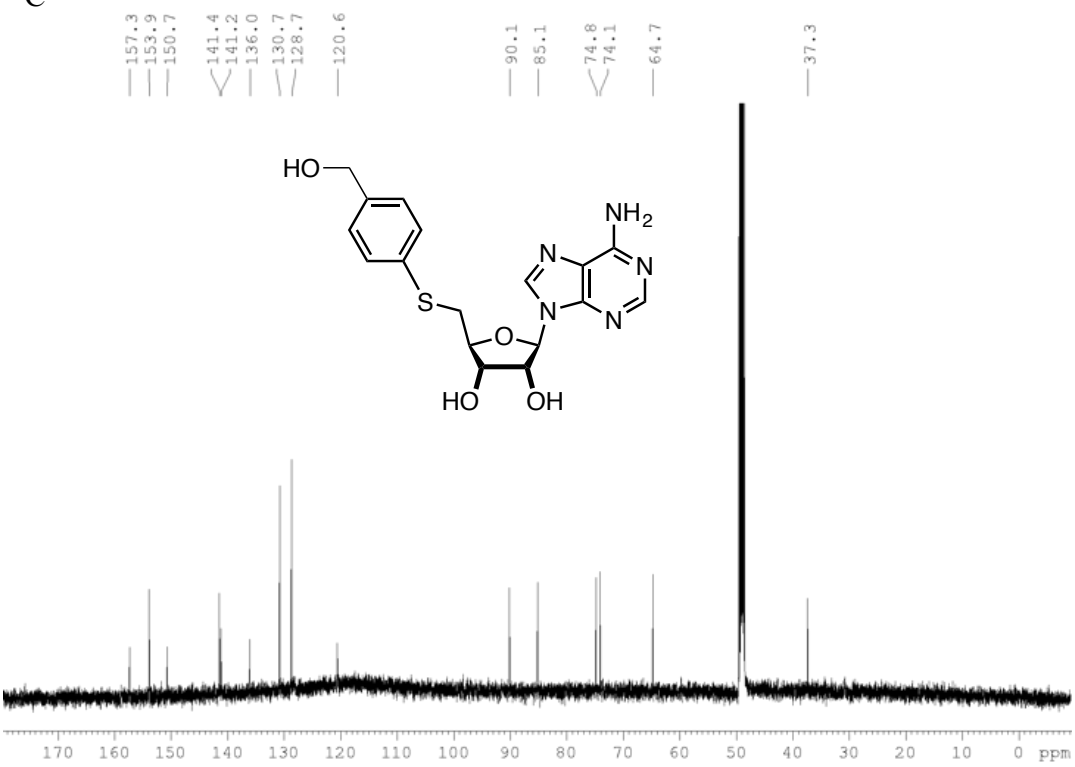


Compound 9 (MeOD)

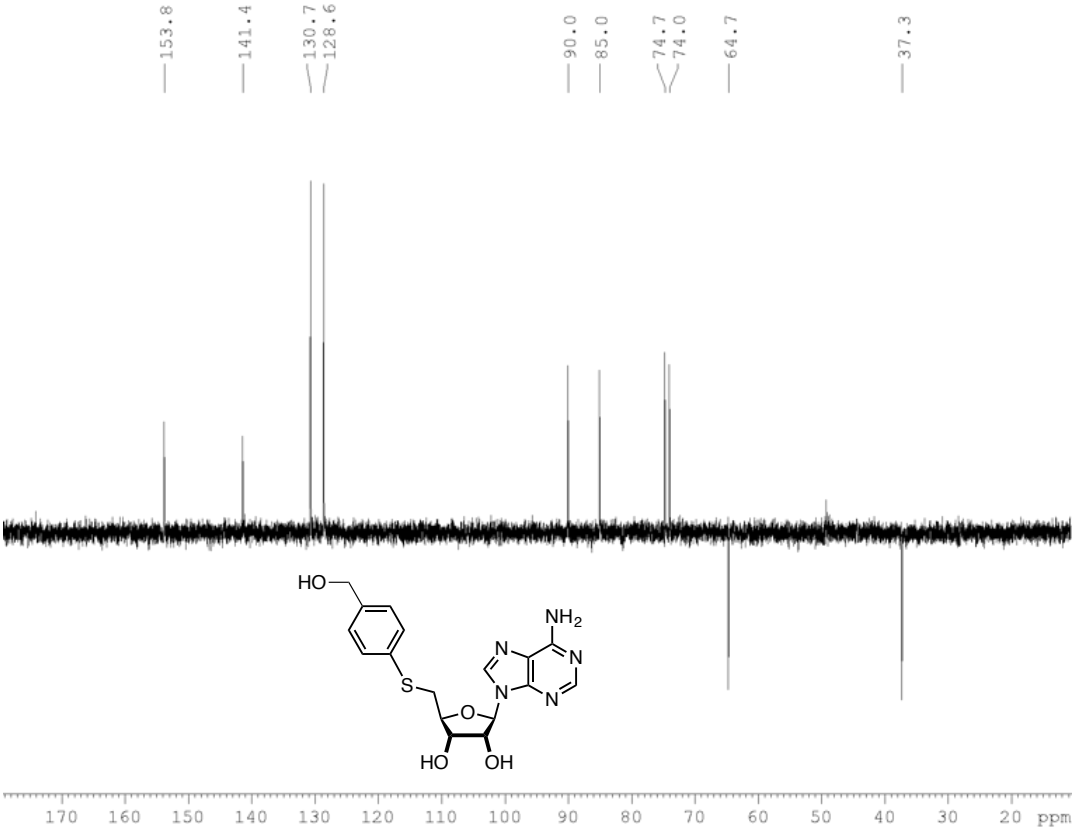
^1H



^{13}C

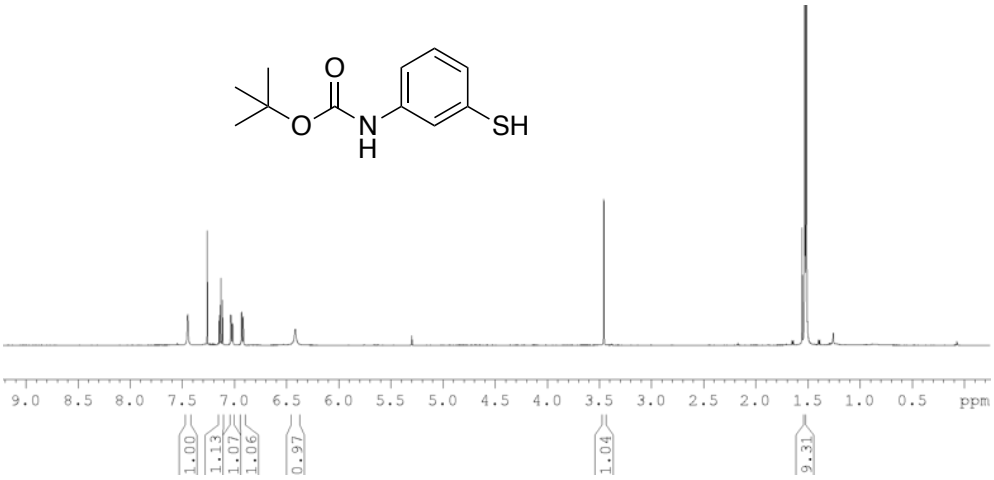

157.3
153.9
150.7
141.4
141.2
136.0
130.7
128.7
120.6
90.1
85.1
74.8
74.1
64.7
37.3
HO
NH2
N
N
S
N
N
O
HO
OH
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

DEPT

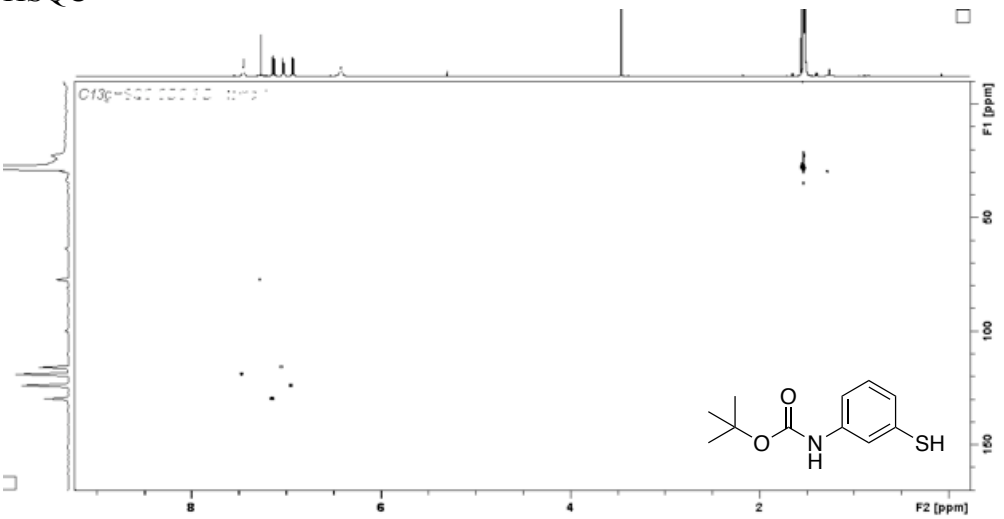

153.8
141.4
130.7
128.6
90.0
85.0
74.7
74.0
64.7
37.3
HO
NH2
N
N
S
N
N
O
HO
OH
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

Compound 35 ($CDCl_3$)

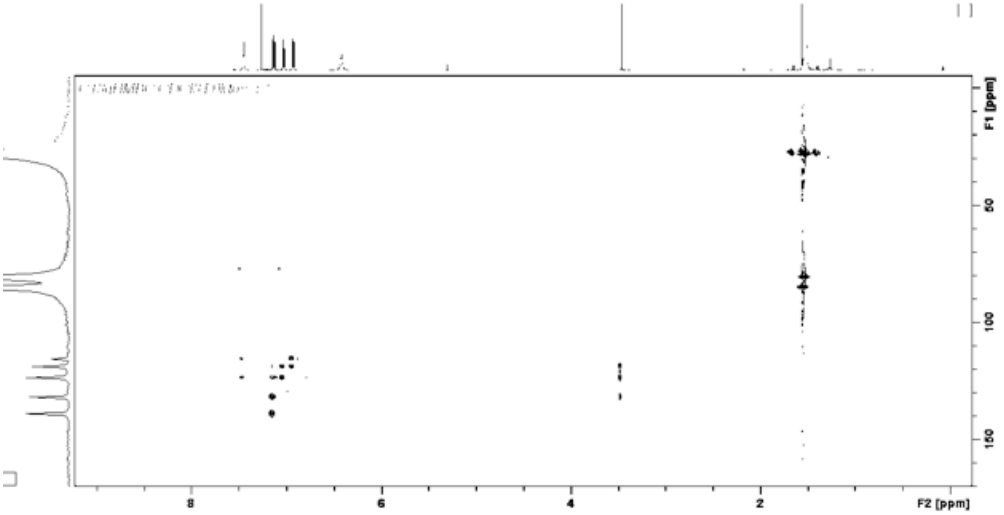
^1H



HSQC

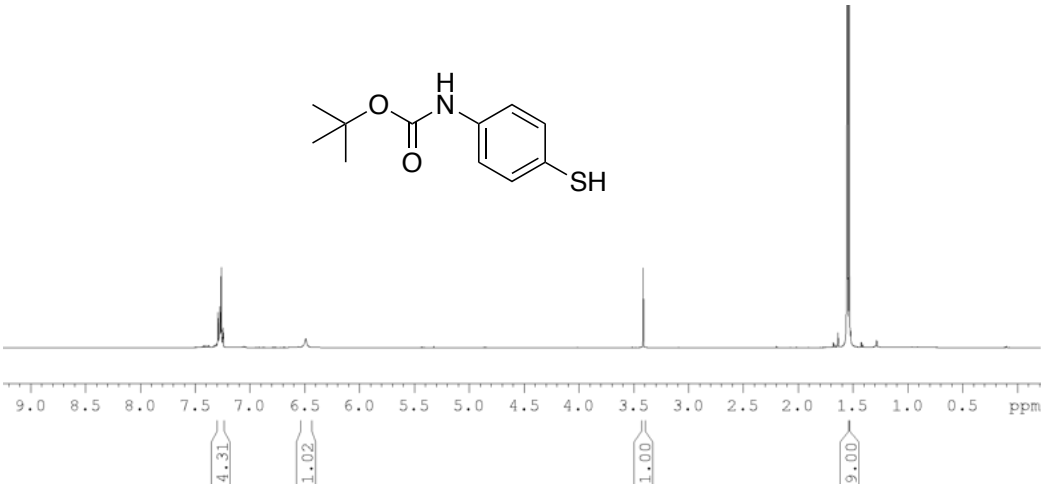


HMBC

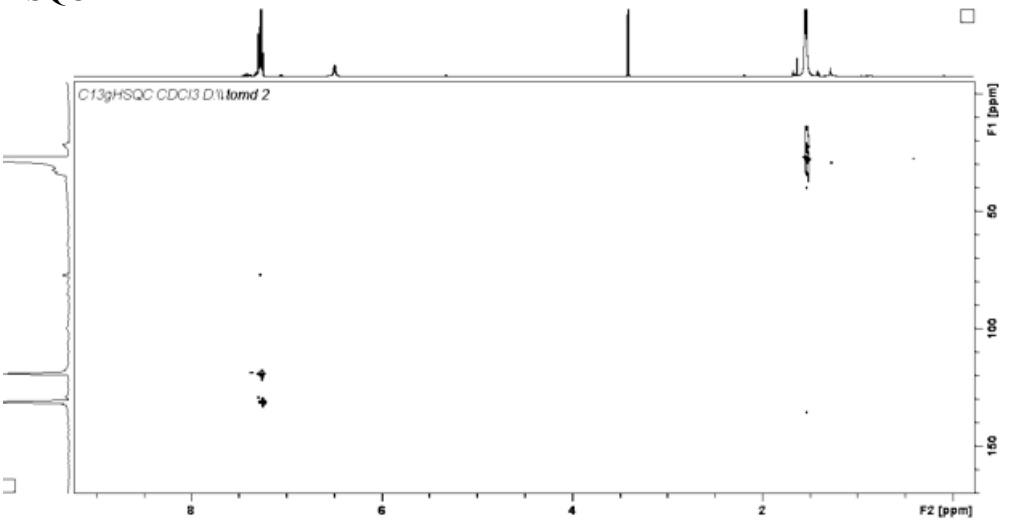


Compound 36 ($CDCl_3$)

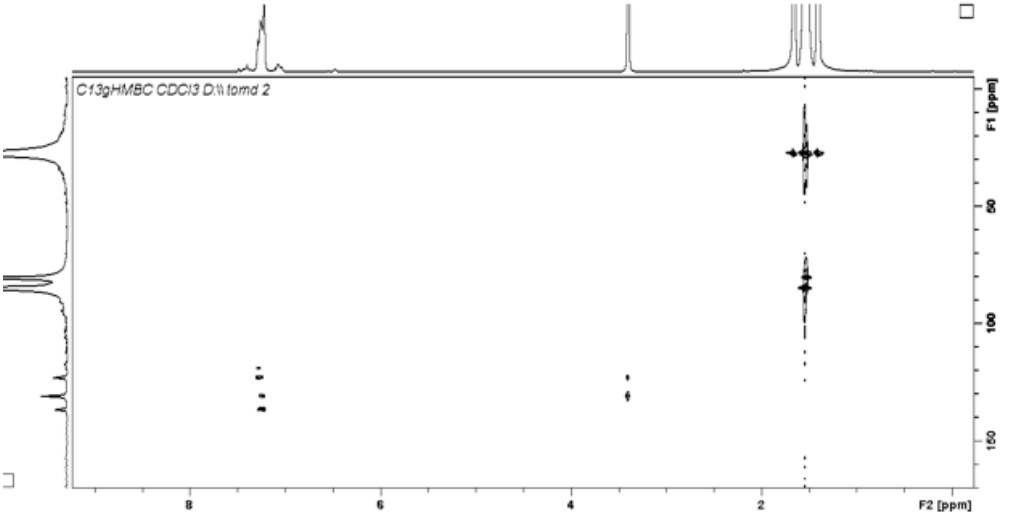
^1H



HSQC

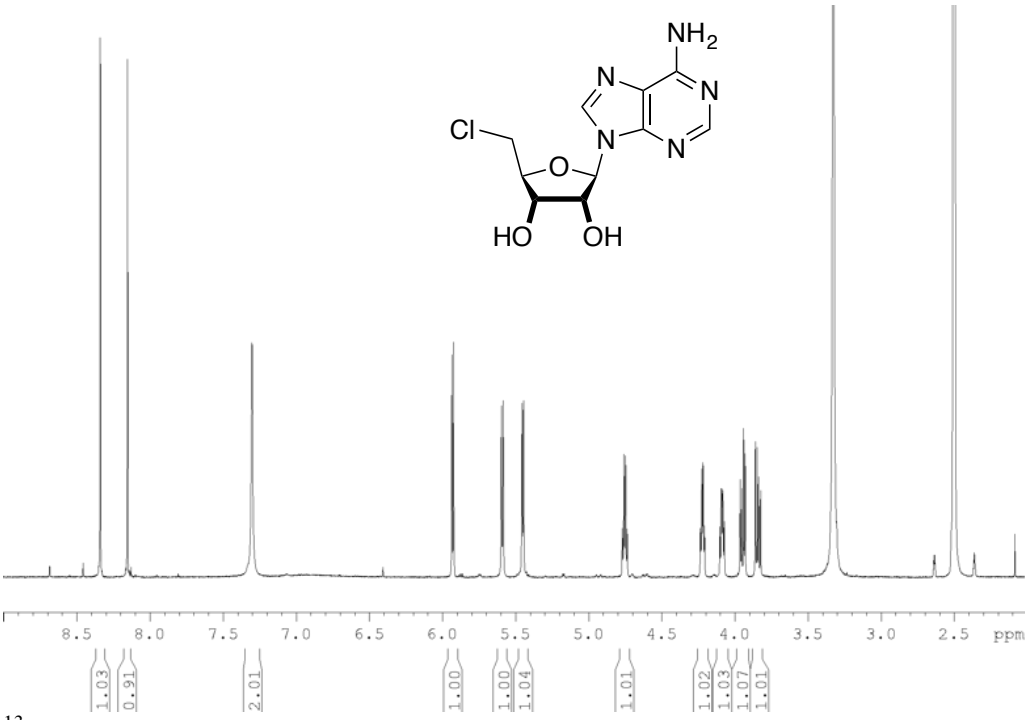


HMBC

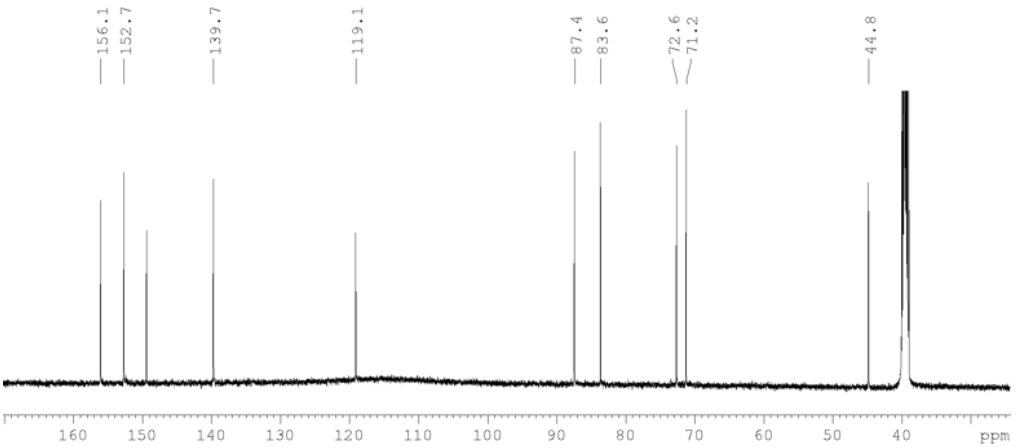


Compound 38 (DMSO)

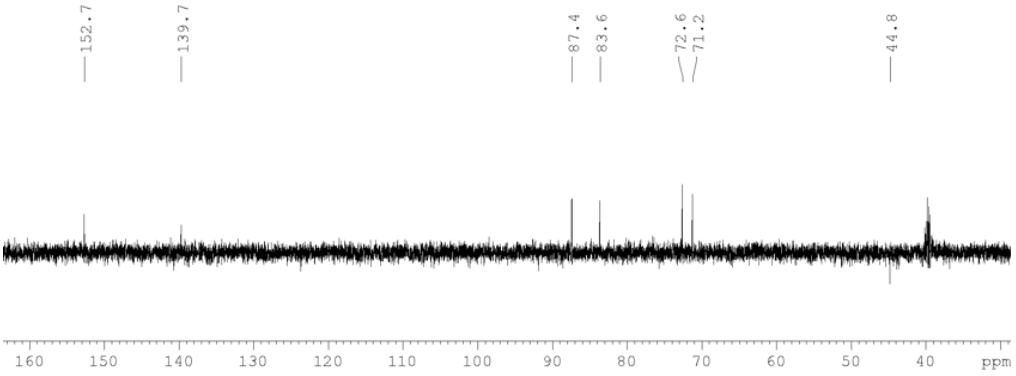
^{1}H



^{13}C

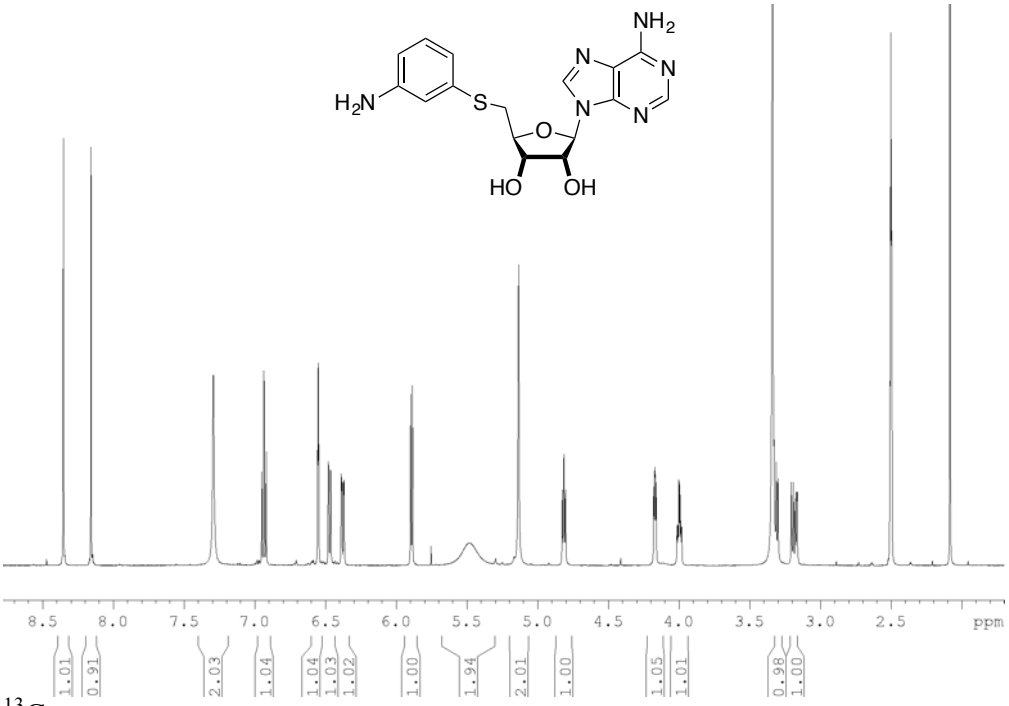


DEPT

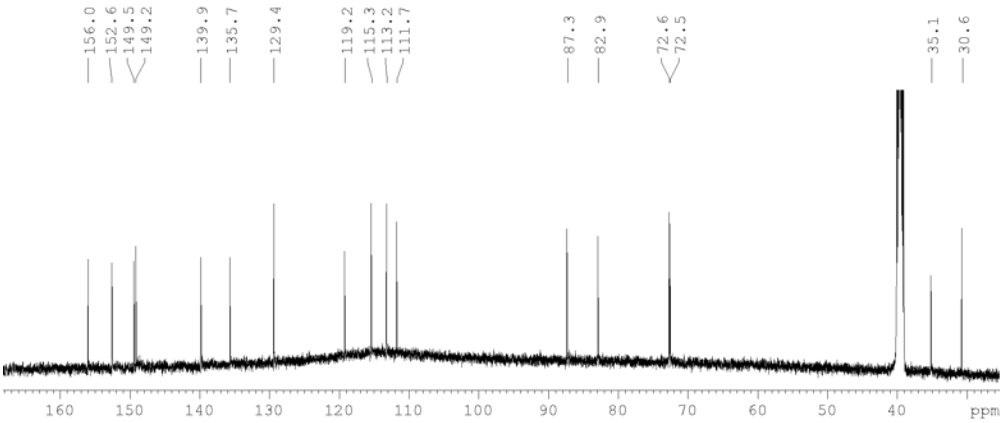


Compound 10 (DMSO)

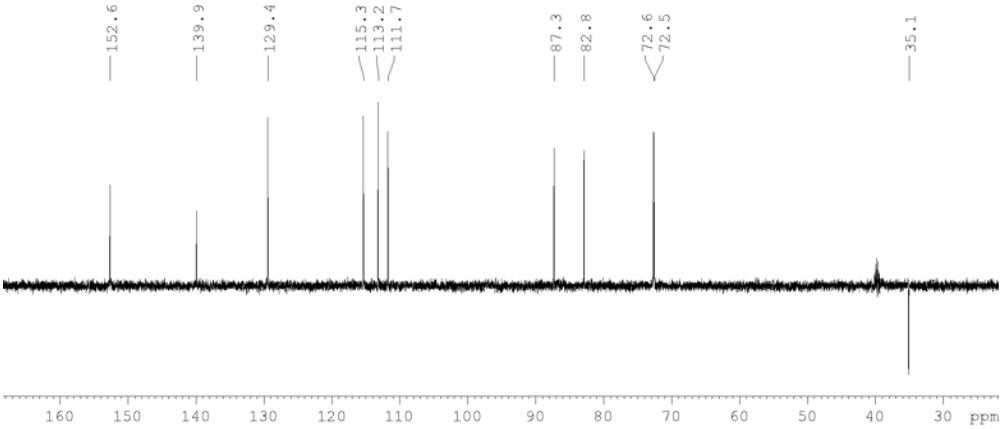
^{1}H



^{13}C

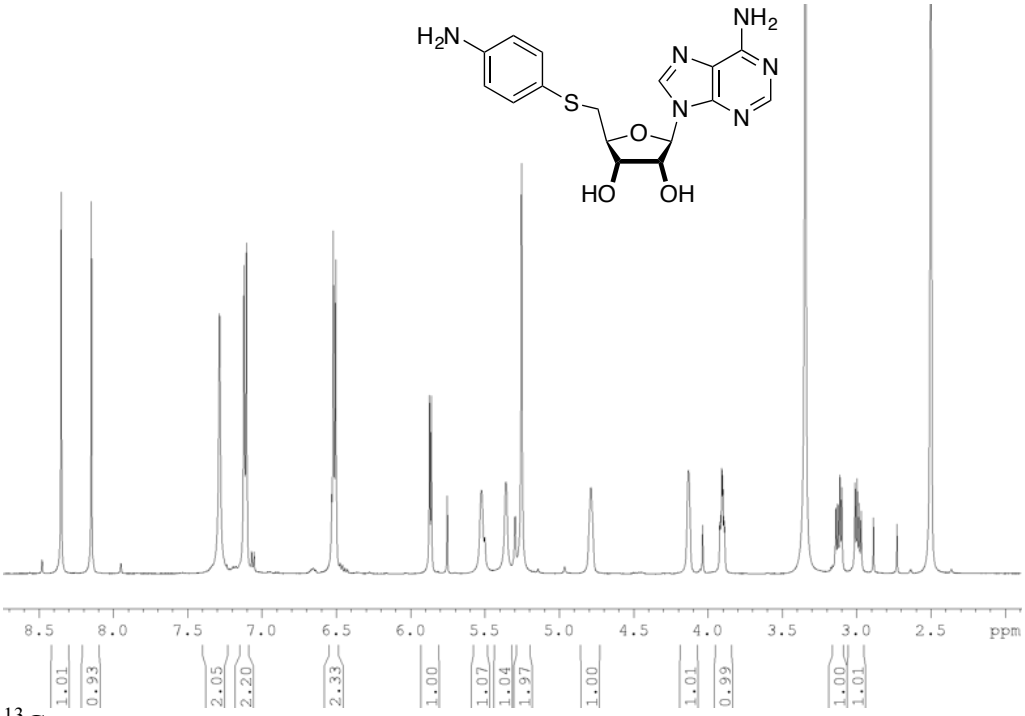


DEPT

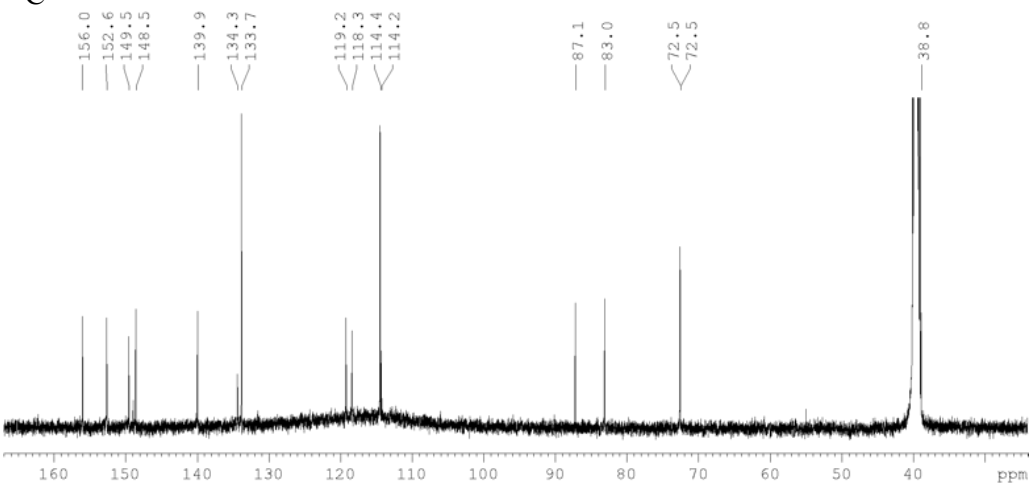


Compound 11 (DMSO)

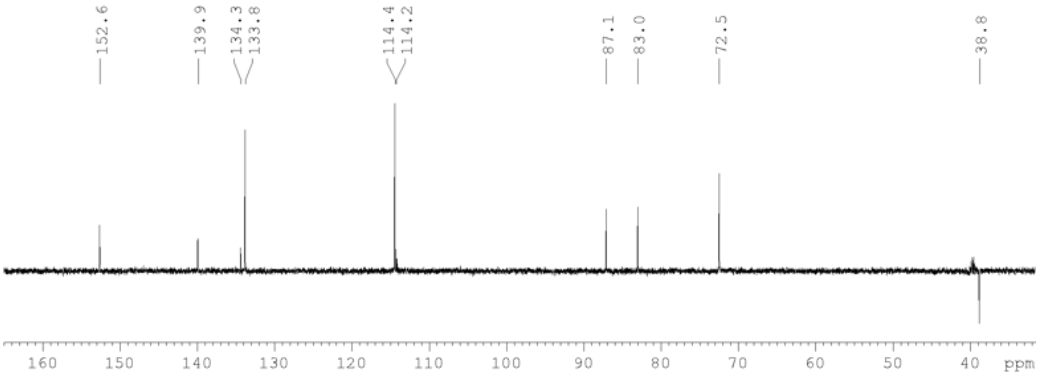
^{1}H



^{13}C

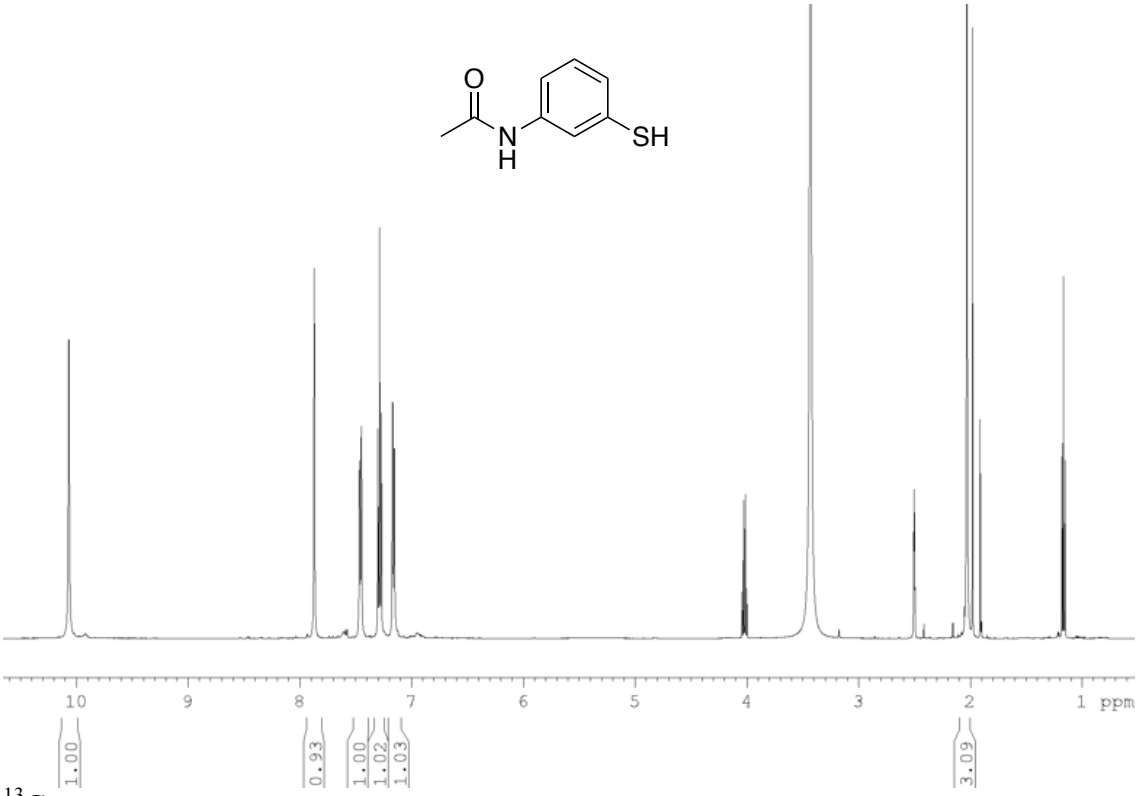


DEPT

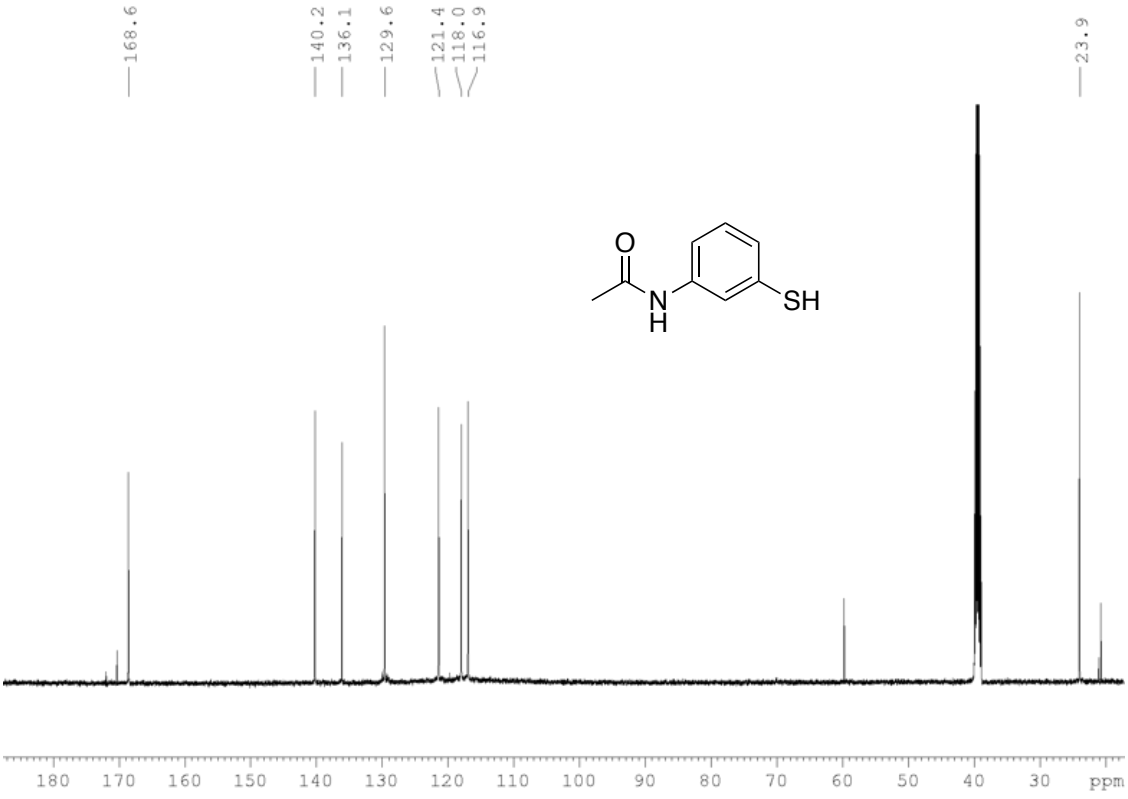


Compound 39 (DMSO)

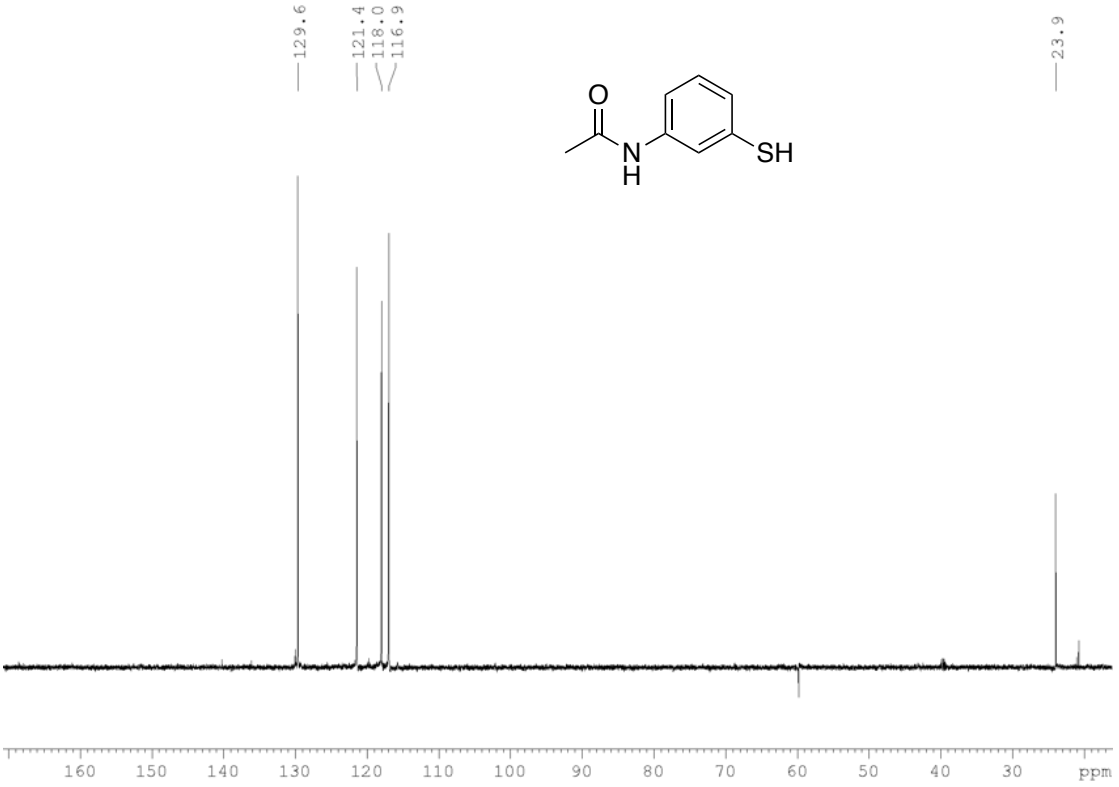
^{1}H



^{13}C

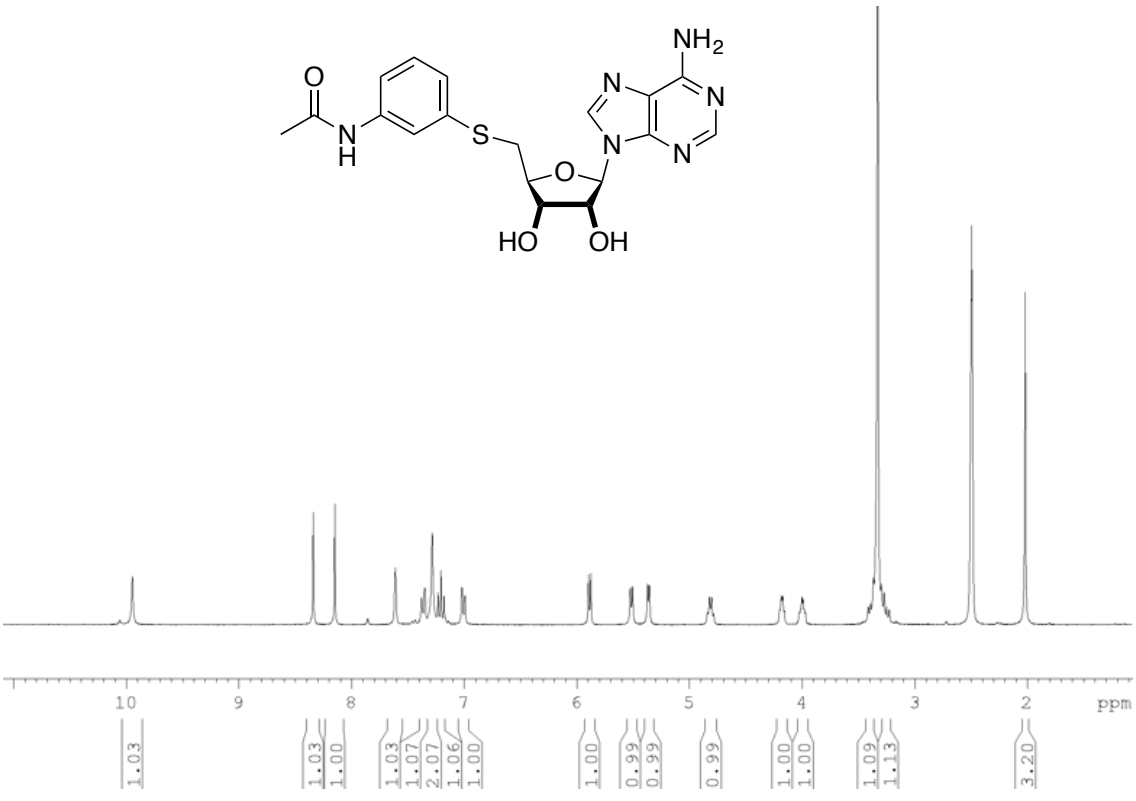


DEPT

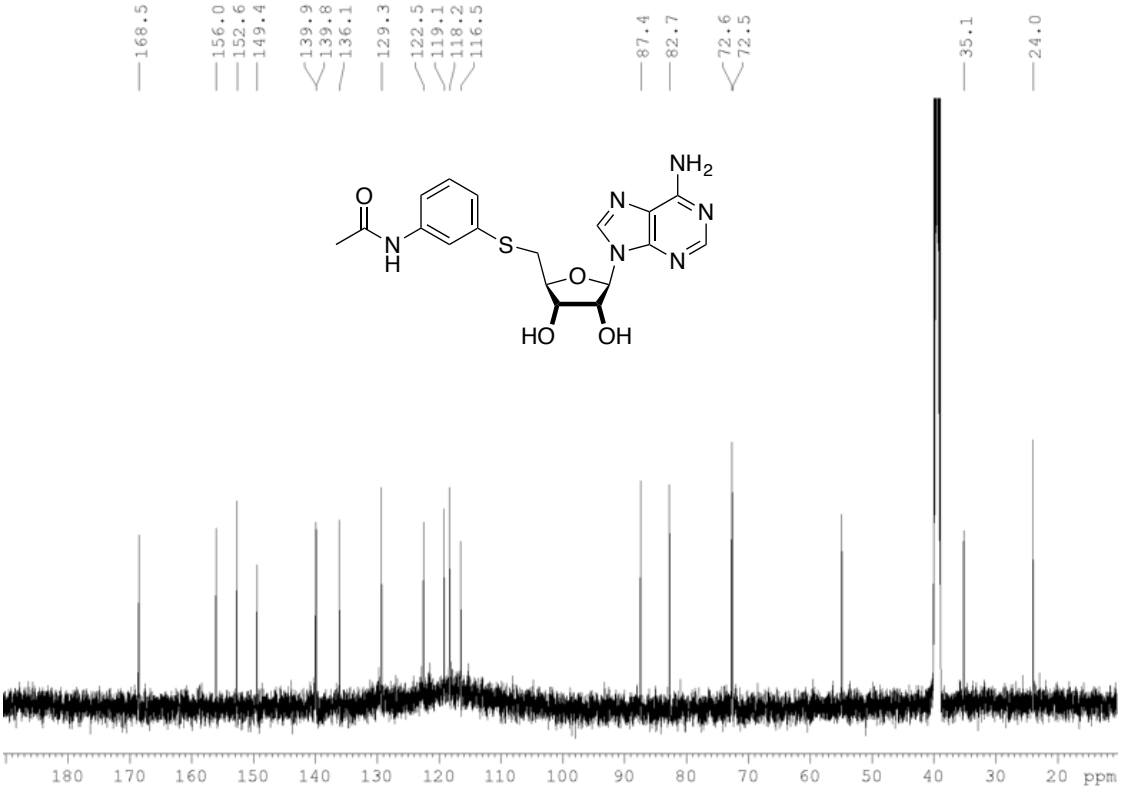


Compound 12 (DMSO)

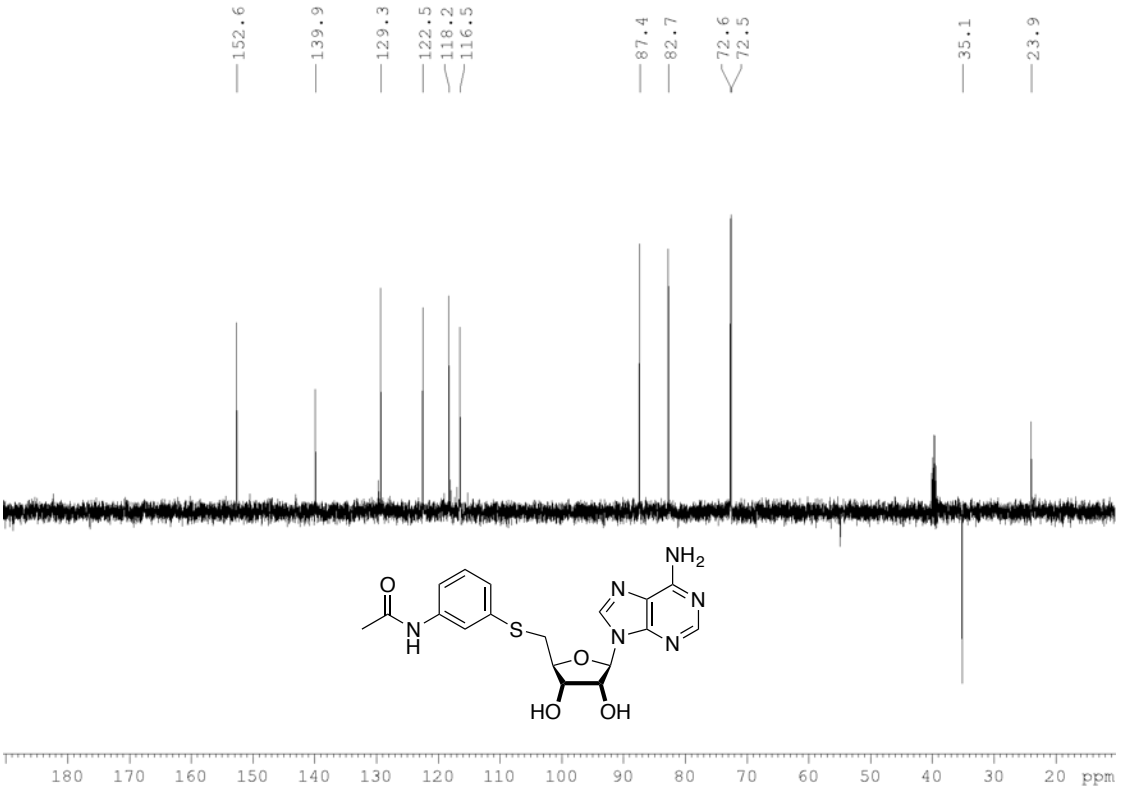
^{1}H



^{13}C


168.5
156.0
152.6
149.4
139.9
139.8
136.1
129.3
122.5
119.1
118.2
116.5
87.4
82.7
72.6
72.5
35.1
24.0
NH2
O
N
H
S
O
HO
OH
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

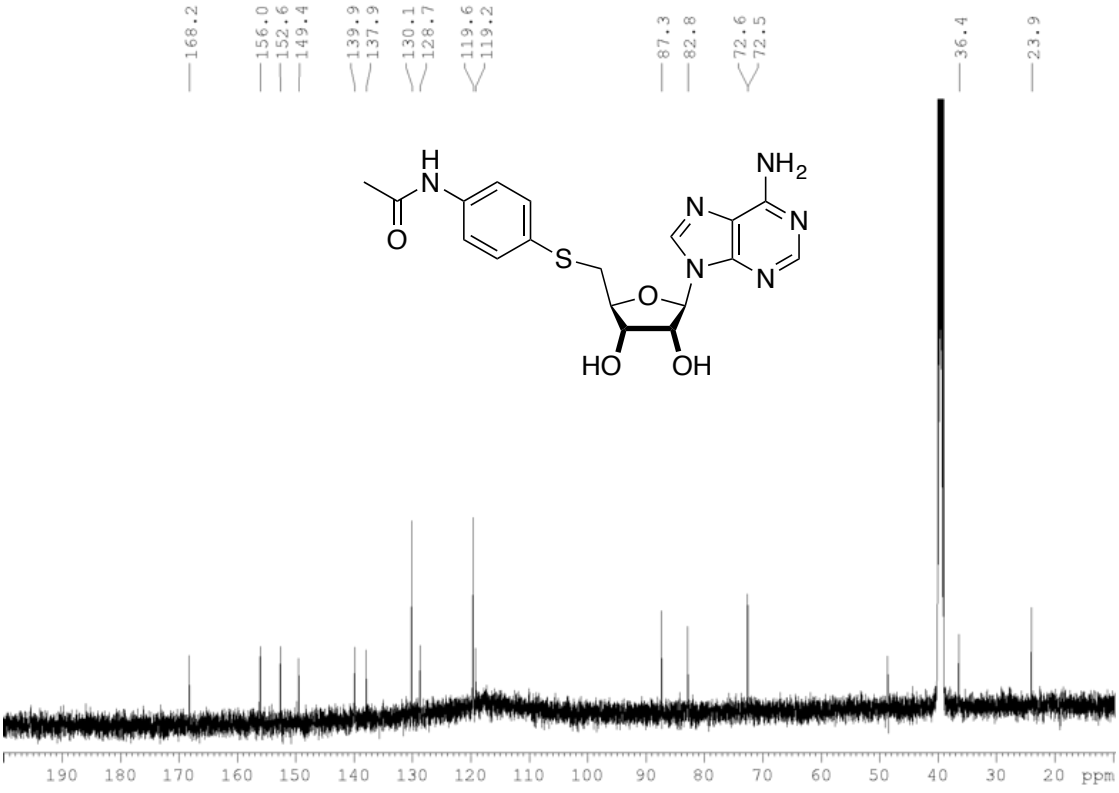
DEPT


152.6
139.9
129.3
122.5
118.2
116.5
87.4
82.7
72.6
72.5
35.1
23.9
NH2
O
N
H
S
O
HO
OH
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

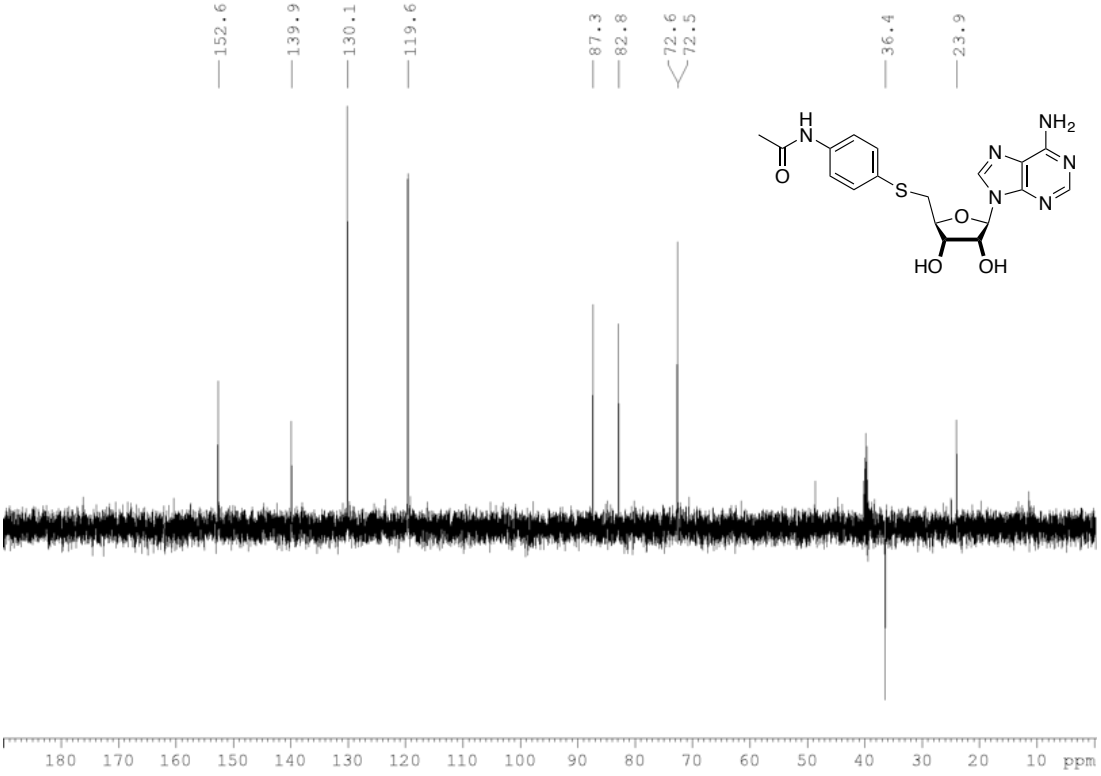
Compound 13 (DMSO)

^{1}H

^{13}C

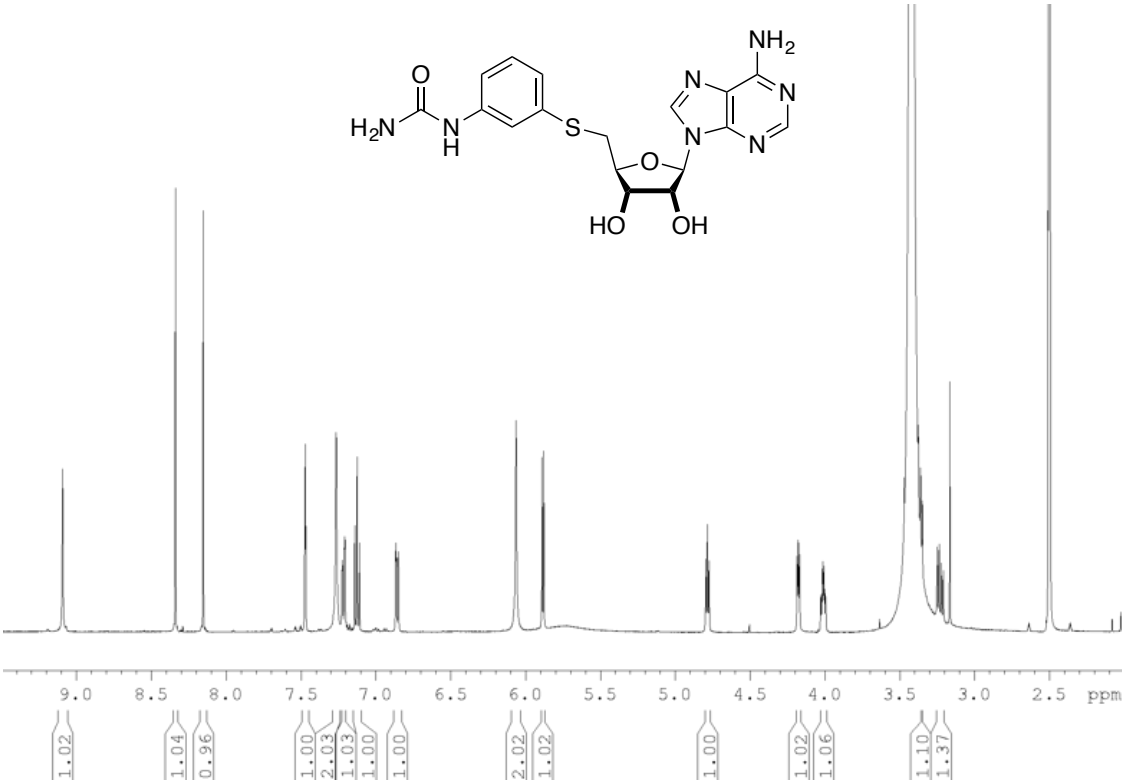


DEPT

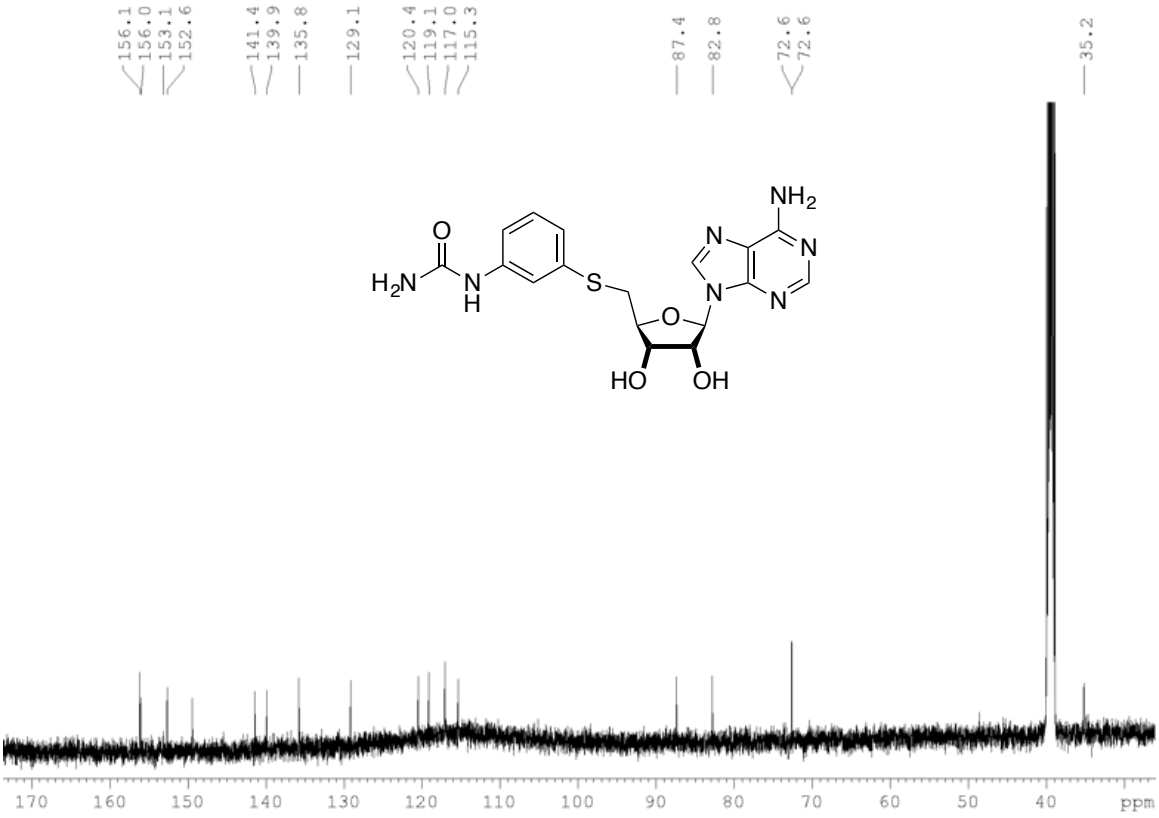


Compound 14 (DMSO)

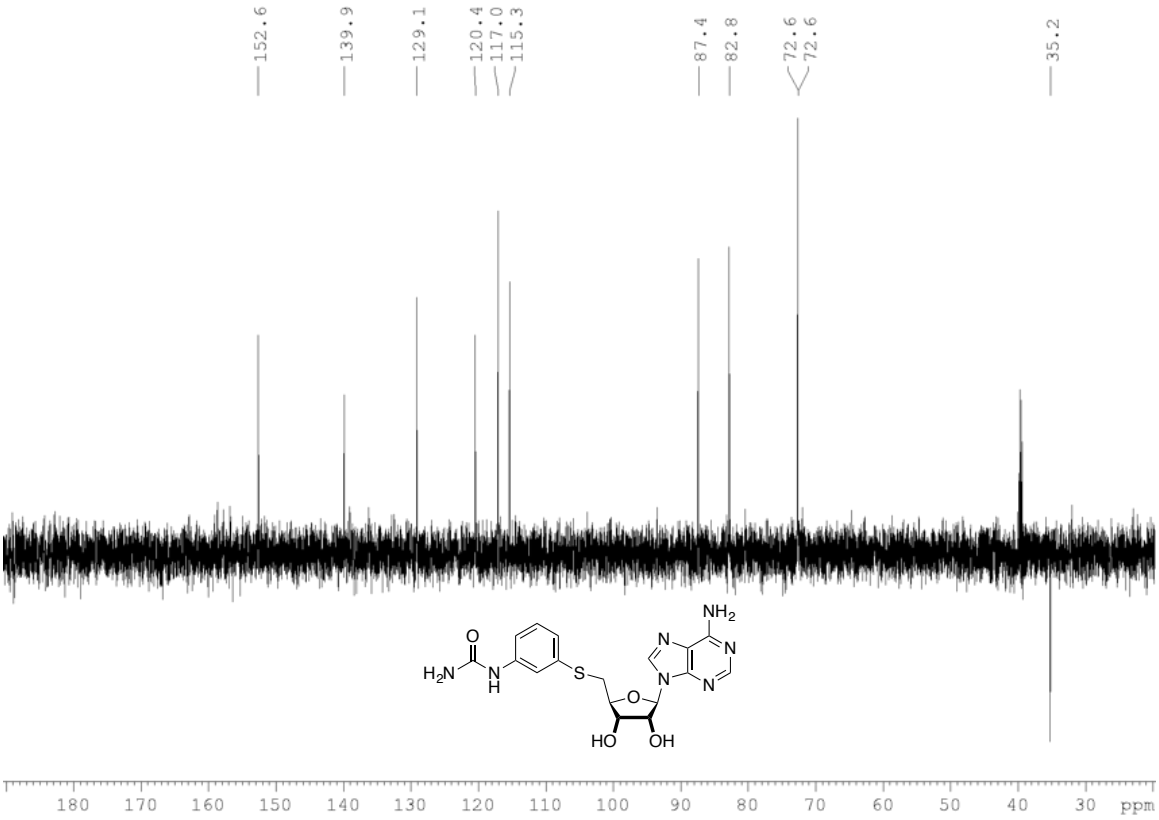
^{1}H



^{13}C

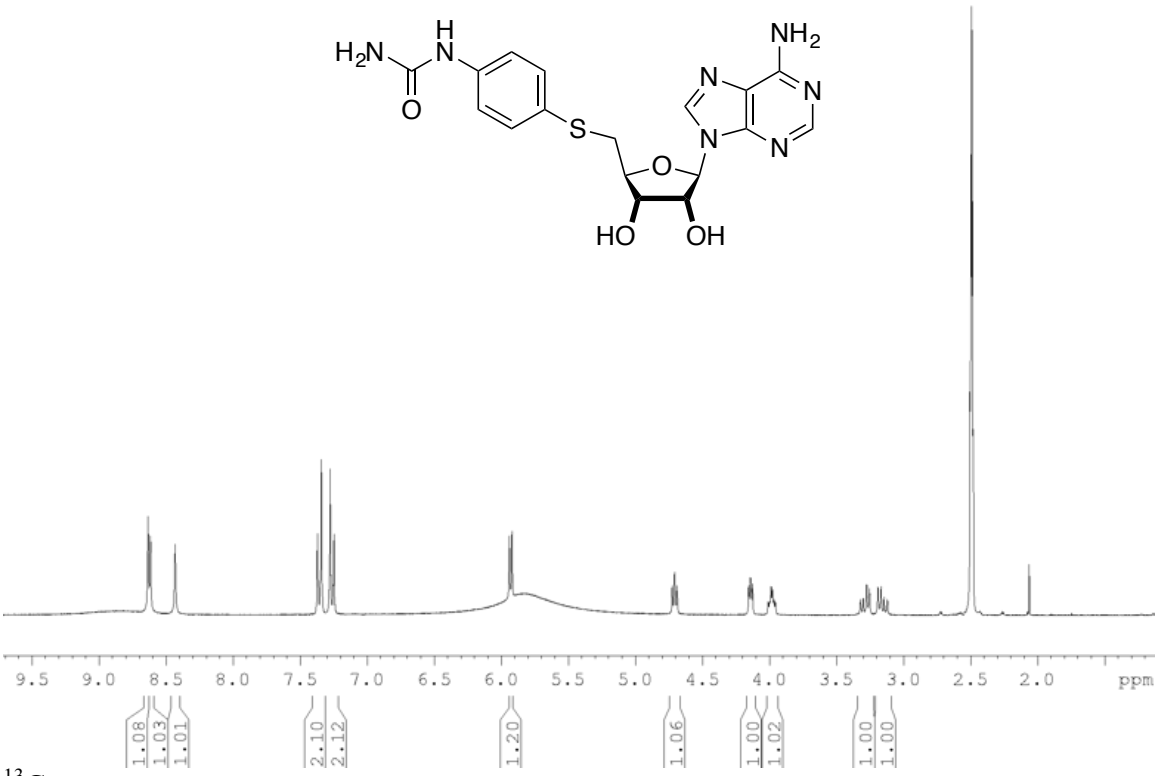

156.1
156.0
153.1
152.6
141.4
139.9
135.8
129.1
120.4
119.1
117.0
115.3
87.4
82.8
72.6
72.6
35.2
NH2
O
H2N
N
H
S
N
N
N
N
O
HO
OH
170
160
150
140
130
120
110
100
90
80
70
60
50
40
ppm

DEPT

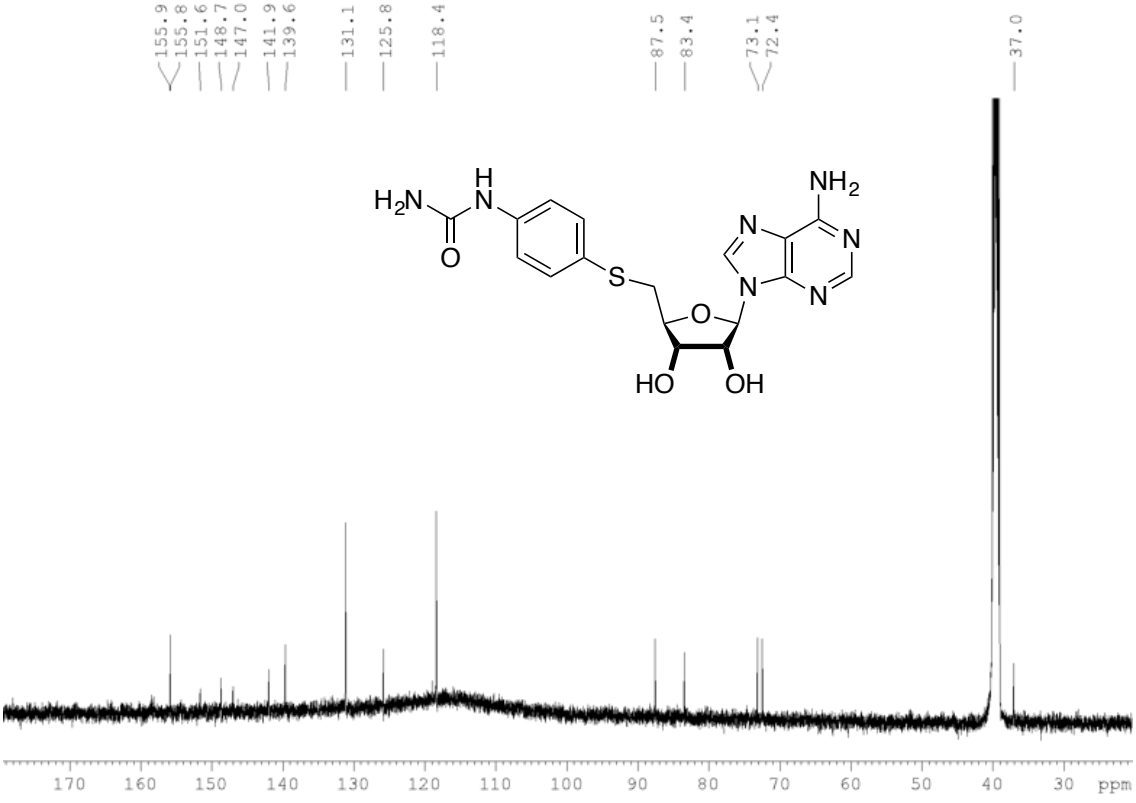

152.6
139.9
129.1
120.4
117.0
115.3
87.4
82.8
72.6
72.6
35.2
NH2
O
H2N
N
H
S
N
N
N
N
O
HO
OH
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm

Compound 15 (DMSO)

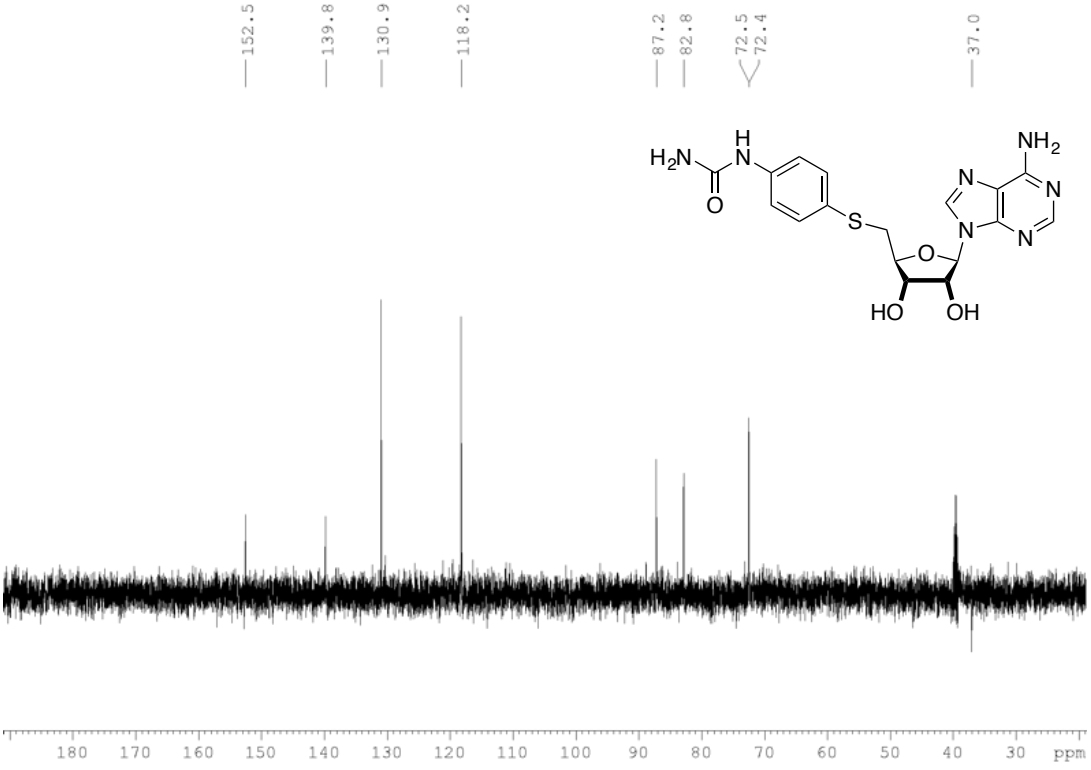
^{1}H



^{13}C

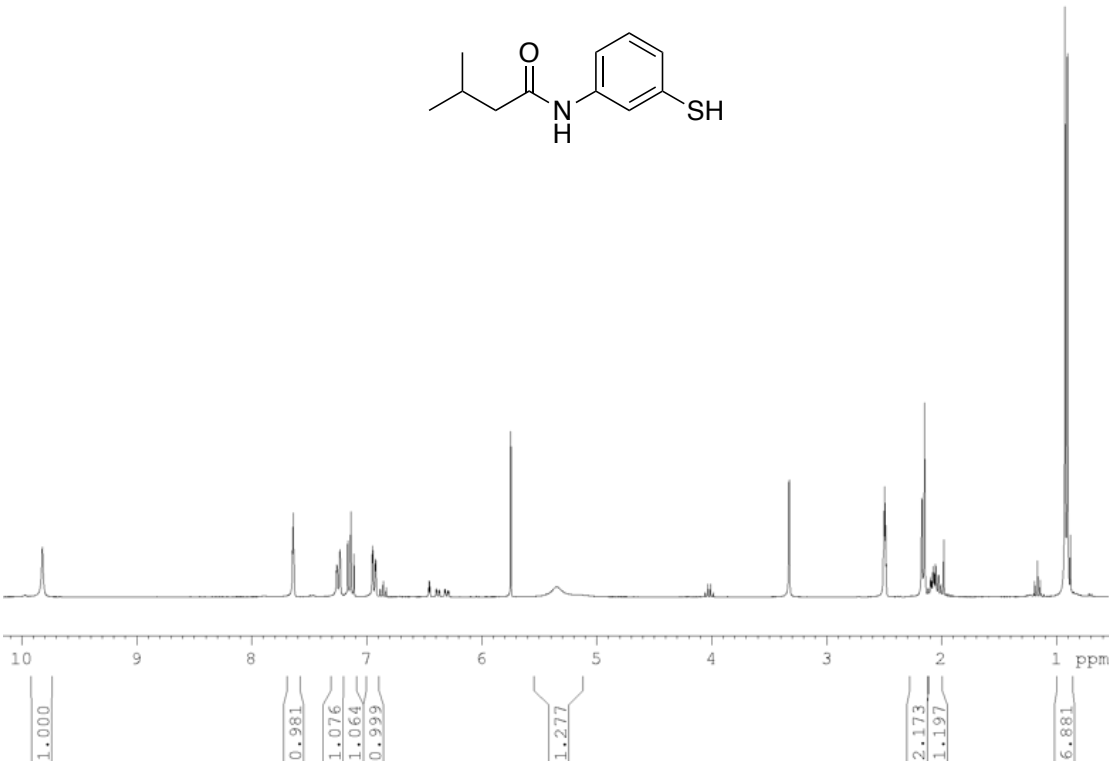


DEPT

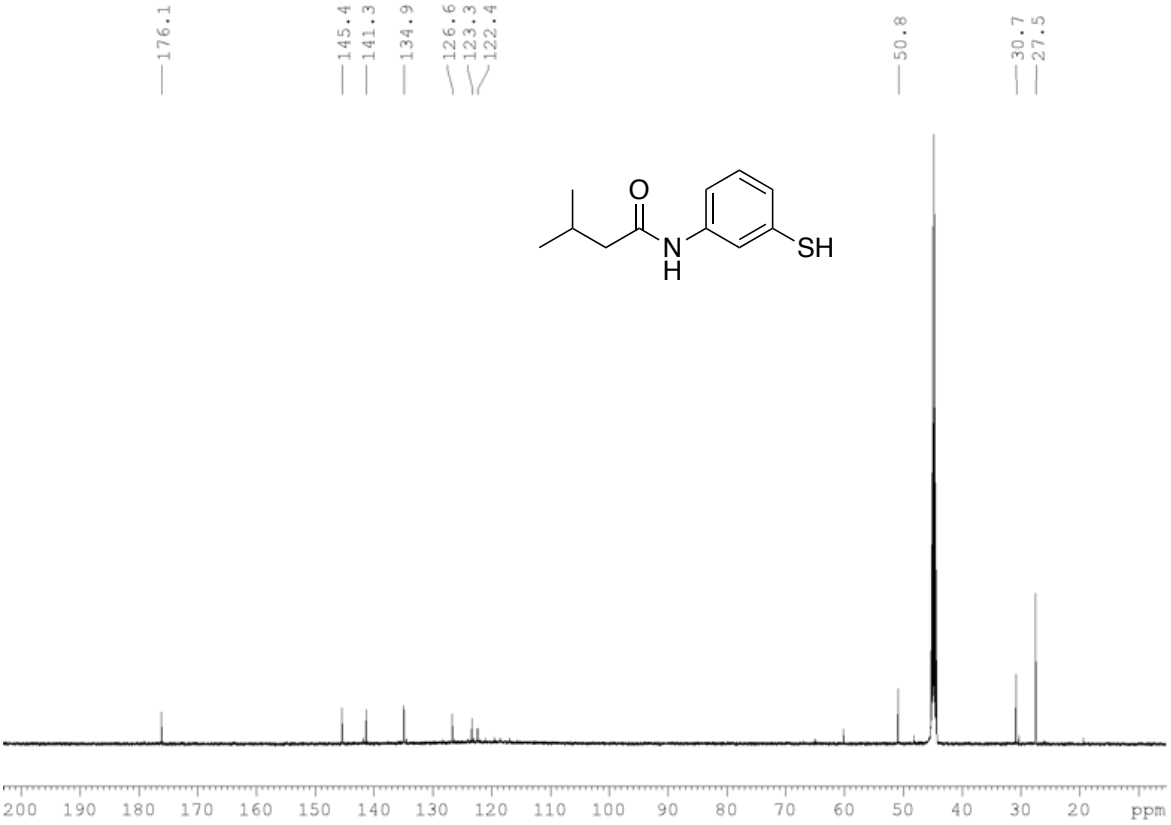


Compound 40 (crude) (DMSO)

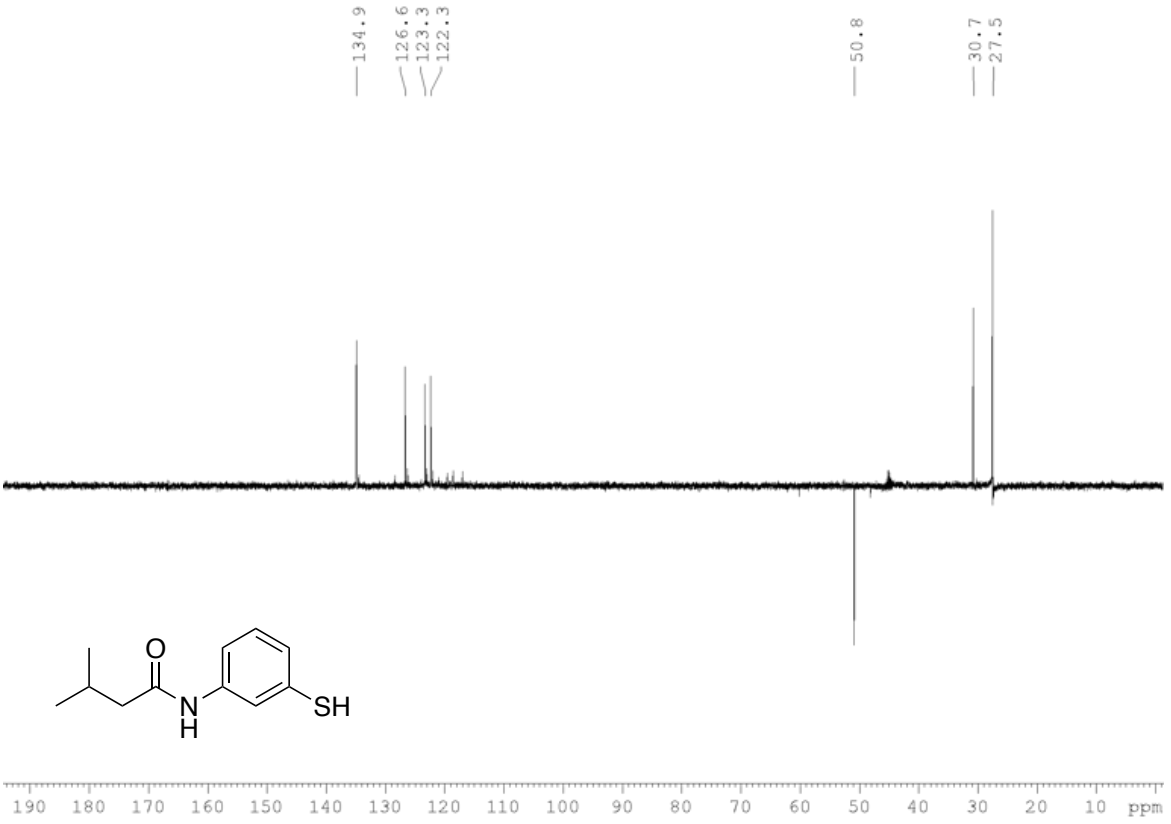
^{1}H



^{13}C

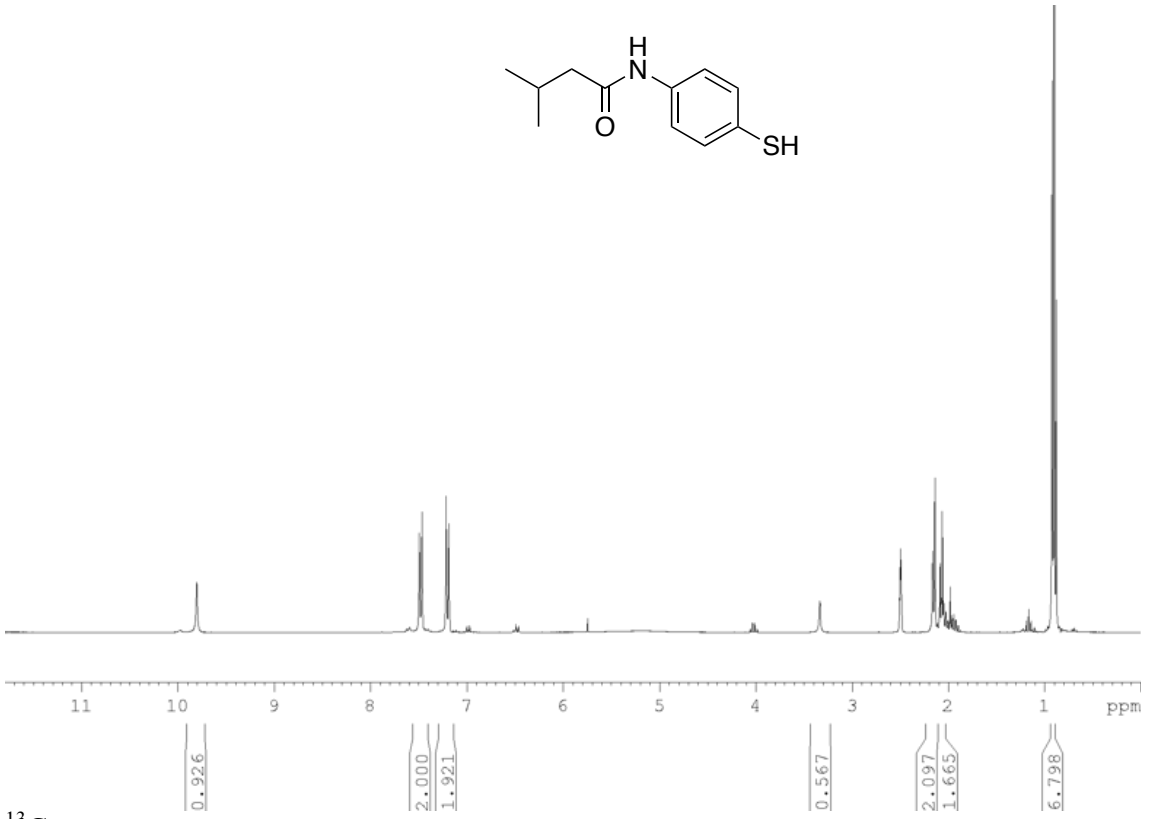

176.1
145.4
141.3
134.9
126.6
123.3
122.4
50.8
30.7
27.5
O
N
H
SH
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

DEPT

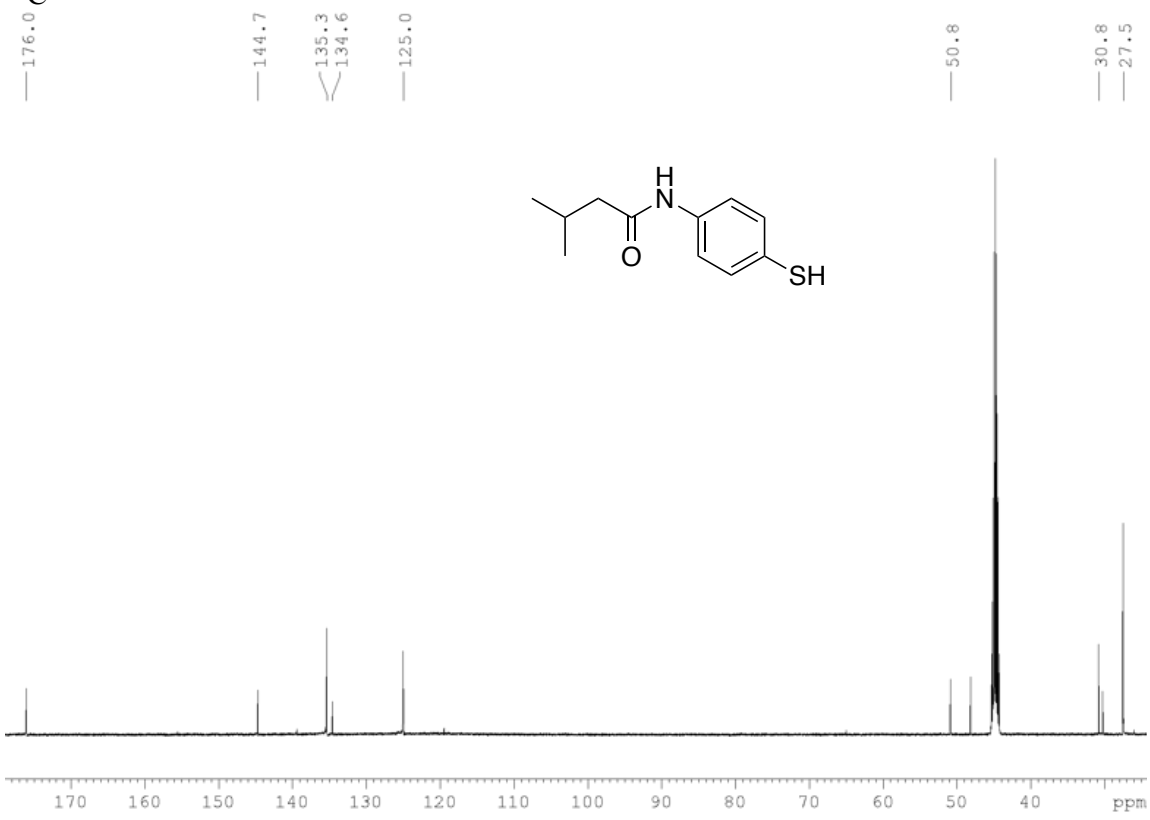

134.9
126.6
123.3
122.3
50.8
30.7
27.5
O
N
H
SH
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

Compound 41 (crude) (DMSO)

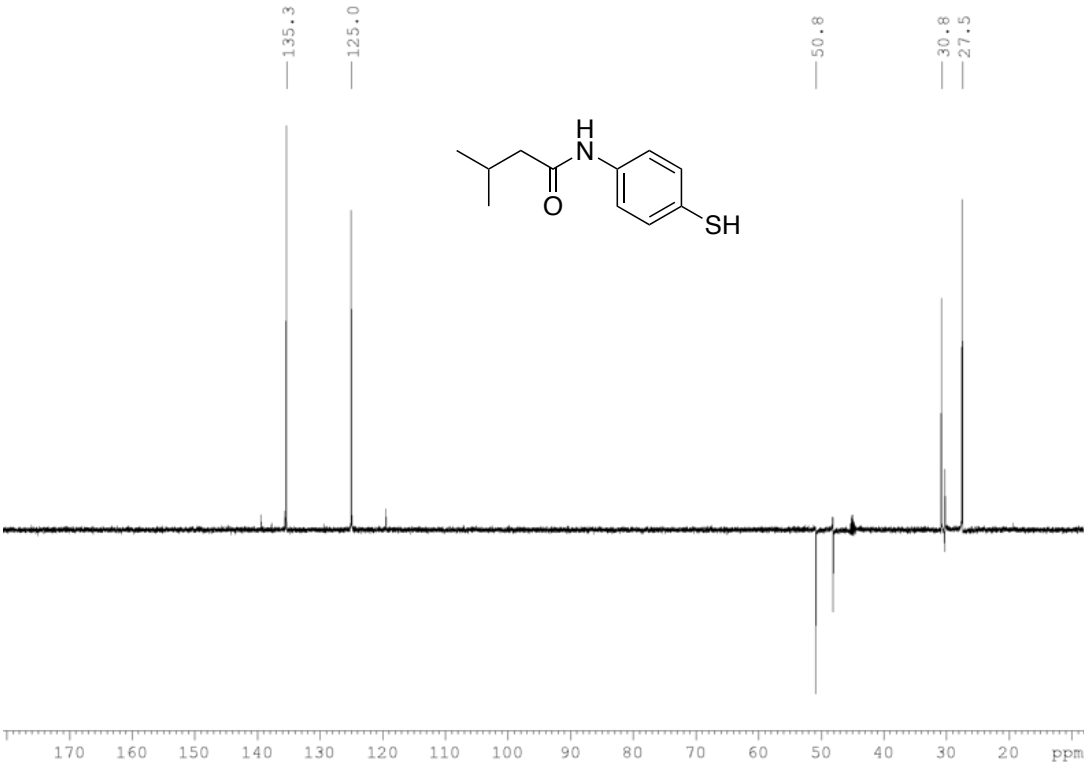
^{1}H



^{13}C

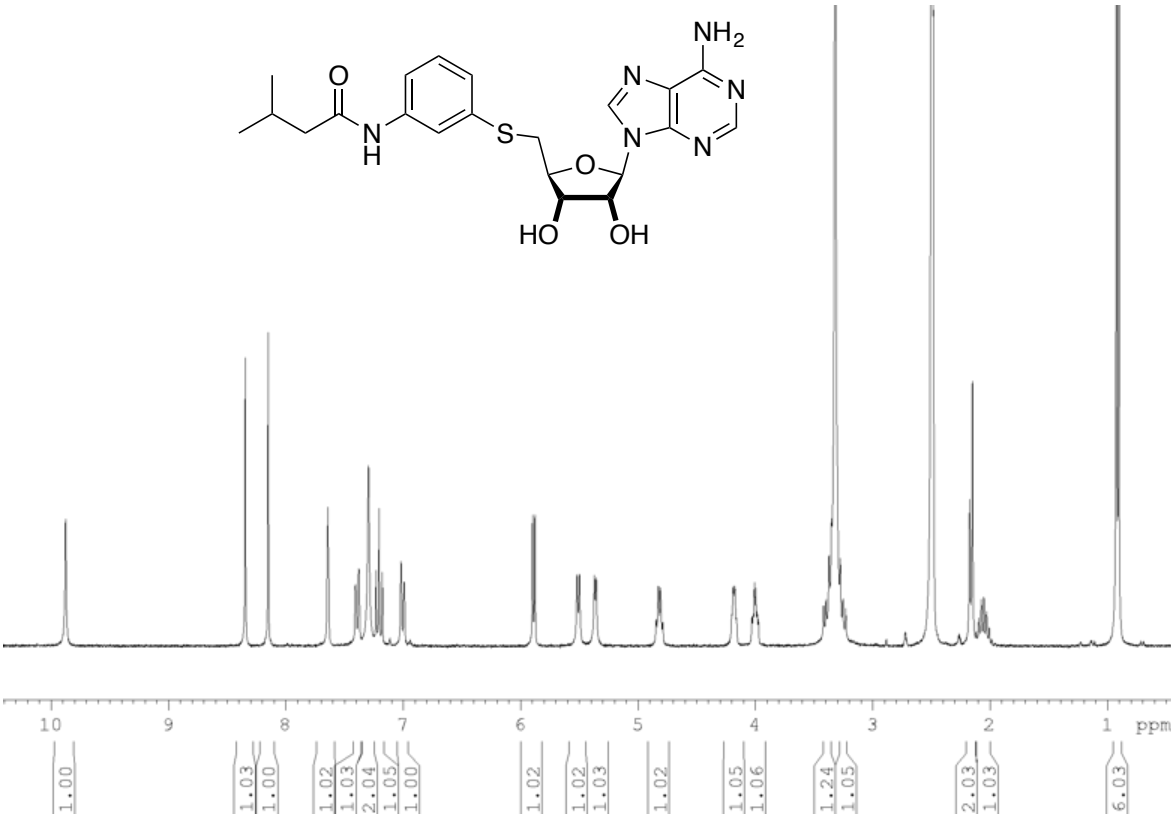


DEPT

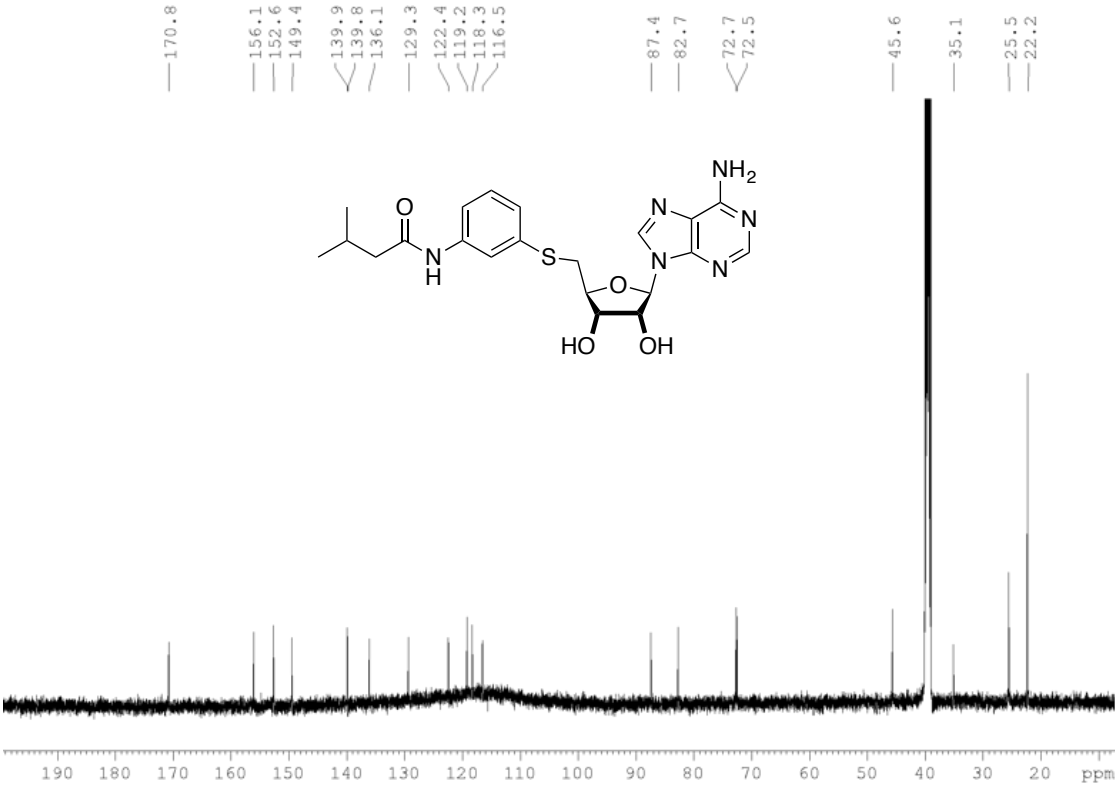


Compound 16 (DMSO)

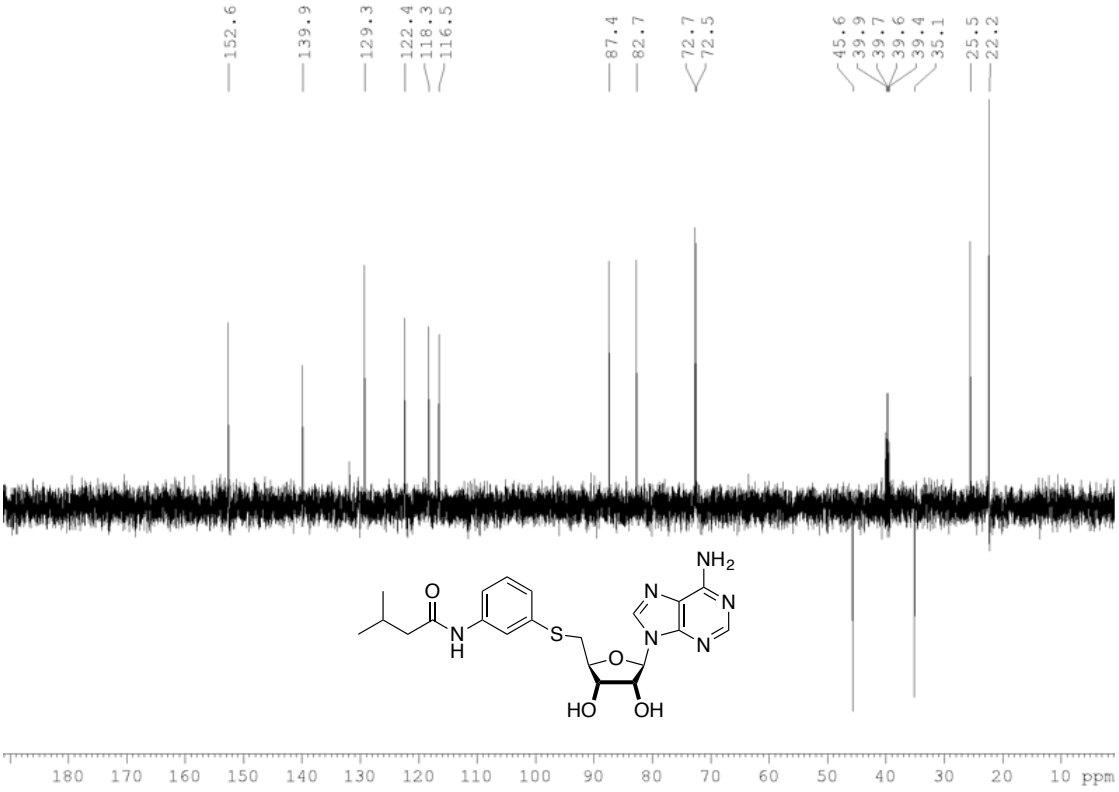
^{1}H



^{13}C

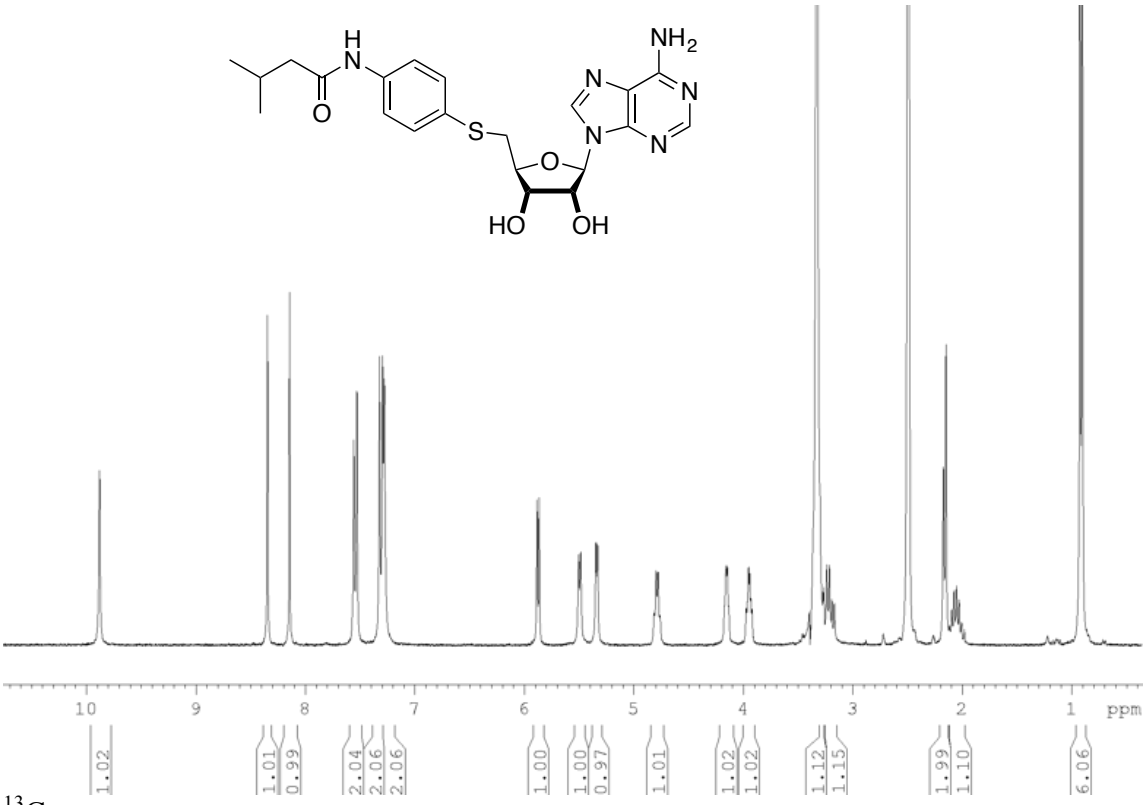

170.8
156.1
152.6
149.4
139.9
139.8
136.1
129.3
122.4
119.2
118.3
116.5
87.4
82.7
72.7
72.5
45.6
35.1
25.5
22.2
NH2
O
N
H
S
N
N
N
N
O
HO
OH
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

DEPT

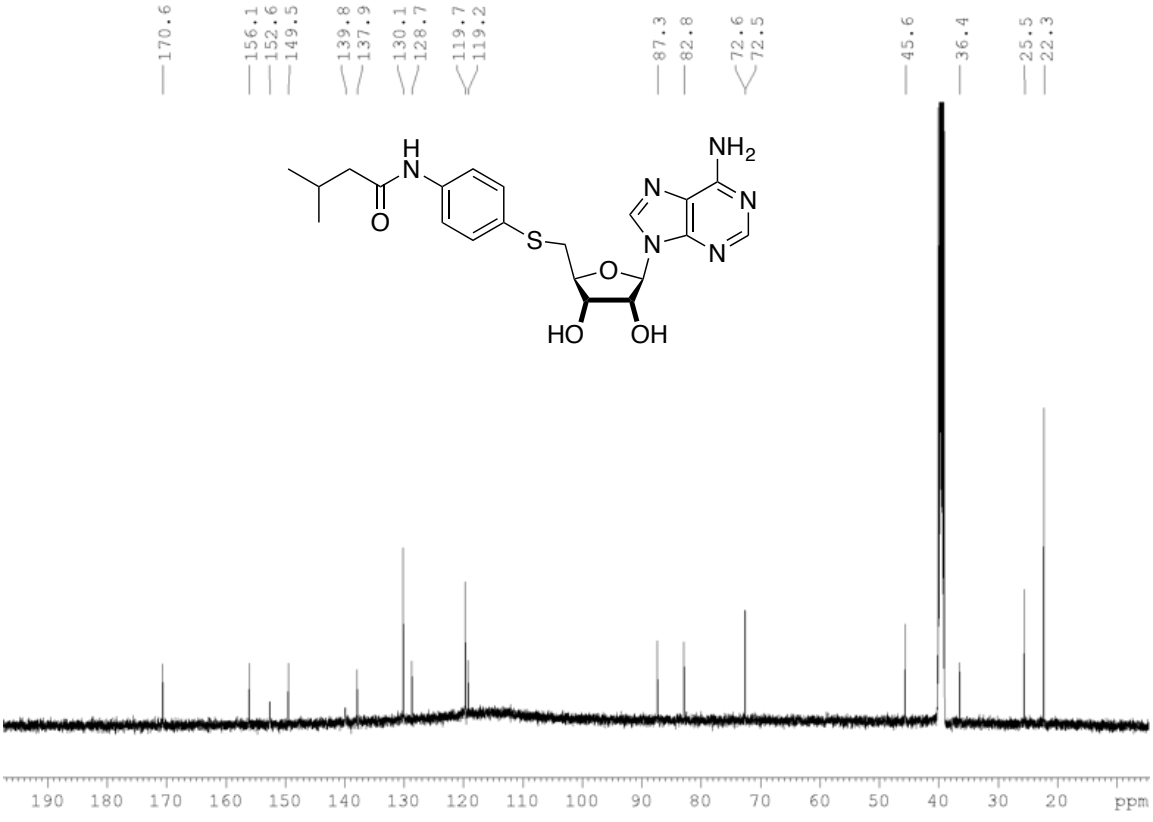

152.6
139.9
129.3
122.4
118.3
116.5
87.4
82.7
72.7
72.5
45.6
39.9
39.7
39.6
39.4
35.1
25.5
22.2
NH2
O
N
H
S
N
N
N
N
O
HO
OH
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

Compound 17 (DMSO)

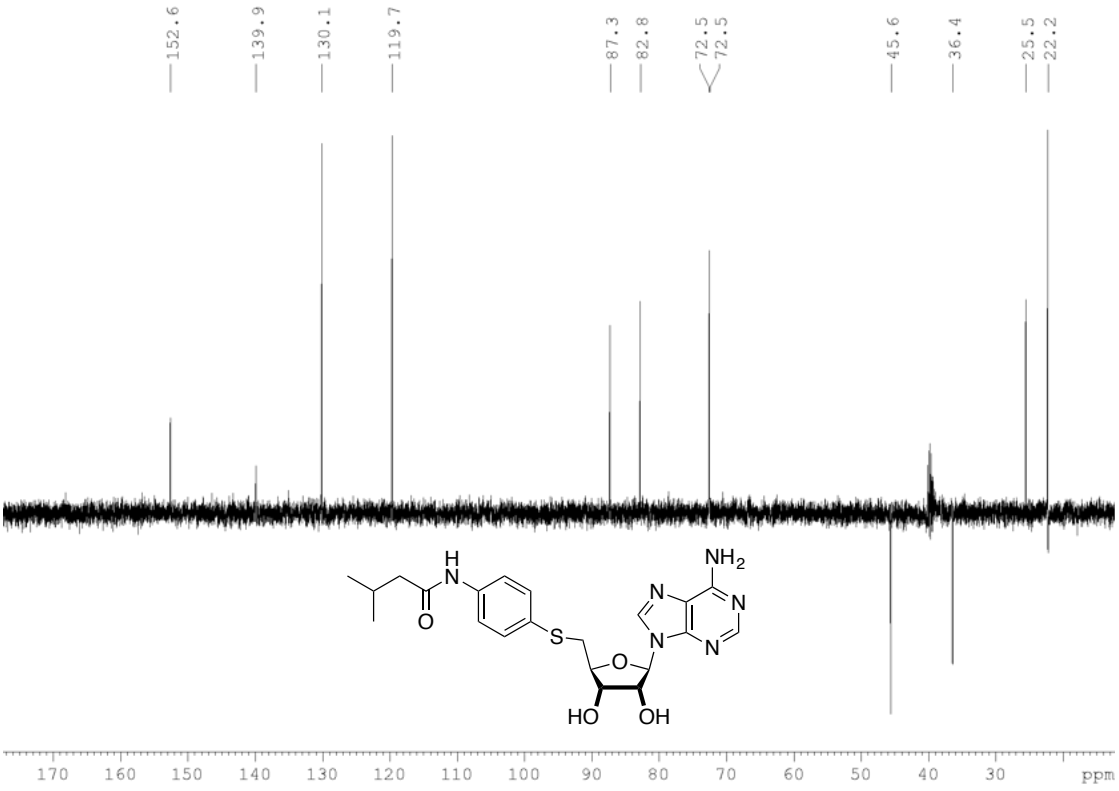
^{1}H



^{13}C

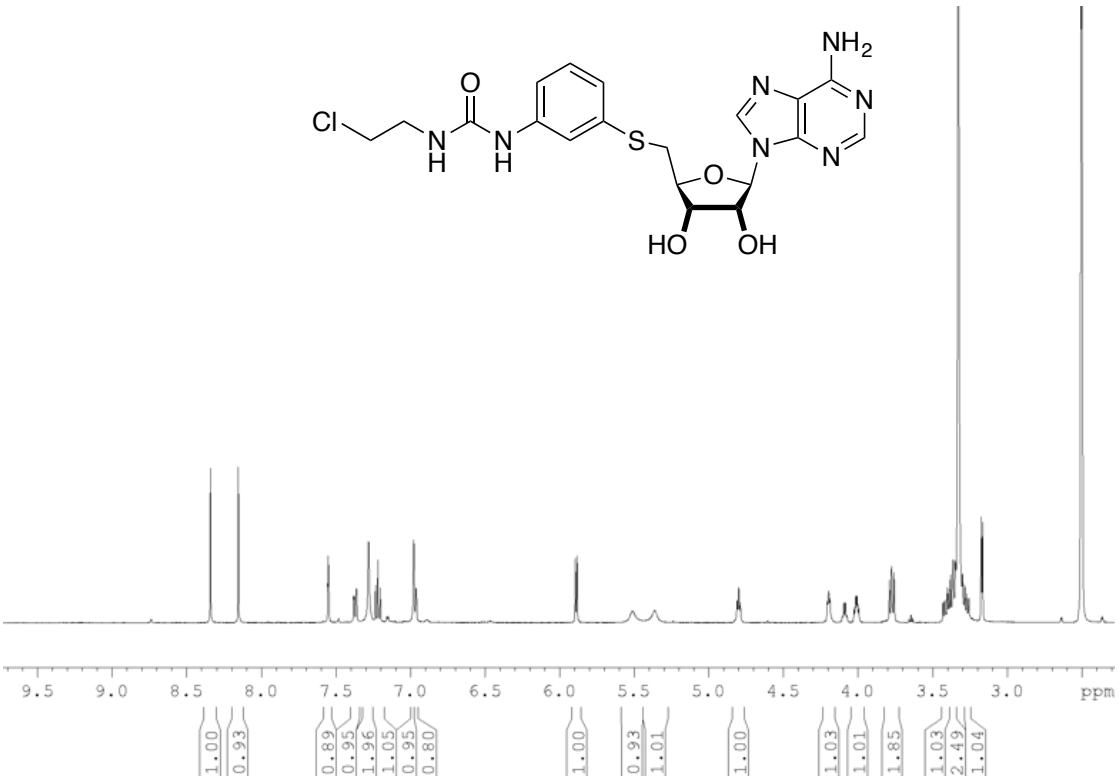


DEPT



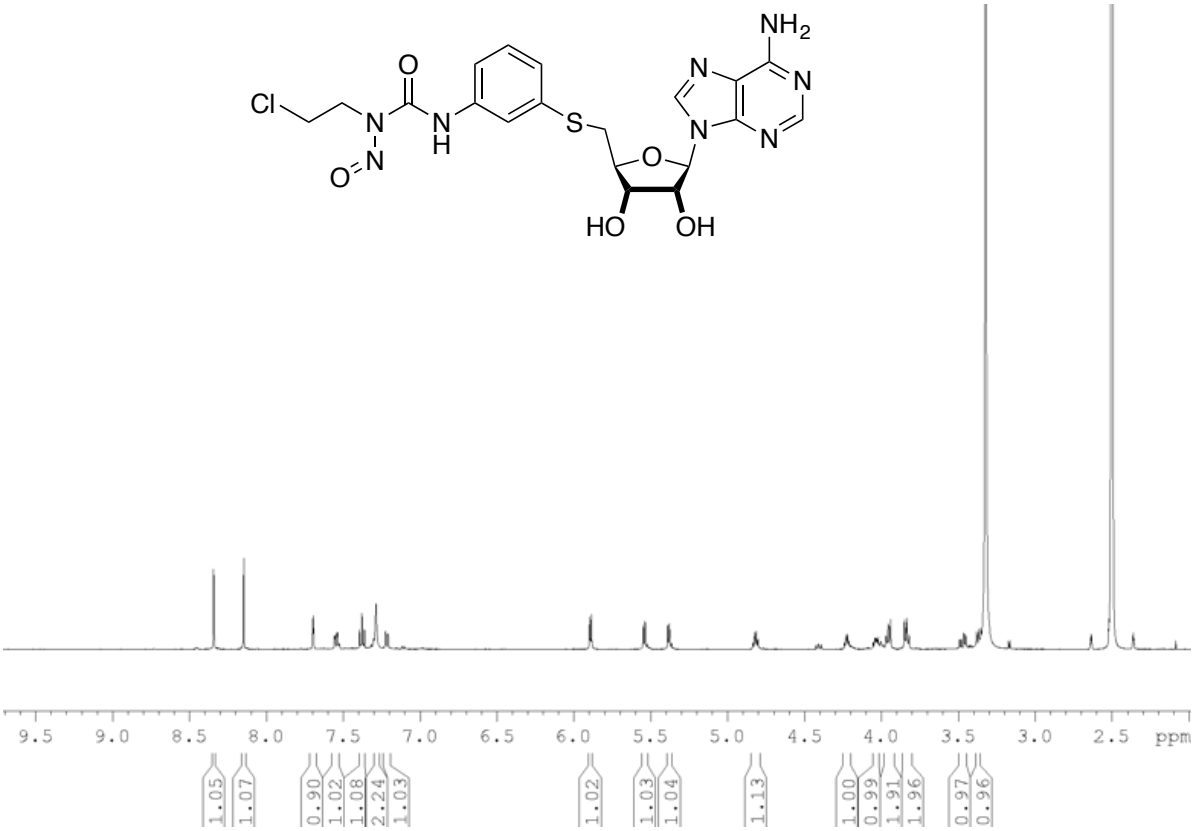
Compound 42 (DMSO)

^{1}H

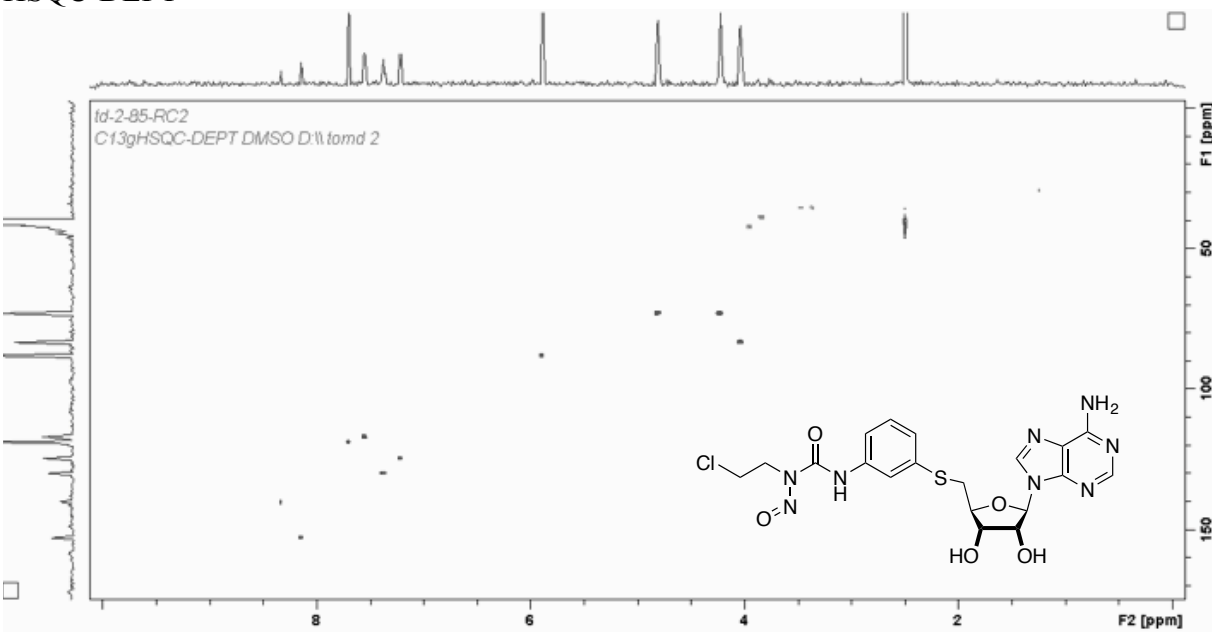


Compound 18 (DMSO)

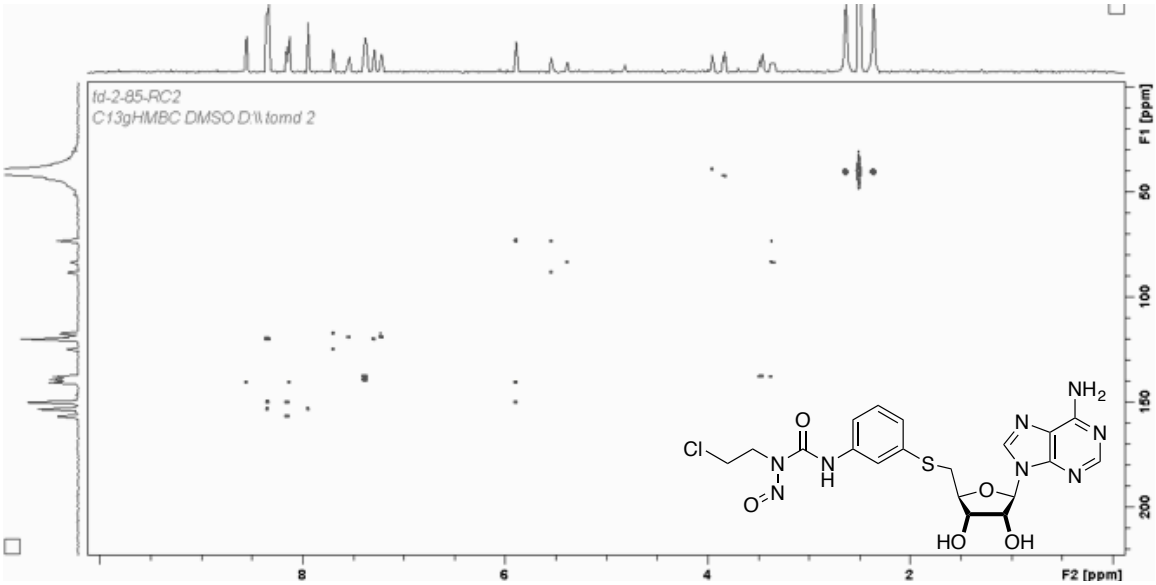
^{1}H



HSQC-DEPT

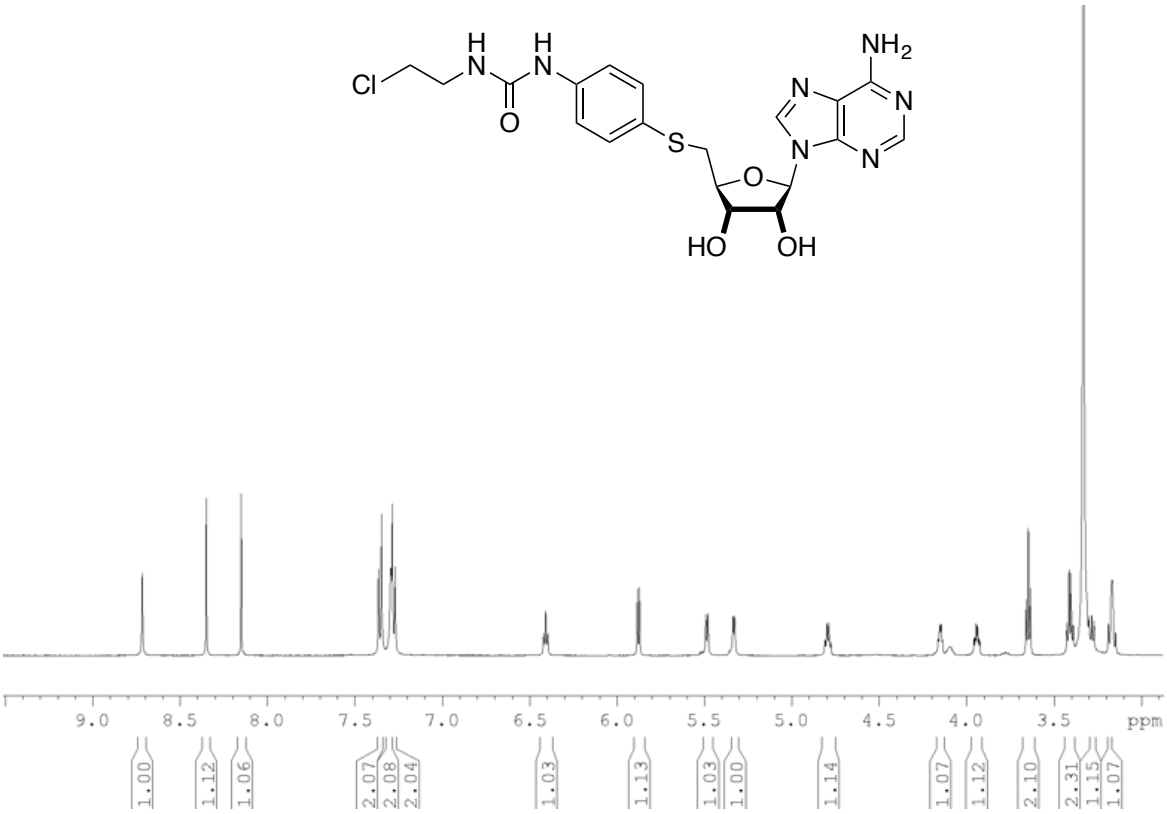


HMBC



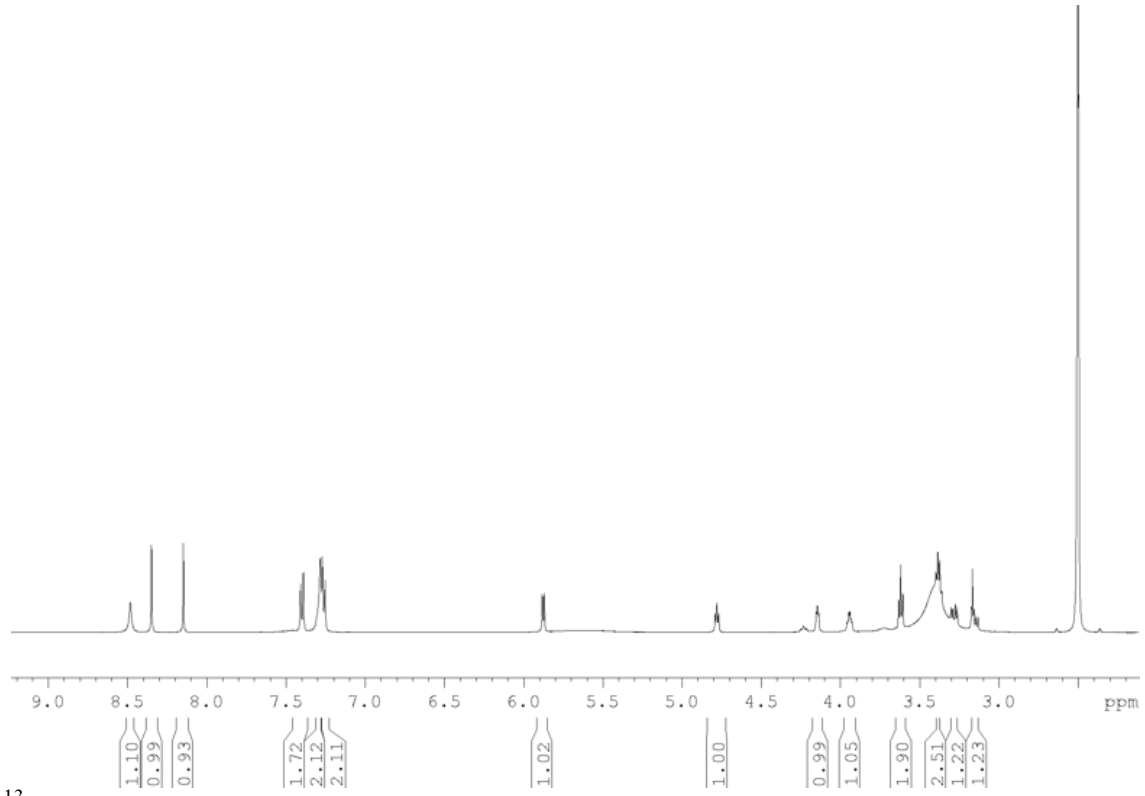
Compound 43 (DMSO)

^{1}H

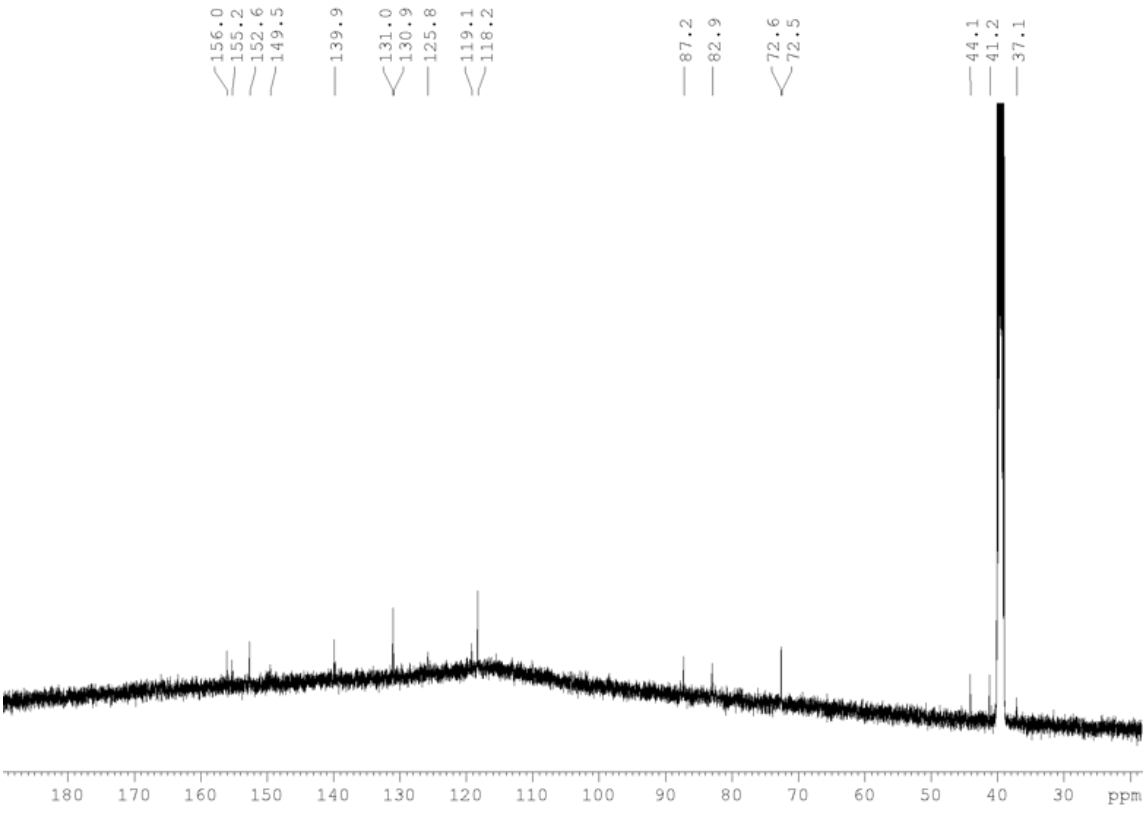


Attempted synthesis of compound 19 (DMSO)

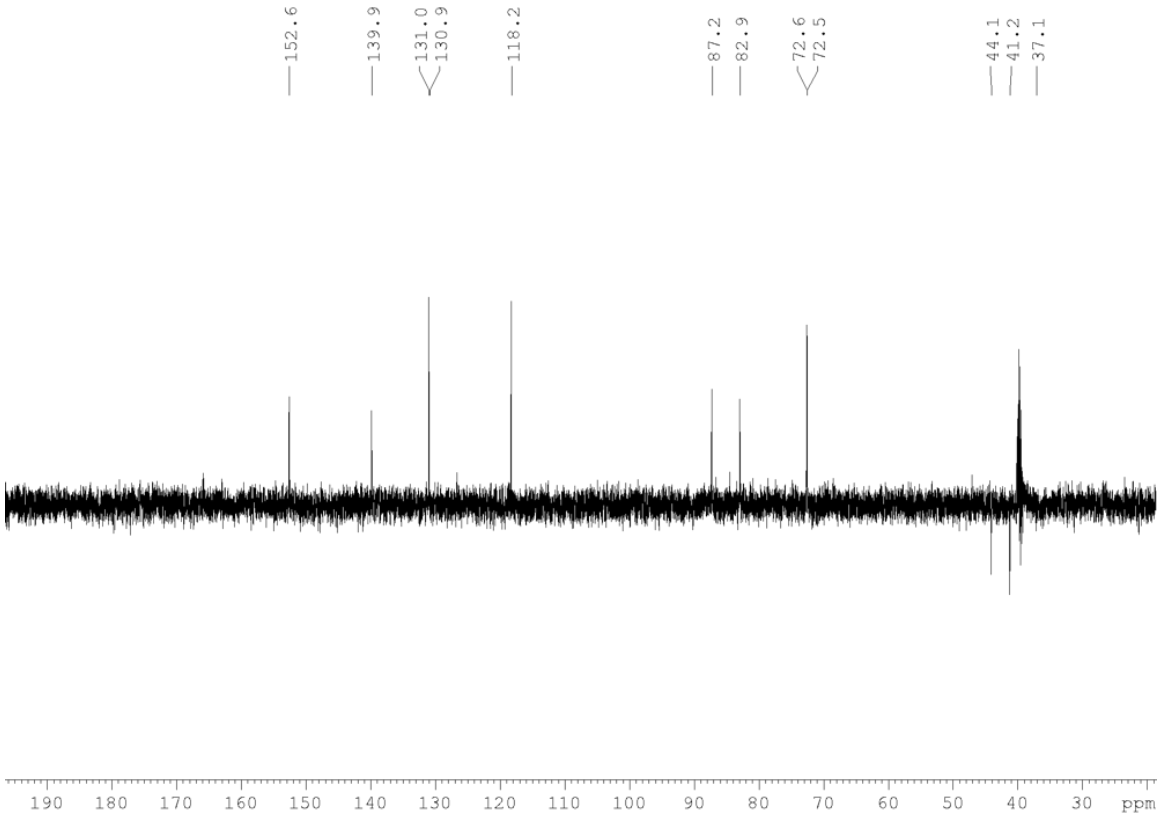
^{1}H



^{13}C



DEPT

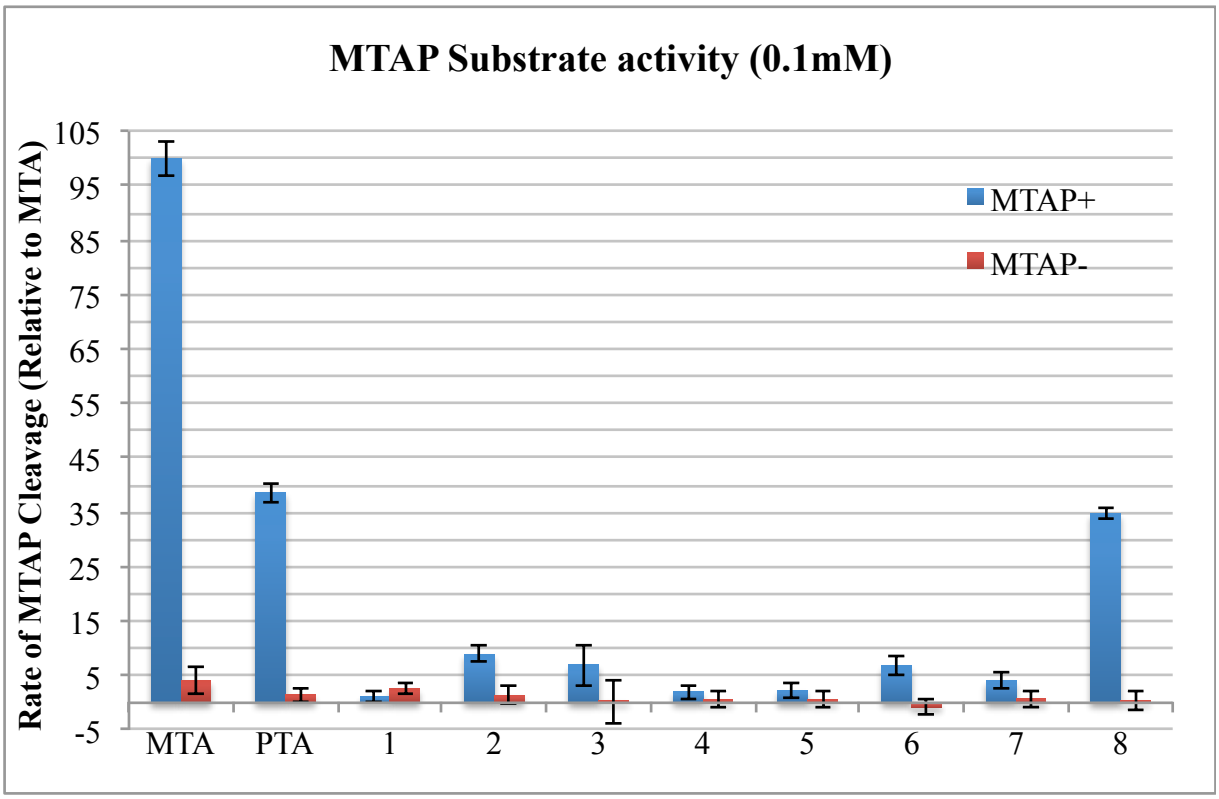

152.6
139.9
131.0
130.9
118.2
87.2
82.9
72.6
72.5
44.1
41.2
37.1
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 ppm

Appendix B Biological Data

MTAP-XO Assay 1 ([MTAP]=0.02μg/μL, [XO]=0.05U/μL)

Table of slopes and uncertainties for MTAP-XO Assay 1, MTAP+, 0.1mM substrate					
Comp-ound	Slope ± slope error, for each replicate			Average slope ± standard error	Slope relative to MTA ± standard error
	1	2	3		
MTA	9.75 ± 0.15	5.86 ± 0.14	4.30 ± 0.15	6.64 ± 0.15	100.0 ± 5.4
PTA	2.95 ± 0.09	2.36 ± 0.10	2.38 ± 0.11	2.56 ± 0.10	38.6 ± 1.8
1	0.14 ± 0.06	–0.03 ± 0.08	0.10 ± 0.08	0.07 ± 0.07	1.1 ± 2.3
2	0.63 ± 0.10	0.53 ± 0.09	0.62 ± 0.09	0.59 ± 0.10	8.9 ± 0.9
3	0.58 ± 0.29	0.13 ± 0.15	0.68 ± 0.30	0.46 ± 0.26	7.0 ± 0.8
4	0.08 ± 0.08	0.20 ± 0.08	0.17 ±0.08	0.13 ± 0.09	2.0 ± 0.3
5	0.13 ± 0.09	0.17 ± 0.10	0.67 ± 0.12	0.15 ± 0.09	2.3 ± 0.1
6	0.30 ± 0.13	0.38 ± 0.09	0.12 ± 0.10	0.45 ± 0.11	6.8 ± 0.4
7	0.16 ± 0.10	0.32 ± 0.11	0.33 ± 0.08	0.27 ± 0.10	4.1 ± 0.7
8	2.31 ± 0.15	2.30 ± 0.10	2.31 ± 0.12	2.31 ± 0.12	34.8 ± 1.4

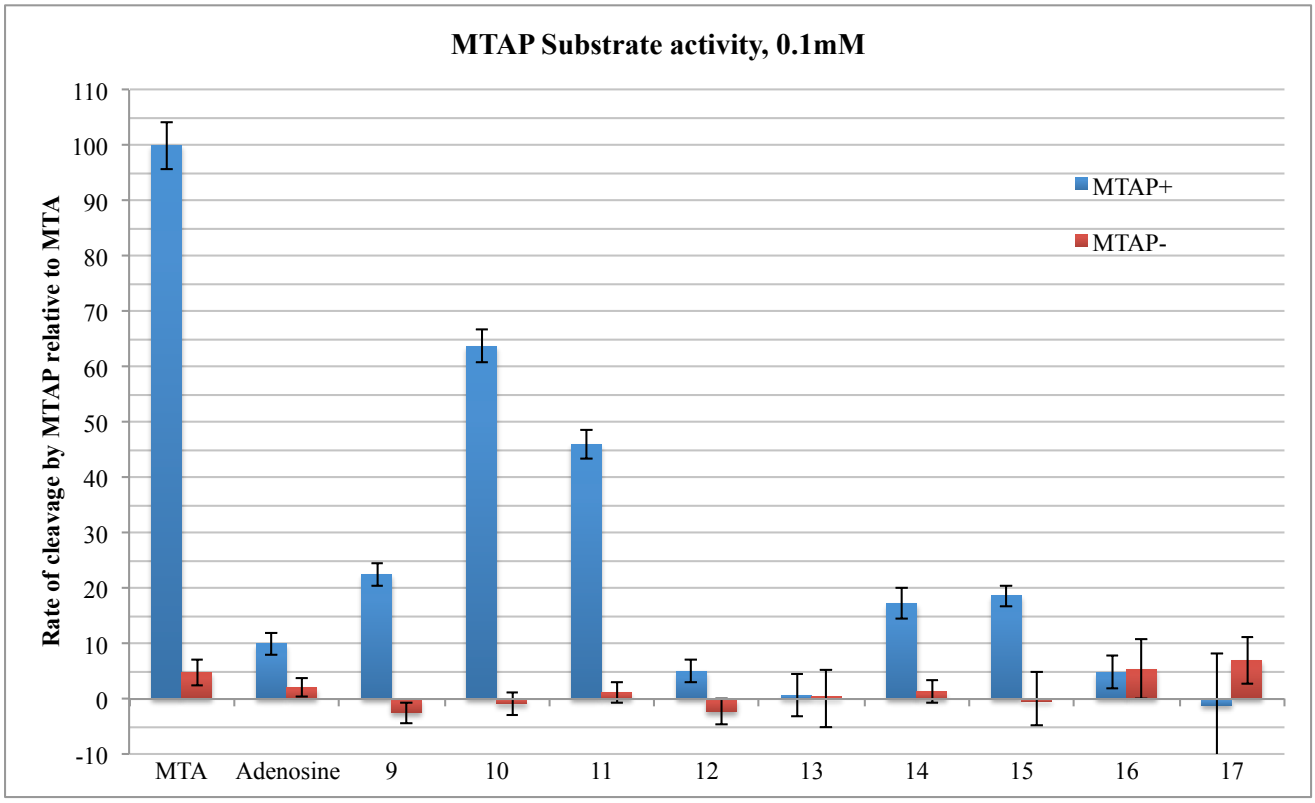
Table of slopes and uncertainties for MTAP-XO Assay 1, MTAP–, 0.1mM substrate					
Comp-ound	**Slope ± slope error, for each replicate**			**Average slope ± standard error**	**Slope relative to MTA ± standard error**
	1	**2**	**3**		
MTA	0.80 ± 0.14	0.24 ± 0.20	–0.22 ± 0.17	0.27 ± 0.17	4.1 ± 2.6
PTA	0.32 ± 0.07	–0.03 ± 0.07	0.02 ± 0.12	0.10 ± 0.09	1.5 ± 1.4
1	0.17 ± 0.09	0.18 ± 0.06	0.17 ± 0.08	0.17 ± 0.08	2.6 ± 1.1
2	0.20 ± 0.09	–0.07 ± 0.13	0.16 ± 0.09	0.09 ± 0.10	1.4 ± 1.6
3	–0.17 ± 0.27	0.04 ± 0.30	0.19 ± 0.23	0.02 ± 0.27	0.3 ± 4.0
4	0.08 ± 0.07	–0.10 ± 0.09	0.06 ± 0.12	0.04 ± 0.09	0.5 ± 1.4
5	0.13 ± 0.10	–0.03 ± 0.09	–0.07 ± 0.09	0.04 ± 0.10	0.5 ± 1.6
6	–0.12 ± 0.10	0.02 ± 0.11	0.06 ±0.06	–0.05 ± 0.09	–0.8 ± 1.3
7	0.10 ± 0.11	0.04 ± 0.11	0.01 ± 0.09	0.05 ± 0.10	0.8 ± 1.6
8	0.12 ± 0.11	0.01 ± 0.13	0.01 ± 0.12	0.03 ± 0.12	0.4 ± 1.8


MTAP Substrate activity (0.1mM)
Rate of MTAP Cleavage (Relative to MTA)
105
95
85
75
65
55
45
35
25
15
5
-5
MTAP+
MTAP-
MTA
PTA
1
2
3
4
5
6
7
8

MTAP-XO Assay 2 ([MTAP]=0.012μg/μL, [XO]=0.05U/μL)

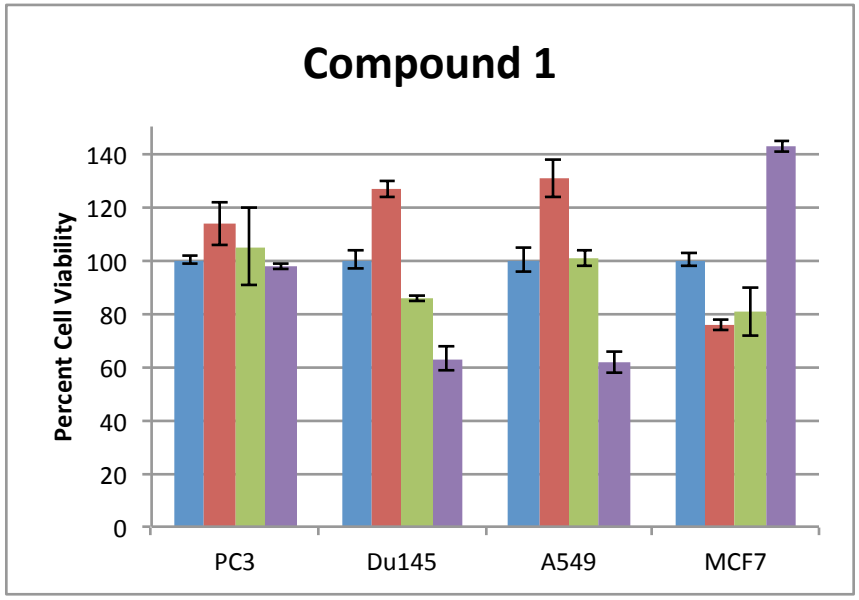
Table of slopes and uncertainties for MTAP-XO Assay 2, MTAP+, 0.1mM substrate												
Repli-cate		**MTA**	**Aden osine**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	slope	1.12	0.43	0.04	0.55	0.57	0.44	1.13	0.30	0.75	-0.08	2.88
	error	0.14	0.19	0.17	0.22	0.15	0.15	0.26	0.21	0.17	0.23	0.90
2	slope	1.75	-0.01	0.19	0.82	0.70	0.24	0.56	0.53	0.32	0.78	-2.75
	error	0.09	0.09	0.07	0.06	0.11	0.08	0.12	0.15	0.06	0.19	0.40
3	slope	2.42	0.03	0.36	1.21	0.70	0.28	0.34	0.12	0.09	-0.24	1.92
	error	0.07	0.10	0.07	0.09	0.14	0.09	0.11	0.09	0.09	0.10	0.36
4	slope	2.46	0.24	0.54	1.14	1.40	-0.02	2.02	0.12	0.25	0.07	2.02
	error	0.16	0.09	0.10	0.06	0.10	0.11	0.39	0.09	0.08	0.10	0.45
5	slope	2.08	0.21	0.33	1.04	0.72	-0.03	0.35	-0.05	0.38	0.42	-1.32
	error	0.33	0.09	0.11	0.14	0.10	0.13	0.13	0.14	0.10	0.14	0.27
6	slope	1.46	0.27	0.30	1.04	0.72	0.20	-0.02	-0.05	0.38	-0.05	0.44
	error	0.21	0.07	0.11	0.11	0.13	0.08	0.12	0.17	0.10	0.11	0.28
7	slope	1.35	0.21	0.60	1.13	0.17	0.21	0.62	-0.05	0.39	0.31	-0.37
	error	0.13	0.06	0.10	0.13	0.14	0.11	0.16	0.21	0.10	0.11	0.44
8	slope	1.29	0.20	0.42	1.36	1.47	-0.22	1.28	0.74	0.32	0.32	-1.00
	error	0.11	0.06	0.10	0.09	0.13	0.14	0.17	0.13	0.08	0.11	0.55
9	slope	1.25	0.16	0.30	0.97	0.35	0.12	-0.08	0.85	0.34	-1.07	1.74
	error	0.10	0.12	0.10	0.10	0.14	0.09	0.17	0.14	0.11	0.28	0.41
10	slope	1.17	0.20	0.39	1.00	0.78	-0.01	1.33	0.44	0.37	0.17	-0.84
	error	0.11	0.10	0.10	0.10	0.08	0.12	0.10	0.11	0.09	0.11	0.58
Average	average slope	1.69	0.17	0.38	1.08	0.78	0.08	0.71	0.29	0.32	0.08	-0.02
	standard error	0.16	0.10	0.11	0.12	0.12	0.11	0.19	0.15	0.10	0.16	0.49
Average relative to MTA	slope	100	9.97	22.5	63.7	46.1	5.00	42.0	17.3	18.6	4.76	-1.14
	standard error	4.29	1.98	2.11	2.96	2.70	2.09	3.82	2.85	2.00	2.96	-9.23

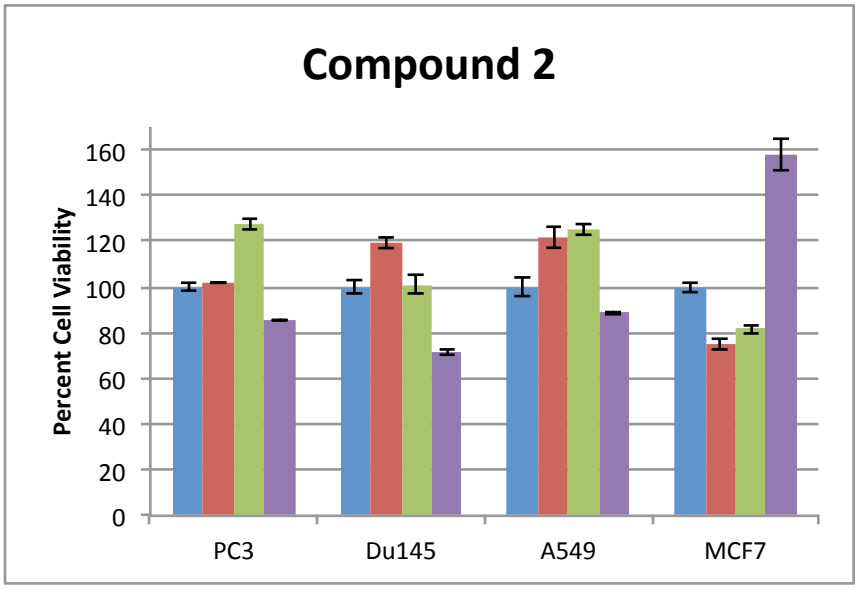
Table of slopes and uncertainties for MTAP-XO Assay 2, MTAP–, 0.1mM substrate												
Repli-cate		**MTA**	**Adenosine**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	slope	-0.02	0.25	0.07	0.37	0.25	-0.20	0.05	-0.05	1.50	0.78	0.68
	error	0.18	0.13	0.16	0.22	0.16	0.16	0.31	0.11	0.34	0.25	0.21
2	slope	0.22	0.13	-0.13	0.17	0.08	0.35	-0.43	0.10	-0.31	0.70	1.19
	error	0.12	0.09	0.09	0.13	0.13	0.10	0.48	0.10	0.16	0.36	0.24
3	slope	0.13	0.05	-0.20	0.00	0.35	0.54	0.36	-0.11	-0.14	-0.04	-0.01
	error	0.13	0.07	0.10	0.08	0.11	0.09	0.31	0.10	0.41	0.27	0.17
4	slope	0.29	0.17	0.01	0.01	-0.01	-0.29	0.87	0.14	-0.80	2.33	0.81
	error	0.09	0.09	0.09	0.08	0.10	0.11	0.30	0.13	0.20	0.19	0.16
5	slope	0.10	0.00	0.04	-0.07	0.03	0.68	-0.61	0.12	-0.54	-1.52	-0.41
	error	0.13	0.11	0.07	0.09	0.09	0.08	0.34	0.12	0.24	0.51	0.29
6	slope	-0.17	-0.04	0.00	-0.12	-0.09	-0.39	0.18	-0.03	-0.96	1.37	0.99
	error	0.10	0.08	0.09	0.09	0.07	0.13	0.15	0.12	0.17	0.35	0.12
7	slope	0.23	0.05	-0.06	-0.01	-0.24	0.70	0.29	-0.01	0.04	-1.33	0.39
	error	0.13	0.08	0.10	0.07	0.08	0.13	0.16	0.11	0.34	0.17	0.23
8	slope	0.15	0.04	0.01	-0.23	0.08	0.02	-0.10	-0.17	-0.82	0.09	-0.56
	error	0.10	0.09	0.11	0.11	0.09	0.09	0.24	0.11	0.30	0.15	0.19
9	slope	0.03	0.09	0.07	0.00	0.02	0.18	-0.43	-0.21	-0.01	0.42	-1.34
	error	0.10	0.09	0.07	0.08	0.09	0.08	0.20	0.08	0.08	0.14	0.36
10	slope	0.16	-0.18	-0.10	0.12	-0.04	-0.40	0.06	-0.17	-0.01	-0.13	0.19
	error	0.09	0.10	0.12	0.09	0.09	0.17	0.09	0.13	0.12	0.22	0.16
Average	average slope	0.11	0.03	-0.04	-0.01	0.02	0.16	0.02	0.00	-0.20	0.21	0.14
	standard error	0.12	0.09	0.10	0.11	0.10	0.12	0.28	0.11	0.26	0.28	0.22
Average relative to MTA	slope	6.68	2.03	-2.46	-0.85	1.25	9.23	1.28	-0.01	-11.9	12.4	8.26
	standard error	2.25	1.74	-1.92	-2.08	1.96	2.24	5.23	-2.11	-4.84	5.31	4.18

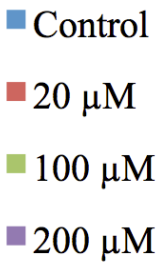

MTAP Substrate activity, 0.1mM
Rate of cleavage by MTAP relative to MTA
110
100
90
80
70
60
50
40
30
20
10
0
-10
MTAP+
MTAP-
MTA
Adenosine
9
10
11
12
13
14
15
16
17

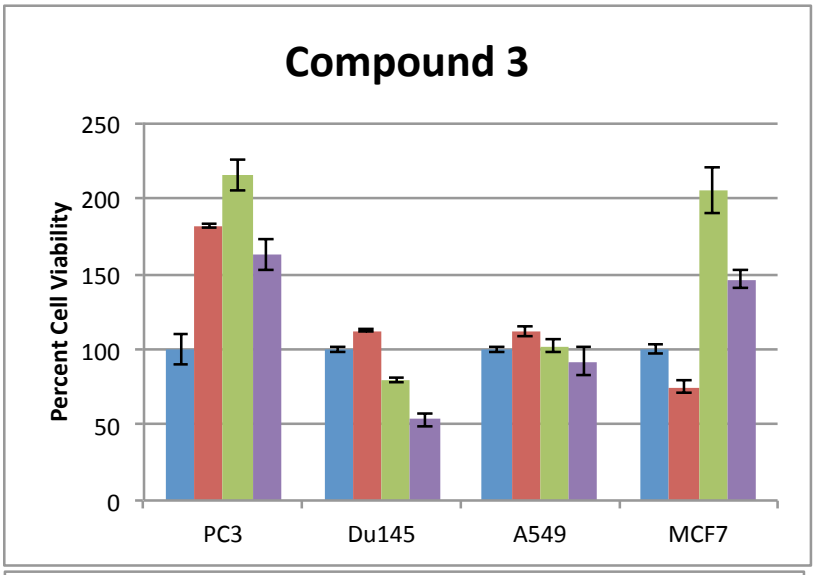
Cytotoxicity Assay 1

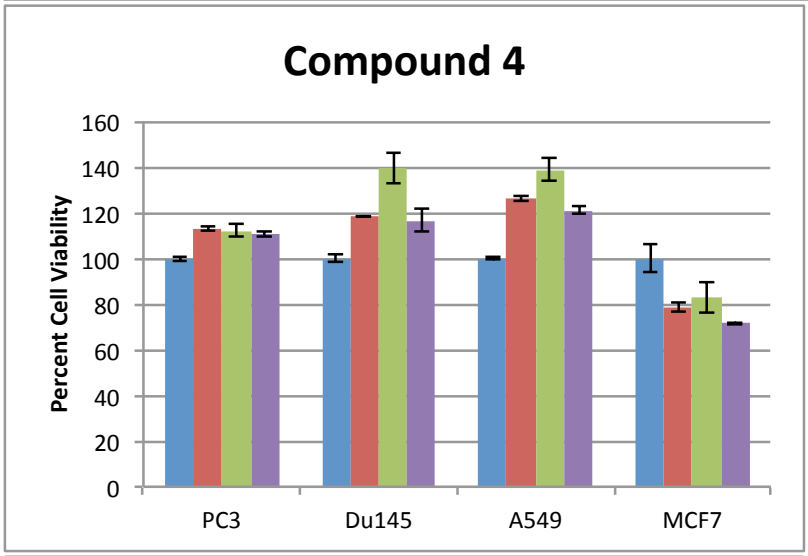
(Work performed by Dr. Hongwei Cheng at the BC Cancer Agency)
Cell lines:
PC3 (prostrate cancer cells, high metastatic potential) (MTAP+)
Du145 (prostrate cancer cells, moderate metastatic potential) (MTAP+)
A549 (adenocarcinomic human alveolar basal epithelial cells) (MTAP–)
MCF7 (breast cancer cells) (MTAP–)

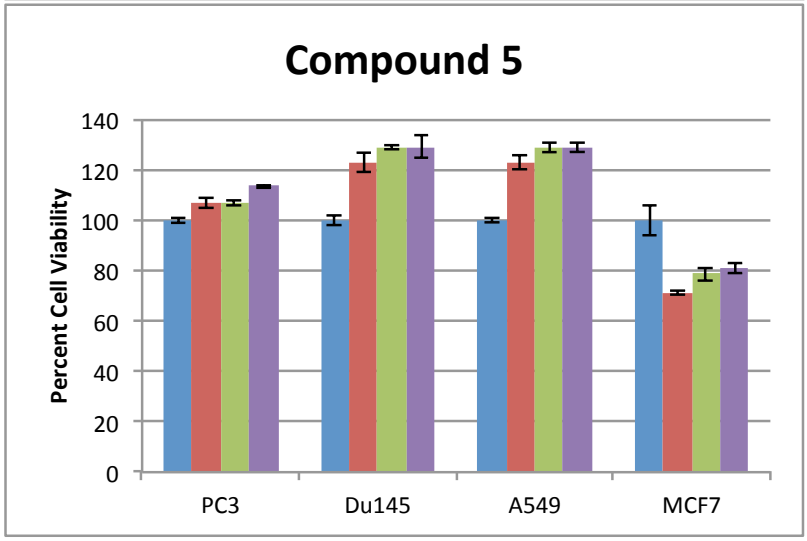


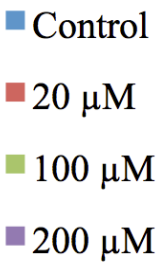


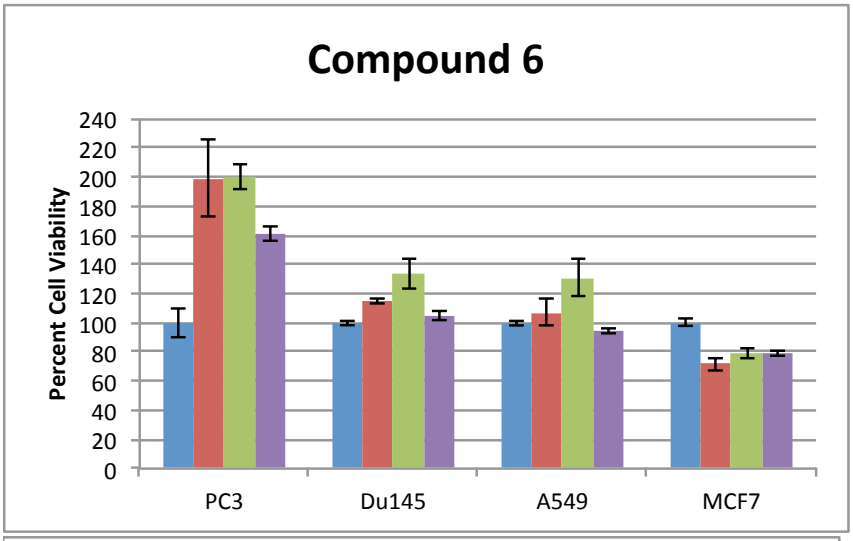


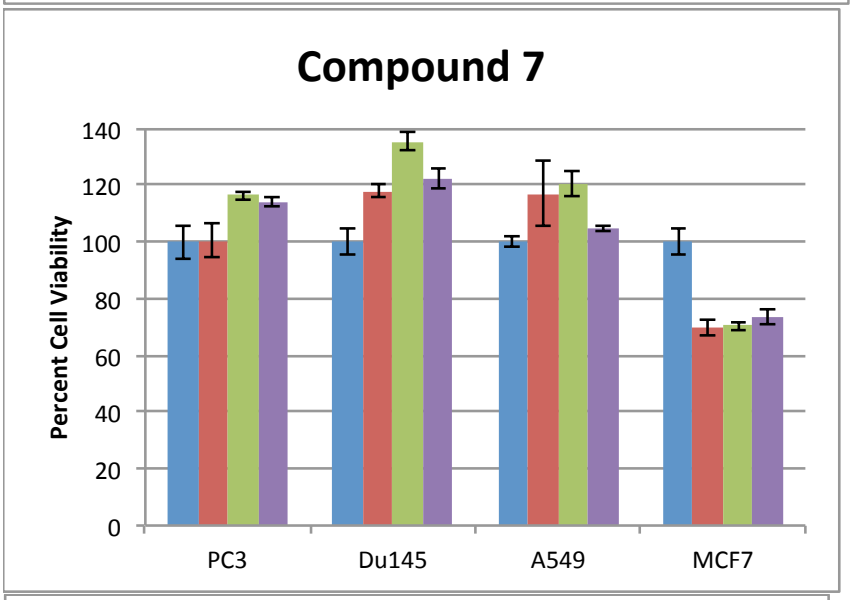

Compound 3
Percent Cell Viability
250
200
150
100
50
0
PC3
Du145
A549
MCF7

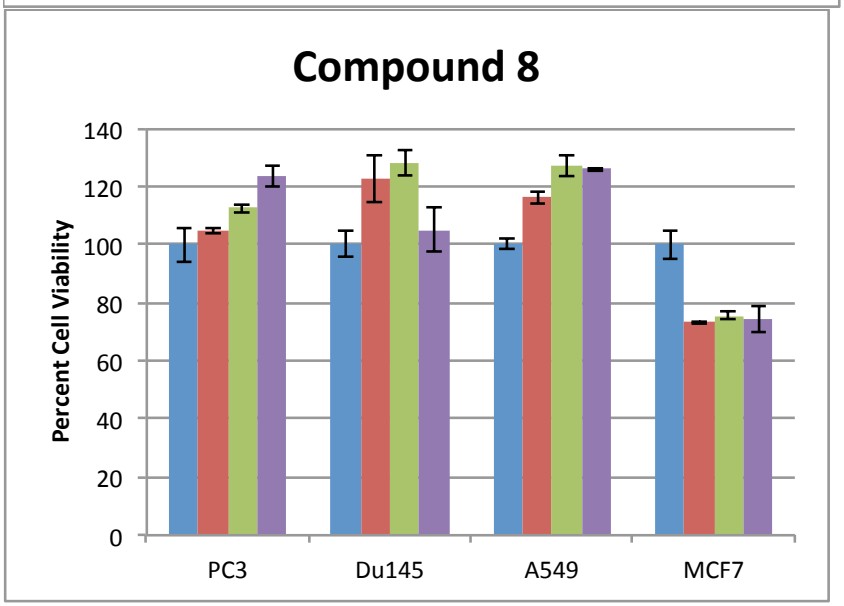

Compound 4
Percent Cell Viability
160
140
120
100
80
60
40
20
0
PC3
Du145
A549
MCF7


Compound 5
Percent Cell Viability
140
120
100
80
60
40
20
0
PC3
Du145
A549
MCF7


Control
20 µM
100 µM
200 µM



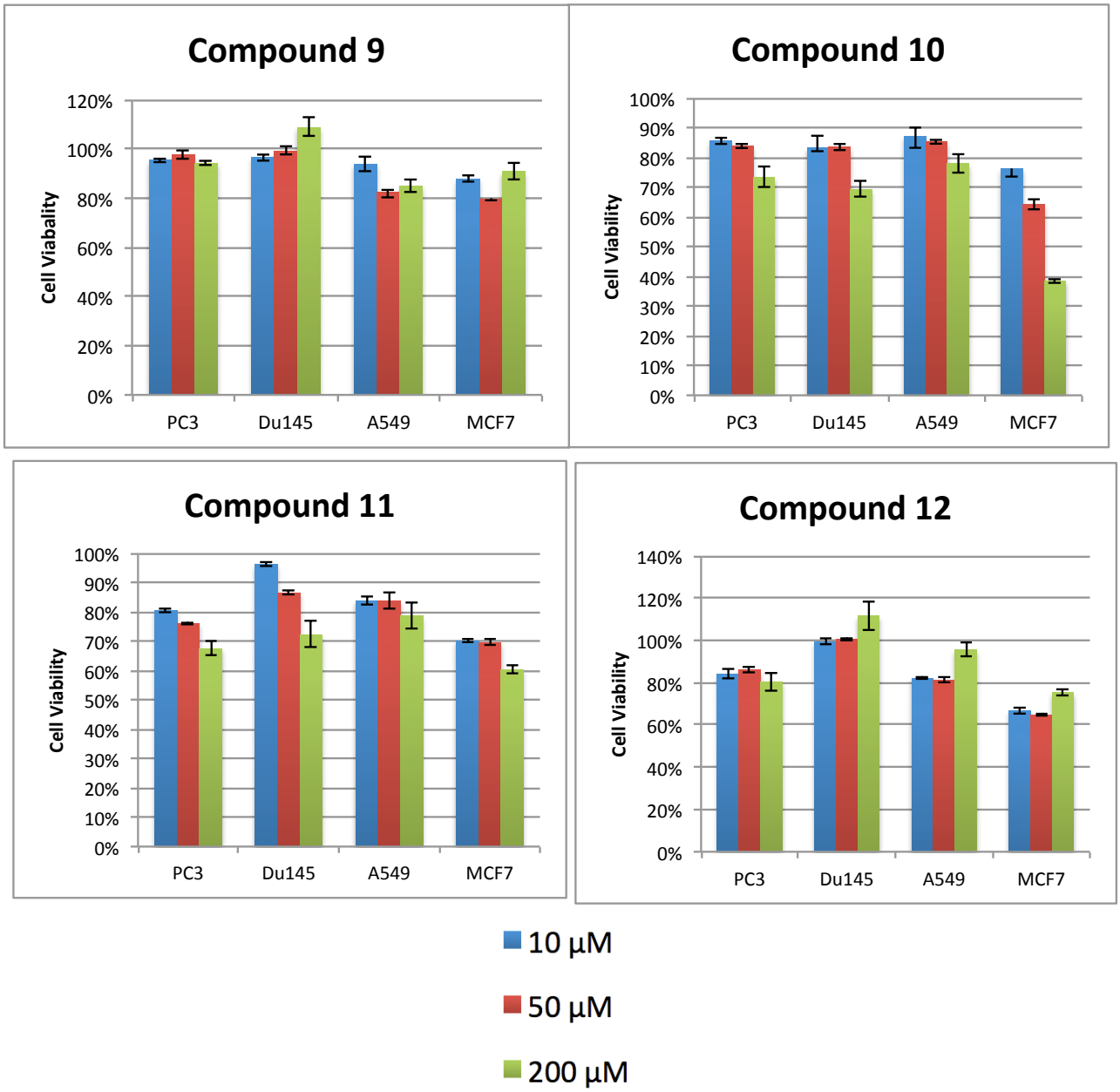


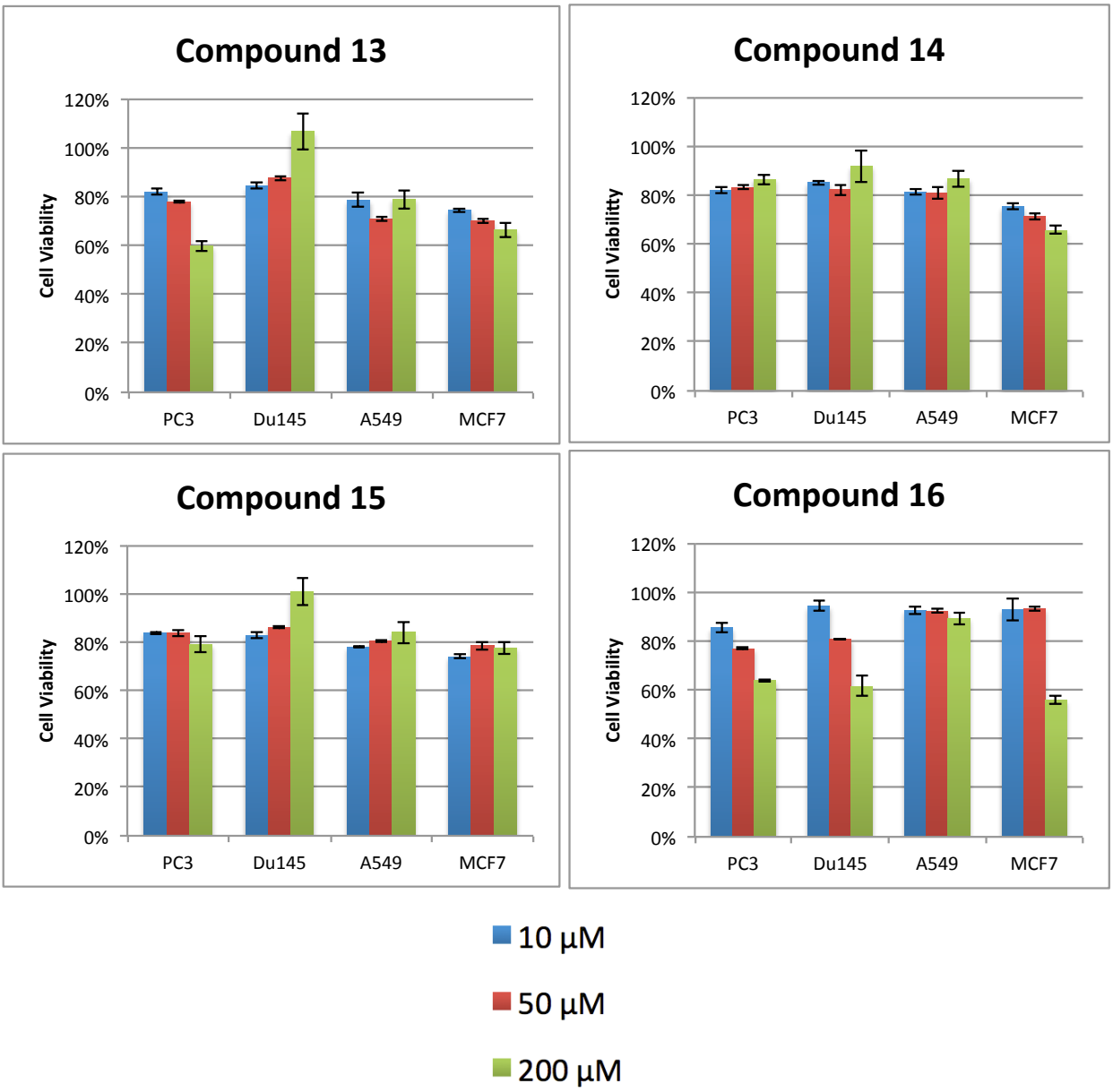


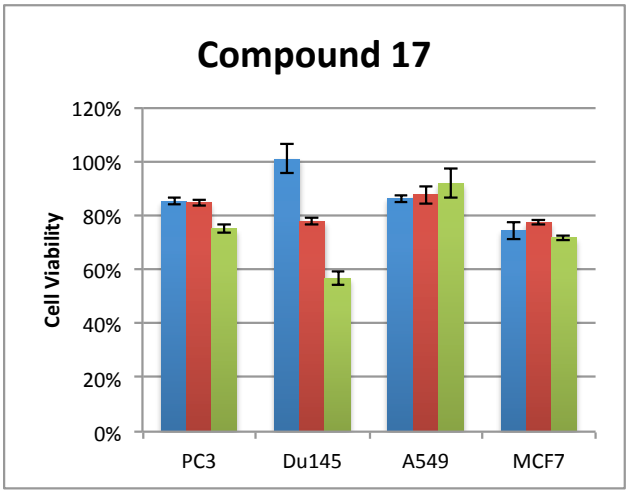
Control
20 μM
100 μM
200 μM

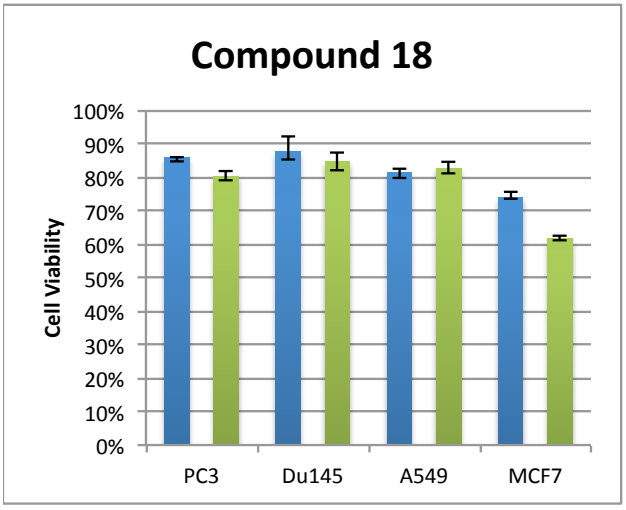
Cytotoxicity Assay 2

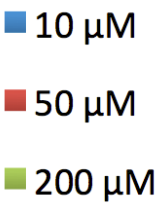
(Work performed by Dr. Hongwei Cheng at the BC Cancer Agency)
Cell lines:
PC3 (prostrate cancer cells, high metastatic potential) (MTAP+)
Du145 (prostrate cancer cells, moderate metastatic potential) (MTAP+)
A549 (adenocarcinomic human alveolar basal epithelial cells) (MTAP–)
MCF7 (breast cancer cells) (MTAP–)

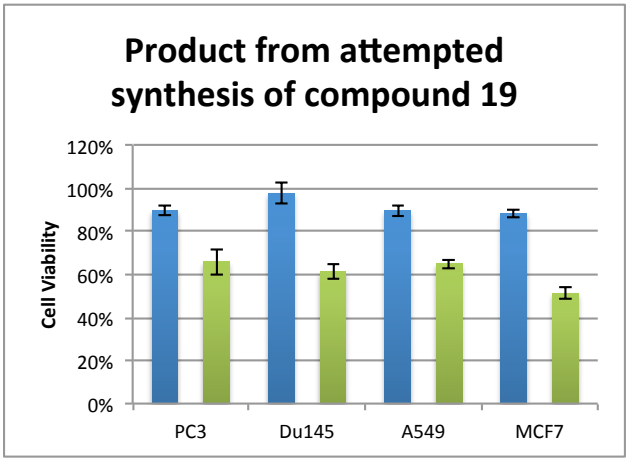



Compound 13
Cell Viability
120%
100%
80%
60%
40%
20%
0%
PC3
Du145
A549
MCF7
Compound 14
Cell Viability
120%
100%
80%
60%
40%
20%
0%
PC3
Du145
A549
MCF7
Compound 15
Cell Viability
120%
100%
80%
60%
40%
20%
0%
PC3
Du145
A549
MCF7
Compound 16
Cell Viability
120%
100%
80%
60%
40%
20%
0%
PC3
Du145
A549
MCF7
10 µM
50 µM
200 µM


Compound 17
120%
100%
80%
60%
40%
20%
0%
Cell Viability
PC3
Du145
A549
MCF7


Compound 18
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Cell Viability
PC3
Du145
A549
MCF7


10 µM
50 µM
200 µM


Product from attempted synthesis of compound 19
120%
100%
80%
60%
40%
20%
0%
Cell Viability
PC3
Du145
A549
MCF7