DESIGN, SYNTHESIS AND EVALUATION OF POTENTIAL MTA PRODRUGS FOR THE TREATMENT OF MTAP-DEFICIENT TUMORS IN COMBINATION WITH 5-FU/6-TG/ANTIFOLATES

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A thesis submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Medicinal Chemistry

Written under the direction of

Professor Longqin Hu

And approved by

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New Brunswick, New Jersey

October, 2018

ABSTRACT OF THE THESIS

DESIGN, SYNTHESIS AND EVALUATION OF POTENTIAL MTA PRODRUGS FOR THE TREATMENT OF MTAP-DEFICIENT TUMORS IN COMBINATION WITH 5-FU/6-TG/ANTIFOLATES

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Many solid tumors and hematologic malignancies are characterized by a deficiency of the enzyme methylthioadenosine phosphorylase (MTAP). MTAP cleaves its natural substrate MTA which is generated during polyamine biosynthesis to adenine via a salvage pathway for AMP production. MTAP deficient cells are unable to salvage adenine from MTA. The difference in the lack of expression of MTAP in tumor cells compared to normal healthy cells has been explored in a therapeutic strategy for selectively killing tumor cells. Antimetabolites such as 5-FU, 6-TG or antifolates disrupt DNA replication and inhibit de-novo purine synthesis through the release of cytotoxic nucleotides generated via phosphorylation using phosphoribosyl-1-pyrophosphate (PRPP). Unfortunately, one major drawback of these cytotoxic nucleotides is that they produce harmful effects on the growth and proliferation of normal cells. To eliminate or reduce the cytotoxicity of these chemotherapeutic drugs and thus increase their therapeutic index, administration of MTA or MTA analogs in conjugation with antimetabolites could prevent damage to healthy

MTAP competent cells. This is because adenine generated in this process by MTAP in healthy cells will block the conversion of 5-FU/6-TG to their cytotoxic nucleotides by competing for the rate-limiting pools of PRPP. Since no adenine is produced in tumor cells due to lack of MTAP, PRPP is present in sufficient levels and the co-administered drug can be readily converted to its toxic metabolite. Thus, a high degree of selectivity can be achieved. There are several MTA prodrugs discussed in the literature for delivering MTA to protect MTAP competent cells and proof of concept exists through in vitro studies emphasizing the protective effects of MTA in the presence of antimetabolites. However, there is still no successful clinical trial reported to have co-administered MTA or its analog with an antimetabolite. Also, the optimum dose of MTA that can rescue normal MTAP competent cells without compromising the ability of antimetabolites to selectively kill MTAP deficient tumor cells remains to be identified. In this thesis work, prodrugs of MTA were designed to be activated by the carboxylesterases to release MTA. We explored two types of prodrugs, namely the *N*-(alkyloxy)carbonyl-MTA derivatives and *N*-[(acyloxy)alkyloxy]carbonyl-MTA derivatives. The *N*-(alkyloxy)carbonyl-MTA prodrugs were stable at physiological pH and showed longer activation half-lives when tested in mouse liver microsomes but failed to be activated in human liver microsomes. Conversely, the *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs showed shorter half-lives ranging from a few minutes to a few hours in the presence of mouse and human liver microsomes but suffered from an inherent instability at physiological pH. As expected, the hydrolytic susceptibility of the ester group in *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs decreased with increasing steric hindrance around the ester bond by replacement with

bulkier alkyl groups. Therefore, the *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs show promise and further modifications can be made to increase stability at physiological pH.

ACKNOWLEDGEMENTS

I would like to extend my gratitude to my advisor Professor Longqin Hu, Department of Medicinal Chemistry, Rutgers University, for his continued support, patience and motivation throughout my studies. I appreciate his guidance and constructive criticism, both of which will surely benefit my future professional development.

My sincere thanks to Professor Joseph. E. Rice and Professor Edmond J. LaVoie for their valued input throughout the completion of my degree. I am also grateful to Professor David J. Augeri for his time and effort spent as part of my thesis committee.

I want to thank my fellow lab colleagues and friends, Haifa Albanyan, Dhulfiqar Abed, Ahmed Ali, Sumi Lee, Husam Zahid, Subhadwip Basu, Jeffrey Yang, Rajlakshmi Sawant and Shravani Barkund for their insight which has certainly helped me excel in my research. I especially thank Akash Taneja, a fellow lab member and close friend for his continuous support and motivation that has helped me succeed at every step of my work. You shall stay in my memory always.

I would like to thank Dr. Michael J. Vitarelli, Department of Chemistry and Chemical Biology for giving me the opportunity to serve as a Teaching Assistant.

A special thank you to Elissa Glinn for being a wonderful person and helping me both personally and professionally.

Last but not least, I cannot thank my parents enough for their support and their faith in me, both of which have been imperative in the pursuit of my goals.

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ABBREVIATIONS

5,10-MTHF	5,10-Methylenetetrahydrofolate
5'-DFCR	5’-Deoxy-5-fluorocytidine
5'-DFUR	5’-Deoxy-5-fluorouridine
5-FU	5-Fluorouracil
5-MTHF	5-Methyltetrahydrofolate
6-MP	6-Mercaptopurine
6-TG	6-Thioguanine
AICARFT	5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase
AMP	Adenosine monophosphate
APRT	Adenine phosphoribosyltransferase
BNPP	Bis(4-Nitrophenyl)phosphate
CAD	Cytidine deaminase
CES	Carboxylesterase
CsF	Cesium flouride
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DIPEA	N,N-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
dUMP	Deoxyuridine monophosphate
dUTMP	Deoxyuridine triphosphate
FdUMP	5-Fluoro-2'-deoxyuridine-5'-monophosphate
FdUTP	5-Fluoro-2'-deoxyuridine-5'-triphosphate
FUTP	5-Fluorouridine-5’-triphosphate
GARFT	Glycinamide ribonucleotide formyltransferase
Glu	Glutamic acid
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
His	Histidine
HLM	Human liver microsomes

IMP	Inosine monophosphate
K_2HPO_4	Dipotassium phosphate
MLM	Mouse liver microsomes
MTA	5'-Deoxy-5'-methylthioadenosine
MTAP	Methylthioadenosine phosphorylase
MTR-1-P	5-Methylthioribose-1-phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
PRPP	Phosphoribosyl-1-pyrophosphate
Ser	Serine
TBAF	Tetrabutylammonium flouride
$TBAHSO_4$	Tetrabutylammonium hydrogensulfate
TBDMSCl	Tert-butyldimethylsilyl chloride
TFA	Triflouroacetic acid
THF	Tetrahydrofolate
TMSCl	Trimethylsilyl chloride
TP	Thymidine phosphorylase
TS	Thymidylate synthase

INTRODUCTION

Each year, approximately 14 million people are diagnosed with cancer all over the world. Being the second leading cause of mortality, it accounted for 8.8 million deaths globally in 2015.[1] The global burden of cancer is significantly increasing and the search for new chemotherapeutic drugs is imperative along with the possibility of repurposing existing drugs. Cancer manifests itself in several different organs and is mainly characterized by abnormal cell growth in a multi-stage process that generally progresses from a pre-cancerous lesion to a malignant tumor. The major risk factors for developing cancer include genetic manipulations, physical, chemical and biological carcinogens and aging.[2] Cancer has a potential to metastasize and hence, is classified as a systemic disease.

The modalities of cancer treatment include surgery, radiation, chemotherapy (traditional and targeted), hormone therapy and immunotherapy. Most traditional chemotherapeutic drugs inhibit the metabolic functions of cancer cells by targeting different phases of the cell cycle. Antimetabolites, one type of these drugs, were among the first effective agents discovered for cancer treatment.[3]

1.1 Introduction to antimetabolites

Depending on the biological pathway that they target, antimetabolites belong to one of three classes: **folate antagonists, pyrimidine-based antagonists and purine-based antagonists.** Inhibitory effects on either the folate metabolism pathway, the pyrimidine

biosynthesis pathway or the purine biosynthesis pathway ultimately hamper the de-novo synthesis of DNA.[3, 4] These three pathways are discussed in detail below.

1.1.1 Targeting the folate metabolism pathway

Folic acid undergoes intracellular reduction by dihydrofolate reductase (DHFR). First, folic acid is converted into dihydrofolate (DHF) and then into tetrahydrofolate (THF). THF can be a substrate for one of two pathways. In one of the pathways, it can be further converted to 5-methyltetrahydrofolate (5-MTHF) via 5,10-methylenetetrahydrofolate (5,10-MTHF). 5,10-MTHF is also a substrate for the enzyme thymidylate synthase (TS) to methylate deoxyuridine monophosphate (dUMP) to deoxyuridine triphosphate (dUTMP) which is a precursor for the de novo synthesis of thymidine nucleotides for DNA synthesis. In a second pathway, THF can be further converted to 10-formyl THF which is used by glycinamide ribonucleotide formyltransferase (GARFT) and subsequently, by 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT) to form inosine monophosphate (IMP). The formation of IMP can serve as a precursor for adenosine monophosphate (AMP). The mode of action of the essential enzymes in these pathways is described in **Figure 1.**[3] Folate antagonists are inhibitors of DHFR, GARFT and TS.[4] Examples of this class of folate antagonists include Methotrexate (DHFR inhibitor), Nolatrexed (TS inhibitor) and Lometrexol (GARFT inhibitor).

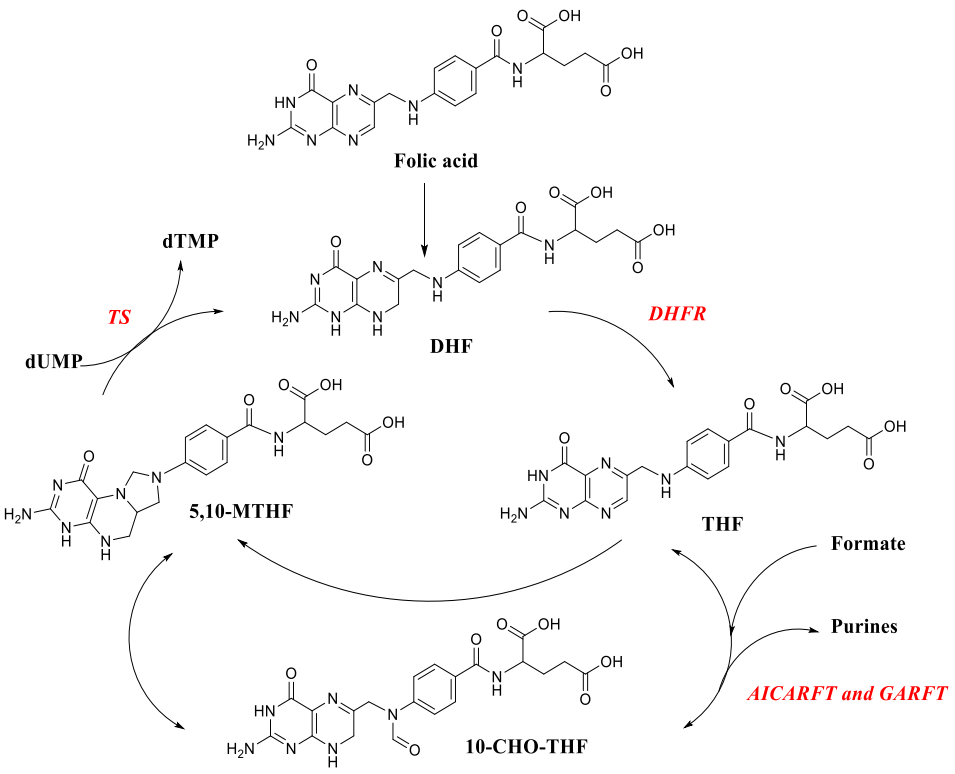


Figure 1. Folic acid metabolism[3]

1.1.2 Targeting pyrimidine biosynthesis pathway

Some antimetabolites mimic the pyrimidine bases, uracil and cytosine, and inhibit one or more enzymes that are critical for DNA synthesis. 5-Fluorouracil (administered in the prodrug form as capecitabine) is structurally similar to uracil, containing a fluorine atom at the C-5 position of the ring. It inhibits TS, preventing the conversion of uracil to thymidine. The active metabolites FUTP, FdUMP and FdUTP inhibit the synthesis of DNA and RNA and thus, cause cell death.[4, 5]

1.1.3 Targeting purine biosynthesis pathway

Some antimetabolites such as 6-thioguanine (6-TG) mimic the purine bases, guanine and adenine, and inhibit production of DNA. 6-TG and its metabolites block the salvage pathway as well as the de-novo purine synthesis pathway as described in **Figure 2.** They compete first with hypoxanthine and guanine for Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and further, inhibit conversion of IMP to XMP.[5, 6] Other FDA approved analogues of purine bases include cladribine, 6-mercaptopurine and clofarabine.

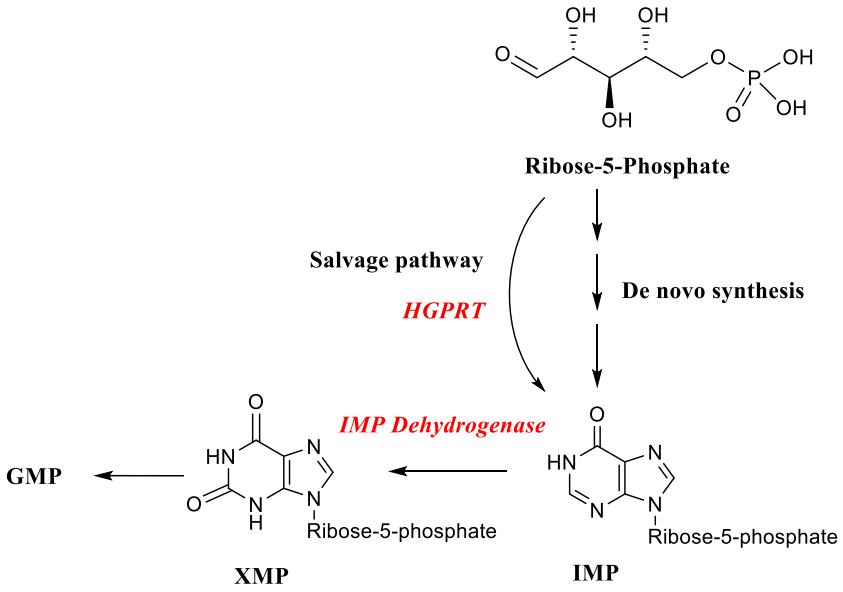


Figure 2. Purine biosynthesis pathway

Although antimetabolites have been found to be successful chemotherapeutic drugs, they cannot differentiate between normal cells of the body and cancer cells, and they have a relatively low therapeutic index.[7] Thus, research efforts are being directed towards the development of tumor specific agents. One approach in developing these tumor specific

agents is based on molecular differences between tumor and normal cells. If this specificity is achieved, a higher dose of antimetabolites could possibly be administered to increase their effectiveness. [8]

1.2 Role of MTAP in polyamine and purine metabolism

Methylthioadenosine phosphorylase (MTAP) is a key enzyme in the methionine salvage pathway that converts the polyamine byproduct 5'-methylthioadenosine (MTA) into adenine and 5-methylthioribose-1-phosphate (MTR-1-P) into methionine as described in **Figure 3.**[9]

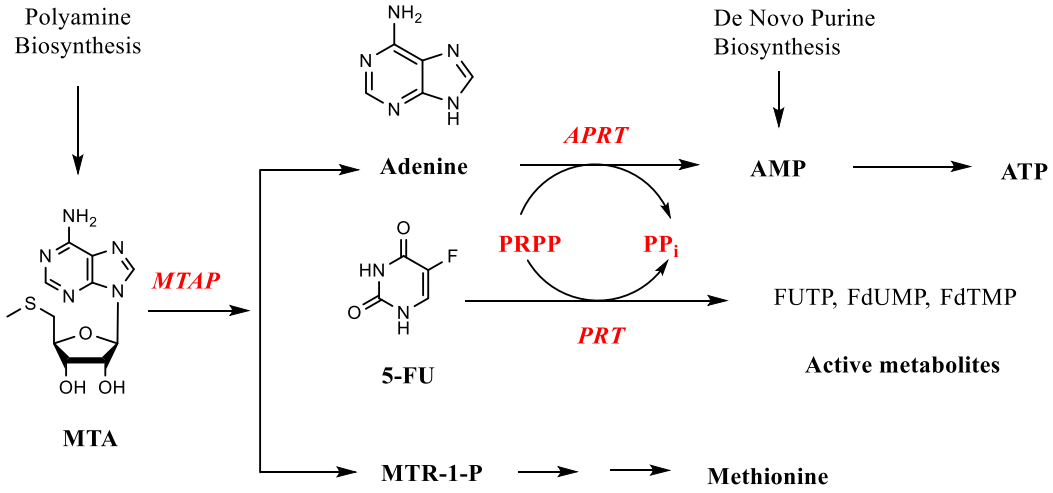


Figure 3. MTAP metabolic pathway [9]

Adenine is converted to AMP by the ubiquitous enzyme adenine phosphoribosyltransferase (APRT) with phosphoribosyl-1-pyrophosphate (PRPP) serving as donor of the phosphoribosyl group. AMP may also be produced in cells by de-novo purine biosynthesis.

MTAP is responsible for the generation of essentially all free adenine in human cells. Without adenine, there would be no AMP production and thus, no DNA synthesis.[10]

1.3 Selective killing of MTAP deficient tumor cells

It has been reported for many years that a variety of tumor cell types have a deficiency of MTAP as listed in **Table 1**.[11] This deficiency can be used for the development of selective cancer therapy. There are several reasons why tumor cells lack MTAP. MTAP gene deletion may occur if the gene is in close proximity (within 100 kb) to CDKN2A, which is homozygously deleted in approx. 15% of tumors. It can also occur if there is a selective deletion of MTAP locus or if methylation of the MTAP promotor occurs.[12] In healthy MTAP competent cells, AMP production relies on one of two pathways: (a) de-novo purine synthesis, or (b) an MTAP mediated salvage pathway as illustrated in **Figure 3**.[9] In tumor cells lacking MTAP, the MTAP mediated salvage pathway is blocked, and as a result, the de-novo purine synthesis pathway is utilized to produce AMP. Accordingly, turning off the de-novo pathway will result in selectively killing MTAP deficient tumor cells. The MTAP deficient nature of certain cancers provides an opportunity to design molecules that selectively kill MTAP deficient cells by preventing toxicity in MTAP competent cells.[11, 13]

Table 1. MTAP deficiency in solid tumors and hematologic malignancies[11]

Tumor type	MTAP deficiency (frequency)
Pancreatic cancer	91/300
Endometrial cancer	7/50
Metastatic melanoma	8/14
Gliomas	9/12
T-cell acute leukemia	28/45
Soft tissue sarcoma	8/21
Mesothelioma	64/95

Data can be found to support the idea that MTAP deficiency makes the tumor cells more sensitive to the effects of purine antagonists.[12-14] Experimental studies have indicated that combining a cytotoxic purine analog or an inhibitor of purine biosynthesis with an MTAP substrate, such as MTA, protected the $MTAP^+$ cells from toxicity of the administered inhibitors. These protective effects are associated with the enzymatic activity of MTAP, which converts MTA to adenine.[10, 15-17] In $MTAP^+$ cells, MTA is converted to adenine, which then converts to AMP via APRT, through a process that utilizes phosphoribosyl pyrophosphate (PRPP). Since cellular PRPP levels are now low, this chain of events would deplete PRPP and there would be less PRPP available for the conversion of toxic antimetabolites, such as 5-FU or 6-TG, to their respective nucleotide forms. Since there would be no conversion to toxic nucleotide forms, these toxic metabolites would not be

able to insert into DNA and inhibit cell proliferation. Thus, it can be deduced that adenine competes with antimetabolites like 5-FU or 6-TG for the rate limiting pool of PRPP. In contrast, $MTAP^-$ cells lack the ability to convert MTA to adenine and therefore, utilize the available PRPP to form toxic nucleotides. Evidence to show that MTAP is indeed responsible for cytoprotective effects comes from the experimental conclusion by Kruger et al. whereby, the addition of adenine protected both $MTAP^+$ and $MTAP^-$ HT1080 cells after administration of 5-FU or 6-TG.[10]

1.4 MTA as a protective agent

MTA is produced as a byproduct of the polyamine biosynthetic pathway during the synthesis of spermidine and spermine. As discussed previously, MTA is a substrate for the enzyme MTAP which metabolizes it to form adenine and methionine. In vitro studies have shown that an accumulation of MTA has a cytostatic effect. This is due to the feedback inhibition of spermidine and spermine synthase which downregulates the polyamine biosynthesis conferring anti-proliferative activity.[18] In experimental models, MTA in high doses has been shown to prevent liver damage.[19, 20] In a study of 50 volunteers, MTA was dosed daily at 600 mg for one month and to an additional 10 volunteers, it was dosed at 1600 mg daily for one month without reports of any toxicity in either group.[21, 22] These results show promise in designing molecules that can either deliver MTA or adenine to rescue $MTAP^+$ cells from toxic effects of antimetabolites.

One potential caveat to this approach is that as the level of MTA in cells increases, the level of adenine generated by normal tissues expressing MTAP increases as well. This increase in adenine can then be utilized by an MTAP deficient tumor cell to form AMP by consuming PRPP thus, decreasing the activation of purine analogs such as 5-FU to their toxic nucleotides. It is, therefore, imperative to optimize the dose of MTA in a cell for protection of normal tissues without compromising anti-tumor effects.[11] In a report by Riscoe et al., MTAP was found to be present in human serum.[23] If the activity of MTAP in serum is high enough to convert administered MTA (or an MTA analog) to significant levels of adenine, it can lead to protection of MTAP deficient tumor cells from purine analogs along with protection of normal cells as discussed above. However, MTAP requires the presence of PRPP for metabolizing MTA or MTA analogs to adenine and as such, in human serum, the presence of phosphate levels are not sufficient enough to observe any considerable MTAP activity.[24]

1.5 Pre-clinical reports on selective rescue of MTAP$^+$ cells

In vitro data from studies by Kruger et al. shows that MTA protection is quite remarkable with at least 10- to 100- fold changes in IC_{50} values when co-administered with 6-TG.[10] These findings suggest that higher doses of 6-TG than currently administered could be given before any side effects are seen. In addition, Bertino et al. have reported that co-administration of 100 mg/kg MTA protected nude mice from a lethal dose of 5-FU, even when the concentration of the inhibitor was increased from 75 to 200 mg/kg. In this experiment, MTA was administered to two groups of 3 female NCr-nu/nu mice each. In

the first group, 100 mg/kg MTA was given two times (60 and 15 min), prior to injection of 5-FU. In the second group, the same dose was administered 30 min before and after injecting 5-FU.[11]

1.6 Clinical trial targeting tumors that lack MTAP

In the only clinical trial to test the effects of selectively targeting MTAP deficient tumors, the de-novo purine synthesis inhibitor, L-alanosine, was used.[25] Patients with known MTAP deficient tumors were infused with the inhibitor at a starting dose of 80 mg/m^2 daily for 5 days, and this dose was then repeated every 21 days. Preclinical studies had demonstrated the selective cytotoxic activity of L-alanosine in MTAP deficient tumor cells.[14, 26] However, the results from the above clinical trial did not show effectiveness of the inhibitor to protect MTAP deficient tumor cells at the given dose and schedule. One possible explanation for these results was that the tumors might have salvaged enough adenine or adenosine from the blood to compete with the L-alanosine and thus, the desired cytotoxic effect of the inhibitor was not produced as expected for the MTAP deficient tumors.[11]

1.7 Design of MTAP substrates to target MTAP deficient tumors

Taking advantage of the MTAP deficiency in tumors, several efforts are being made to design MTAP substrates, with different physical and biological properties than the enzyme's natural substrate MTA, to act as anti-toxicity agents.[27] These substrates would

be converted after metabolism by MTAP to adenine. The adenine would then enter a de-novo AMP synthesis pathway and increase AMP production in $MTAP^+$ cells (non-tumor) but not in $MTAP^-$ cancer cells. Alternatively, MTA prodrugs can be designed to release MTA upon activation. Bloom L.A et al. have reported several MTA prodrug designs that have functional groups such as carbamates, phosphates and esters to improve MTA's solubility and bioavailability as shown in **Figure 4**.[28]

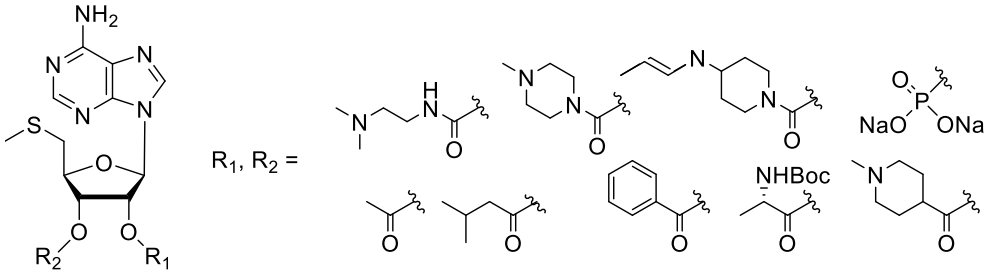


Figure 4. MTA prodrug designs reported in literature[28]

1.8 Carboxylesterases in the activation of prodrugs

Carboxylesterases (CESs) belong to the α/β hydrolase family and are present throughout the body in the blood, intestines, lungs and tumors. They play an important role in metabolizing several carbonate and carbamate containing substrates.[29] CESs use a catalytic triad (Ser-His-Glu) for hydrolysis. Although this triad is highly conserved in many different species, CESs are known to efficiently hydrolyze small substrates but, there may be a vast difference as to their ability to hydrolyze large substrates. Prodrugs designed to be activated by CESs have been reported in the literature.[30-32]

1.9 Rationale

The strategy of taking advantage of MTAP deficiency in tumors to achieve targeted treatment has proven to be challenging. We pursued the MTA prodrug approach which can deliver MTA at an optimal dose in order to rescue $MTAP^+$ normal cells from toxic effects of purine analogs. In the literature, several MTA prodrugs have been reportedly utilizing functional groups such as amides, esters and carbamates.[28] We focused our research on the carbamate prodrugs of MTA which are susceptible to cleavage by a carboxylesterase enzyme.[30, 32] Esterases are ubiquitously present in most animals including humans, and several types are present in blood, liver and other tissue. The initial design of the prodrug being tested was based on the activation of the marketed prodrug of 5-FU known as Capecitabine, as described in **Figure 5**. Clinically, Capecitabine passes intact through the intestinal mucosa and is converted to its active form, 5-FU, by a 3-step enzymatic activation in liver and tumor tissues. First, the carbamate moiety is hydrolyzed to 5'-deoxy-5-fluorocytidine (5'-DFCR) by carboxylesterase (CES) primarily in the liver with a reported half-life of 0.55 to 0.87 hours. Secondly, 5'-DFCR is further converted by cytidine deaminase (CAD) to 5'-deoxy-5-fluorouridine (5'-DFUR) which is then converted to 5-FU by thymidine phosphorylase (TP).[33]

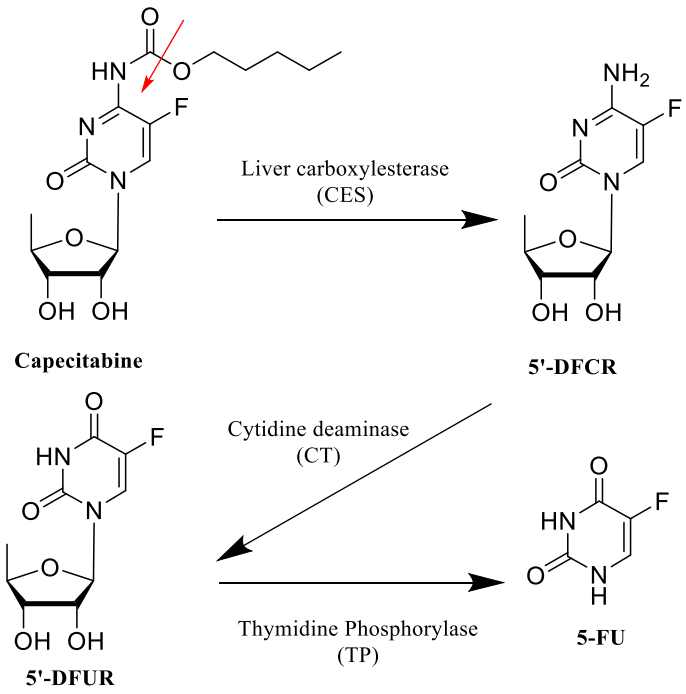


Figure 5. Capecitabine activation pathway

We used a similar strategy to design MTA carbamate prodrugs that can get activated by the liver carboxylesterases for systemic release of MTA. In the process of designing MTA carbamate prodrugs, we began by varying the alkyl groups of the promoiety as depicted in **Figure 6** to discern what effect the alkyl group had on the rate of cleavage of the carbamate moiety by the liver carboxylesterase. The carbamate functional group can impart chemical stability and since carbamates do not ionize, they can provide better bioavailability for the prodrugs. Upon hydrolysis, the prodrugs would release alcohol and carbamic acid that would be unstable under physiological pH and the carbamic acid would decompose spontaneously to an amine (in our case, MTA) and CO_2.

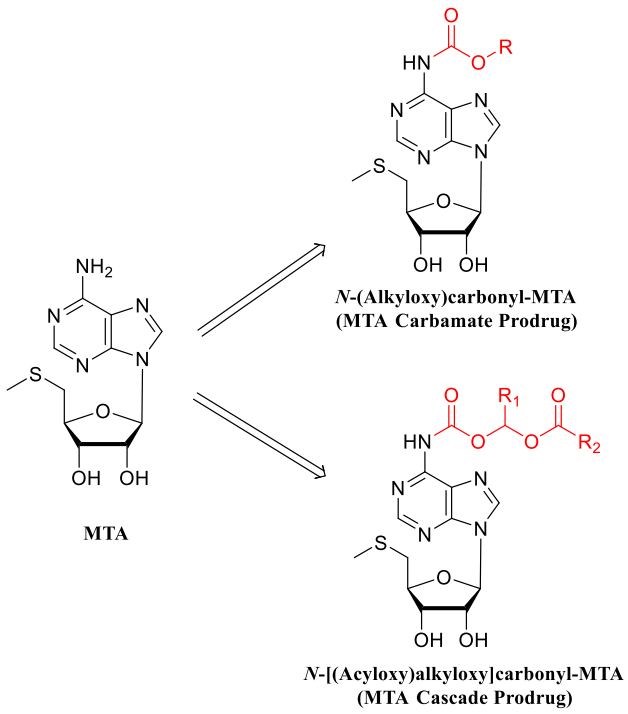


Figure 6. MTA prodrug designs

In a comparative study with the marketed prodrug Capecitabine, two compounds **LH1201** (*N*-(n-hexyloxy)carbonyl-MTA) and **LH1205** (*N*-(n-butoxy)carbonyl-MTA) were initially synthesized and tested for activation by CESs in mouse and human liver microsomes. Both compounds were found to be activated by the mouse liver microsomes only. The compounds were found to be stable in human liver microsomes. In order to test the viability of the human liver microsomes, we performed a parallel study with compounds **LH1201/LH1205** and Capecitabine in the presence of human liver microsomes. It was found that Capecitabine was activated by the human liver microsomes. We thus concluded that the human liver microsomes were active towards Capecitabine, however failed to activate the *N*-(alkyloxy)carbonyl-MTA prodrugs. To validate the role of CESs in carbamate prodrug hydrolysis, an activation assay was performed in the presence of bis(4-

nitrophenyl)phosphate (BNPP), an inhibitor of esterase. It was observed that no MTA was formed in the presence of BNPP and thus, we concluded that the alkyl carbamate prodrugs were activated by liver microsomal esterases only. A series of these *N*-(alkyloxy)carbonyl-MTA prodrugs were synthesized and tested for activation.

Our next approach was aimed at exploring the *N*-[(acyloxy)alkyloxy]carbonyl derivatives as depicted in **Figure 6,** taking advantage of the hydrolytic susceptibility of an ester group. Carboxylesterase would hydrolyze this modified double ester leading to a hemiacetal form of the prodrug (**I)** as shown in **Figure 7.** In an aqueous environment, (**I)** would decompose to form a carbamic acid **(II)** which would spontaneously decompose to give a free amine (in our case, MTA) and CO_2. A series of the above-mentioned MTA cascade prodrugs were synthesized modifying the ester groups as a measure of hydrolytic susceptibility.

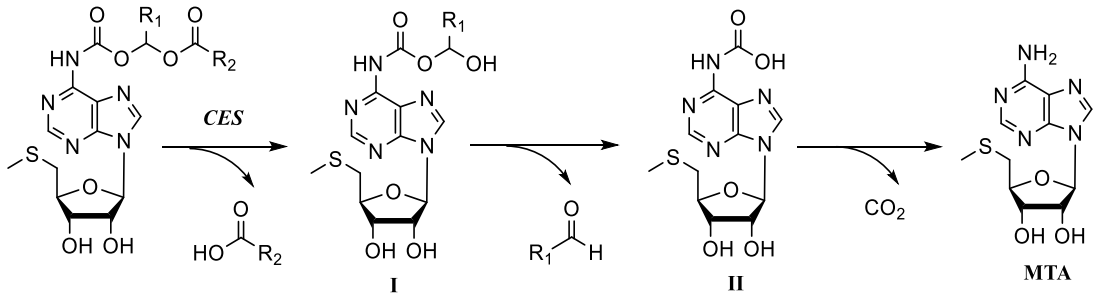


Figure 7. Cascade activation of *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs

Although several MTA prodrugs and treatments for administering MTA have been developed through our research and the research of others, at present, there are no such treatments available to patients. Studies continue in the development of more of these MTA analogs and prodrugs.

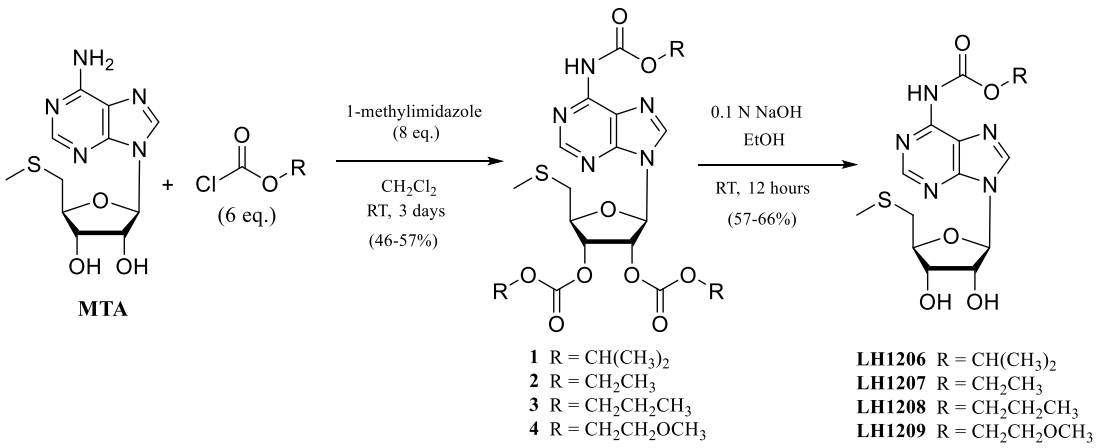
RESULTS AND DISCUSSIONS

2.1 Synthesis and evaluation of *N*-(alkyloxy)carbonyl-MTA Prodrugs

The results from our activation studies on previously synthesized compounds, **LH1201** and **LH1205,** showed promise in developing similar carbamate prodrugs that can be hydrolyzed in the mouse liver microsomes. In the microsomes, these prodrugs, which would deliver MTA for selectively targeting MTAP deficient tumor cells, were found to have a half-life of 1.2 hours and 9.4 hours respectively. Our initial studies were focused on synthesizing and evaluating simple *N*-(alkyloxy)carbonyl-MTA prodrugs for activation in both human and mouse liver microsomes.

The carbamate moiety belonging to compounds **LH1201** and **LH1205** was modified with shorter chain alkyl groups to analyze the half-life. The *N*-(alkyloxy)carbonyl-MTA prodrugs were synthesized in two steps as shown in **Scheme 1.**

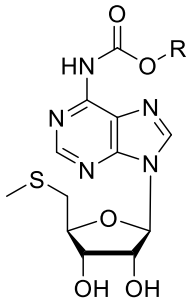
Treatment of commercially available 5'-methylthioadenosine (MTA) with commercially available alkyl chloroformates in anhydrous dichloromethane gave the intermediates as shown in **Scheme 1**. Further hydrolysis using 0.1 N NaOH yielded the desired prodrugs.



Scheme 1. Synthetic route for *N*-(alkyloxy)carbonyl-MTA prodrugs

Experimental results from an activation study in mouse liver microsomes on the above-mentioned prodrugs indicated a half-life ranging from 1.2 hours to 115 hours depending on the type of alcohol leaving group in the carbamate moiety as discussed in **Table 2**. Unfortunately, none of the prodrugs showed activation in human liver microsomes.

Table 2. Stability and activation of *N*-(alkyloxy)carbonyl-MTA prodrugs



Compound ID	R	$t_{1/2}$ (h)[a]		
		Buffer[b]	MLM	HLM[c]
LH1201	$CH_2CH_2CH_2CH_2CH_2CH_3$	>72 (< 10 %)	1.2	>72 (< 5 %)
LH1205	$CH_2CH_2CH_2CH_3$	>72 (< 10 %)	9.4	>72 (< 5 %)
LH1206	$CH(CH_3)_2$	>72 (< 10 %)	>72 (< 5 %)	>72 (< 5 %)
LH1207	CH_2CH_3	>72 (< 10 %)	>72 (< 5 %)	>72 (< 5 %)
LH1208	$CH_2CH_2CH_3$	>72 (< 10 %)	40	>72 (< 5 %)
LH1209	$CH_2CH_2OCH_3$	>72 (< 10 %)	115	>72 (< 5 %)

[a]The half lives of prodrugs were measured using HPLC by following their disappearance at 100 μM and 37 °C in 50 mM Phosphate buffer containing 1 mM EDTA, pH=7.4, in the absence (buffer) or presence of 0.5 mg/ml mouse liver microsome (MLM) or human liver microsome (HLM).
[b]All compounds showed less than 10% change in measured peak area over 72 hours in buffer.
[c]All compounds showed less than 5% change in measured peak area over 72 hours in HLM.

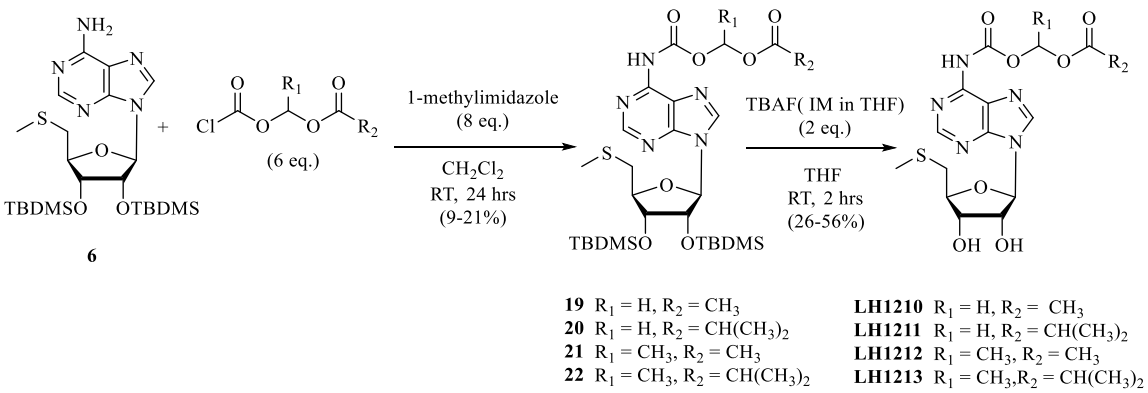
LH1208 and **LH1209** showed prolonged activation times in mouse liver microsomes but none of the synthesized prodrugs were found to be a better alternative to either **LH1201** or **LH1205**. All the above carbamate prodrugs were stable in phosphate buffer, pH=7.4 at 37 °C with less than 10% change in 72 hours. We also conducted a study to analyze the stability of MTA in both human and mouse liver microsomes as well as in phosphate buffer,

pH= 7.4 at 37 °C. It was found that the half-life in human and mouse liver microsomes was 19 hours and 14 hours respectively.

We then tested the activation of the prodrugs by an oxidative pathway in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). **LH1201** and **LH1205** when incubated with mouse liver microsomes in presence of NADPH were activated by this mechanism, however when compared with control (presence of mouse liver microsomes only) there was no enhanced activation in presence of NADPH. Compounds **LH1206, LH1207 and LH1209** did not show activation in presence of NADPH. Compound **LH1208** showed slight activation after 24 hours however, this was not high enough to be comparable. When tested in human serum for activation, all prodrugs were stable with less than 1.5% hydrolysis in 48 hours at physiological pH 7.4, 37 °C.

2.2 Synthesis and evaluation of *N*-[(acyloxy)alkyloxy]carbonyl-MTA Prodrugs

Following our previous studies, we then changed our focus to developing prodrugs having a *N*-[(acyloxy)alkyloxy]carbonyl moiety. The general synthetic scheme for these compounds was optimized as shown in **Scheme 2.**



Scheme 2. Synthetic route for *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs

Two different approaches were tried for the synthesis of this class of prodrug molecules. However, before the synthesis of the prodrug molecules, it was necessary to protect the hydroxyl groups of MTA. Since the prodrug moiety contained an ester functional group, these protected hydroxyl groups should be cleaved under acidic conditions. Several attempts were made to protect the two hydroxyl groups as represented in **Figure 8**. Silyl protection was the best choice of protection since it can be easily cleaved using a fluoride containing reagent.

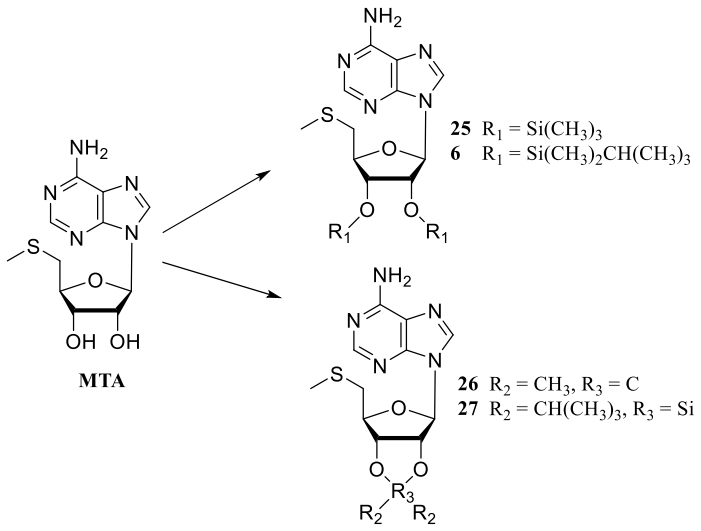
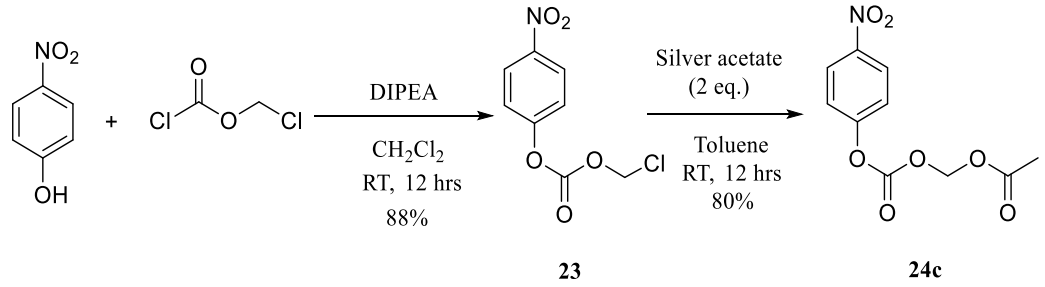


Figure 8. Comparison of hydroxyl protecting groups

Throughout our experimentation, we considered several silyl protecting groups. The TMS group formed using trimethylsilyl chloride (TMSCl) to protect the MTA was unstable through the course of the reaction and as a result, the formed compound **25** would reversibly change back to the starting material MTA. Reaction of MTA with a different silyl ether protecting group, 2 eq. di-*tert*-butylsilyl bis (trifuoromethanesulfonate), failed to provide the desired product **27,** even after using polar solvents such as DMF. Because of its stability, the optimum protecting group chosen was *tert*-butyldimethylsilyl chloride (TBDMSCl), although an excess amount of reagent was needed. In testing TBDMSCl as a protecting group, we added $AgNO_3$ to a solution of MTA in anhydrous dichloromethane to promote the attack of the silyl group on the alcohol. 1-Methylimidazole was added as a base followed by the addition of a solution of excess *tert*-butyldimethylsilyl chloride in dichloromethane at 0 °C and was stirred at RT for 48 hours to yield the compound **6** in

50% yield. Once the protecting group was selected, we explored two different approaches for the synthesis of N-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs.

Our first approach was to utilize [(acyloxy)alkyl]4-nitrophenyl carbonates as described by Moha-Lerman et al.[34] Treatment of commercially available 4-nitrophenol with chloromethyl chloroformate in the presence of DIPEA afforded the chloromethyl 4-nitrophenol ester **23** in 88% yield as depicted in **Scheme 3**. We tried several routes to form the (acetyloxy)methyl 4-nitrophenyl carbonate as shown in **Table 3**. The desired reagent needed to form the prodrug was ultimately obtained in 80% yield by exchanging the chlorine with an iodine in a Finkelstein swap reaction and further reacting it with the silver salt of acetic acid to obtain the compound **24c** in a two-step reaction.



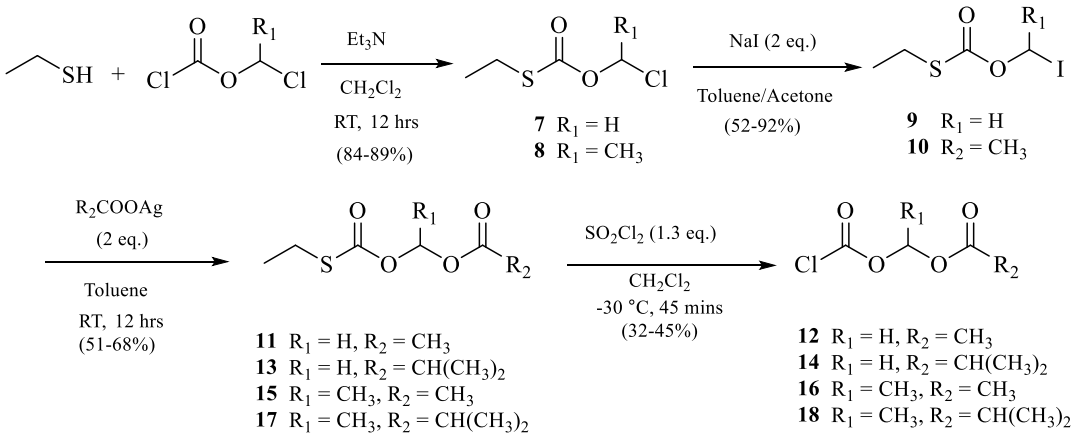
Scheme 3. Synthetic route for preparation of (acetyloxy)methyl 4-nitrophenyl carbonate

Table 3. Conditions used for synthesis of (acetyloxy)methyl 4-nitrophenyl carbonate

Compound	Conditions	Yield
24a	Glacial acetic acid (2 eq.), ZnO (3 eq.), NaBr (1 eq.)[34]	0%
24b	Glacial acetic acid (1.4 eq.), $TBAHSO_4$ (1.4 eq.), $NaHCO_3$ (2.7 eq.)[33]	0%
24c	1. NaI (2 eq.), $NaHCO_3$ (0.1 eq.) 2. Silver acetate (2.5 eq.)[33]	80%

Further reaction of compound **24c** with compound **6,** in the presence of DIPEA, failed to give the desired *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrug, despite the use of the nucleophilic catalyst, DMAP. It was hypothesized that the carbonate moiety was not sufficiently electrophilic. A more reactive electrophilic group was needed that would provide a pathway for the reaction, and it was hypothesized that (acyloxy)alkyl chloroformates could be a substitute for the (acyloxy)alkyl 4-nitrophenyl carbonate.

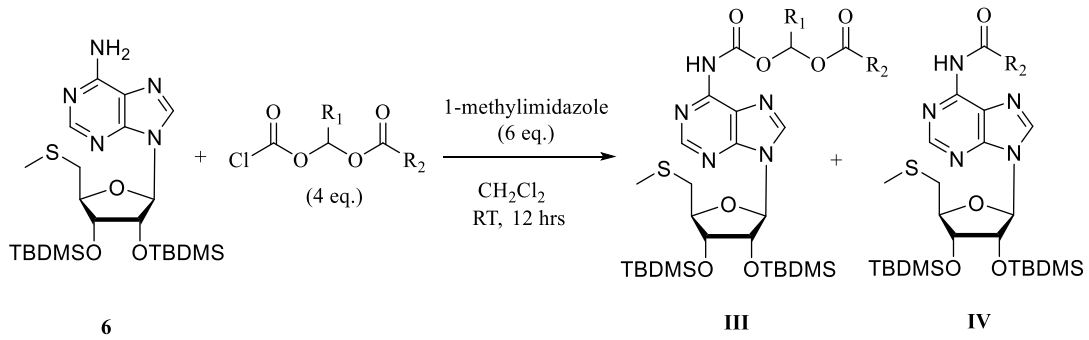
The general procedure for the synthesis of these (acyloxy)alkyl chloroformates is as shown in **Scheme 4.**



Scheme 4. Synthesis of (acyloxy)alkyl chloroformates

Starting from commercially available compounds, intermediates **7** and **8** were synthesized by reacting ethyl mercaptan with chloromethyl chloroformate and 1-chloroethyl chloroformate, respectively in the presence of triethylamine. These were further converted via a Finkelstein swap reaction to their corresponding iodo forms giving compounds **9** and **10** in 92% and 52% yield, respectively. Subsequently, compounds **9** and **10** were converted to compounds **11, 13, 15** and **17** by reacting them with silver salts of either acetic acid or isobutyric acid. The respective chloroformates, **12, 14, 16** and **18,** were obtained by reacting the individual intermediates from the previous step with sulfuryl chloride at -30 °C under a continuous stream of N_2 and strictly anhydrous conditions. Further reaction of the (acyloxy)alkyl chloroformate with compound **6** in the presence of 1-methylimidazole afforded the corresponding *N*-[(acyloxy)alkyloxy]carbonyl-2',3'-bis-*O*-(*tert*-butyldimethylsilyl)-MTA as shown in **Scheme 2.** The conversion yield for this step was found to be low, due to the instability of the synthesized chloroformates and formation of the byproduct **IV** as shown in **Scheme 5.** Formation of the byproduct was impossible to avoid, even though the reactions were run at very low temperatures. This byproduct could

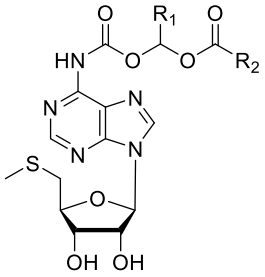
be a result of the reaction between sulfuryl chloride and *O*-(acyloxy)alkyl *S*-ethyl ester to form the acyl chloride of the acyloxy functional group. Further deprotection of compound **III** using TBAF (1 M in THF) afforded the desired *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrug.



Scheme 5. Synthesis of *N*-[(acyloxy)alkyloxy]carbonyl-2',3'-bis-*O*-(*tert*-butyldimethylsilyl)-MTA

MTA prodrugs, **LH1210, LH1211, LH1212 and LH1213,** were further tested for activation in mouse and human liver microsomes and their results are summarized in **Table 4.** The incorporation of an isobutyl ester group helped in prolonging the activation time from a few minutes to a few hours. The additional methyl group also helped improve stability in both mouse and human liver microsomes, although, when compared with that in phosphate buffer pH= 7.4, the difference was not considerable. When compounds **LH1211 and LH1213** were tested for activation in human serum, the activation time did not differ significantly from that of mouse or human liver microsomes. Compound **LH1213** showed a modest rate of hydrolysis in all assays and was chosen as an optimized molecule for further study in animal models.

Table 4. Stability and activation of *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs



Compound ID	R_1	R_2	$t_{1/2}$ (h)[a]		
			Buffer	MLM	HLM
LH1210	H	CH_3	3.50	0.02	0.05
LH1211	H	$CH(CH_3)_2$	3.20	2.20	2.30
LH1212	CH_3	CH_3	4.50	0.80	2.80
LH1213	CH_3	$CH(CH_3)_2$	4.60	3.30	3.70

[a]The half lives of prodrugs were measured using HPLC by following their disappearance at 100 μM and 37 °C in 50 mM Phosphate buffer containing 1 mM EDTA, pH=7.4, in the absence (buffer) or presence of 0.5 mg/ml mouse liver microsome (MLM) or human liver microsome (HLM).

Optimization of Scheme 2

The product yield from the deprotection of *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs was found to be very low due to the formation of MTA as a byproduct. The formation of this byproduct was a result of the presence of water, even in commercially available TBAF solutions, rendering them basic. The basicity of these reagents makes the ester group susceptible to hydrolysis, giving MTA as the byproduct of the deprotection step as illustrated from **Figure 9**.

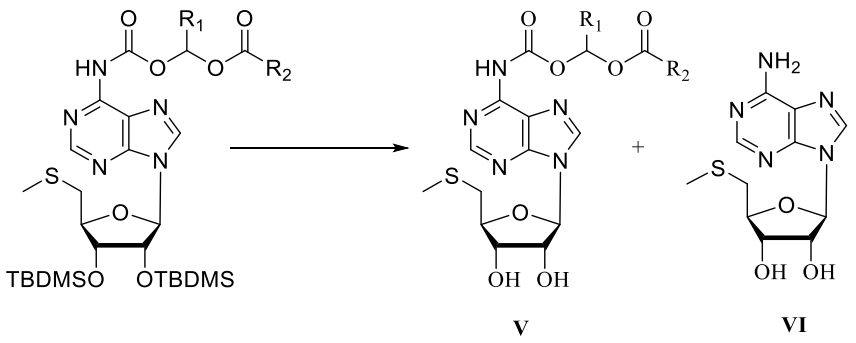


Figure 9. Deprotection of *N*-[(acyloxy)alkyloxy]carbonyl-2',3'-bis-*O*-(*tert*-butyldimethylsilyl)-MTA prodrugs

To circumvent this issue, deprotection under neutral conditions was desired. From a reported procedure by Philips et al., the deprotection procedure was modified to include a phosphate buffer that can maintain neutral conditions.[35] Using 0.2 eq. of TBAF, deprotection was carried out at pH=6.7, 7.1 and 7.4, maintaining the THF-Phosphate buffer ratio (100:1). For optimizing reaction conditions, compound **22** was chosen as the reference prodrug. The progress of the reaction was monitored by LCMS and TLC. At pH=6.7, the desired product was not obtained as evidenced from LCMS, even after 24 hours. Deprotection of a single silyl ether group was the only product to be formed. Formation of the byproduct MTA was detected at a significant rate within 2 hours at pH=7.4 whereas, it was slower at pH=7.1. As had been previously stated in the reported procedure by Philips et al., the ratio of the THF-Buffer was also found to be crucial to the rate of the reaction.

Another approach discussed by Philips et al. applied CsF as a fluoride source for silyl ether deprotection.[35] With 2.2 eq. CsF as a fluoride source in DMSO:MeOH (100:1) when buffered conditions were not used, there was a significant amount of byproduct MTA **(VI)**

formed as was expected. With the use of the phosphate buffer, pH=7.1, along with DMSO and methanol as reaction solvents in the ratio DMSO:Buffer:MeOH=100:1:1, there was no presence of byproduct MTA detected on the LCMS, even after 24 hours. The optimized procedure would then be the use of CsF under buffered conditions.

SUMMARY

We successfully synthesized and conducted activation studies of prodrugs of 5'-methylthioadeosine (MTA), namely *N*-(alkyloxy)carbonyl-MTA prodrugs and *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs, in human and mouse liver microsomes. We also analyzed the *N*-(alkyloxy)carbonyl-MTA prodrugs for oxidative activation in the presence of NADPH, and found them to be activated. When tested in human serum, it was found that only the *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs were hydrolyzed while the *N*-(alkyloxy)carbonyl-MTA prodrugs showed no activation.

From our results, we were able to conclude that the *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs were more susceptible to hydrolysis than the *N*-(alkyloxy)carbonyl-MTA prodrugs. However, the former prodrugs exhibited instability at physiological pH. Further modifications in the (acyloxy)alkyloxy carbonyl promoiety were made to increase stability. We were able to achieve a good hydrolysis profile with *N*-[(2-methylpropanoyloxy)ethoxy]carbonyl-MTA in human and mouse liver microsomes with a half-life of 3.7 hours and 3.3 hours, respectively. Further studies on the *N*-[(acyloxy)alkyloxy]carbonyl-MTA prodrugs are aimed at studying in-vivo rate of hydrolysis.

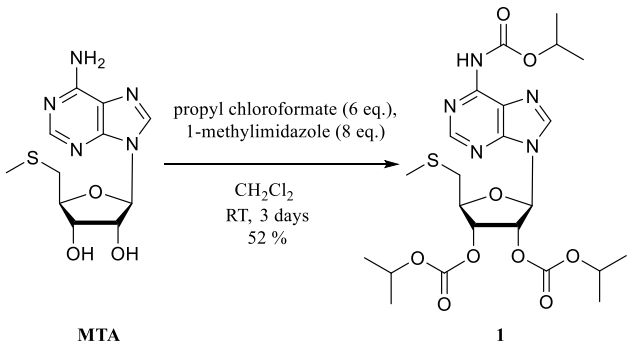
EXPERIMENTAL SECTION

GENERAL

All reactions were performed in oven-dried glassware. All solvents used were either ACS reagent grade or HPLC grade. All reactions were performed using anhydrous solvents. Moisture sensitive reactions were performed under N_2 atmosphere. Air sensitive reagents were transferred to a reaction flask using a cannula under N_2 atmosphere. All reactions were monitored by Thin Layer Chromatography (TLC) using 0.25 mm Whatmann precoated silica gel plates. TLC plates were visualized by either UV absorbance or potassium permanganate solution. Purification was done by using automated flash column chromatography using Teledyne ISCO CombiFlash Companion System.

Analytical LC-MS was obtained using Agilent 1200 Series LC system equipped with an ODS column (3 x 33 mm). Solvent A was 0.1% formic acid/H_2O and solvent B was 0.1% formic acid in methanol and the gradient used was 10-90% B in 5 mins at a flow rate of 0.8 ml/min. NMR spectra were recorded on a Bruker Ultrashield 400 MHz at ambient temperature. High Resolution Mass Spectrometry data was obtained from the Center for Integrative Proteomics Research (CIPR) at Rutgers University.

***N*-(Isopropoxycarbonyl)-2′,3′-bis(isopropylcarbonate)-MTA (1)**

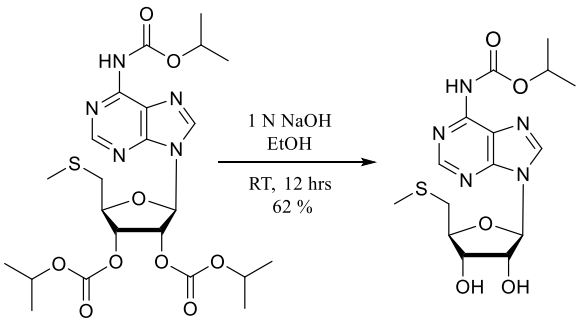


MTA 1

Methylthioadenosine (MTA) (200 mg, 0.67 mmol) was suspended in 20 ml anhydrous dichloromethane. 1-methylimidazole (441.8 mg, 5.38 mmol, 8 eq.) was added and the suspension was stirred at 0 °C for 5 mins. Isopropyl chloroformate (494.5 mg, 4.03 mmol, 6 eq.) was added dropwise to the cold suspension over 5 mins while maintaining the temperature at 0 °C. The reaction mixture was allowed to warm to room temperature and was stirred for an additional 3 days. The reaction progress was monitored using TLC and LCMS. The reaction mixture was diluted with dichloromethane (20 ml) and washed successively twice with saturated $NaHCO_3$ (10 ml) and brine (10 ml). The organic phase was separated and dried over Na_2SO_4 and filtered. The solvent was evaporated, and the crude product was purified by column chromatography using 10% methanol in dichloromethane as an eluent to give compound **1** as a pure oily white product (190 mg, 52%). ^{1}H NMR (400 MHz, Methanol-d_4) δ (ppm): 8.52 (s, 1H), 8.38 (s, 1H), 6.19 (d, J = 5.3 Hz, 1H), 5.96 (t, J = 5.5 Hz, 1H), 5.54 – 5.49 (m, 1H), 4.98 (dt, J = 12.5, 6.2 Hz, 1H), 4.81 (dd, J = 12.5, 6.2 Hz, 1H), 4.67 (dt, J = 12.5, 6.2 Hz, 1H), 4.33 (q, J = 5.9 Hz, 1H), 2.92 (dd, J = 14.3, 5.8 Hz, 1H), 2.86 (dd, J = 14.3, 6.1 Hz, 1H), 2.00 (s, 3H), 1.17 (ddd, J = 46.6, 20.1, 6.3 Hz, 18H); ^{13}C NMR (100 MHz, Methanol-d_4,) δ (ppm): 155.02, 154.68,

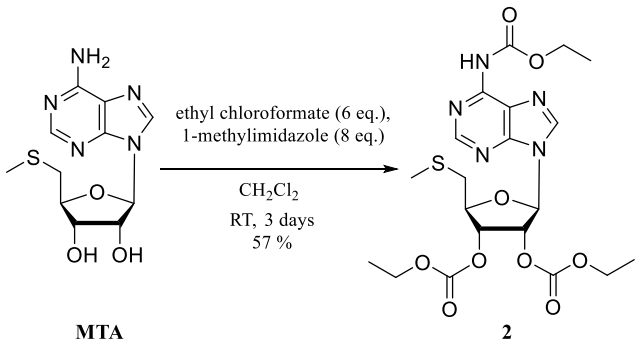
153.58, 153.06, 152.45, 151.43, 144.29, 123.80, 87.73, 83.19, 76.78, 76.64, 74.49, 74.23, 70.96, 36.97, 22.35, 16.69. LCMS (ESI^+): 556.32 $[M + H]^+$.

***N*-(Isopropoxycarbonyl)-MTA (LH1206)**



0.1 N NaOH (3 ml, 0.3 mmol) was added to a solution of compound **1** (170 mg, 0.3 mmol) in 5 ml ethanol and stirred at room temperature for 12 hours. On completion of the reaction, 1 N HCl was added to the reaction mixture until the pH was neutral. Dichloromethane (10 ml) was then added to the reaction mixture and extracted with brine (5 ml). The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated. The crude product was purified using column chromatography in 3% methanol in dichloromethane to give a white solid **LH1206** (72 mg, 62). ^1H NMR (400 MHz, Methanol-d_4) δ (ppm): 10.38 (bs, 1H), 8.67 (s, 1H), 8.64 (s, 1H), 6.01 (d, J = 5.8 Hz, 1H), 5.59 (s, 1H), 5.39 (s, 1H), 4.93 (hept, J = 6.2 Hz, 1H), 4.83 – 4.77 (m, 1H), 4.19 (s, 1H), 4.11 – 4.05 (m, 1H), 2.91 (dd, J = 13.9, 5.9 Hz, 1H), 2.81 (dd, J = 13.9, 6.9 Hz, 1H), 2.06 (s, 3H), 1.28 (d, J = 6.2 Hz, 6H); ^{13}C NMR (100 MHz, Methanol-d_4) δ (ppm): 152.51, 150.68, 150.61, 149.58, 141.50, 122.07, 89.67, 84.30, 74.62, 73.09, 70.27, 36.82, 21.89, 21.83, 16.71; HRMS (ESI^+) m/z calcd for $C_{15}H_{22}N_5O_5S$ $[M + H]^+$: 384.1336, found: 384.1350.

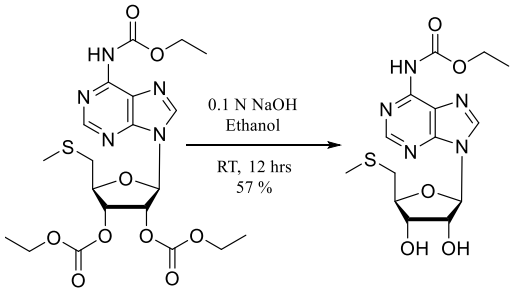
***N*-(Ethoxycarbonyl)-2′,3′-bis(ethylcarbonate)-MTA (2)**



MTA **2**

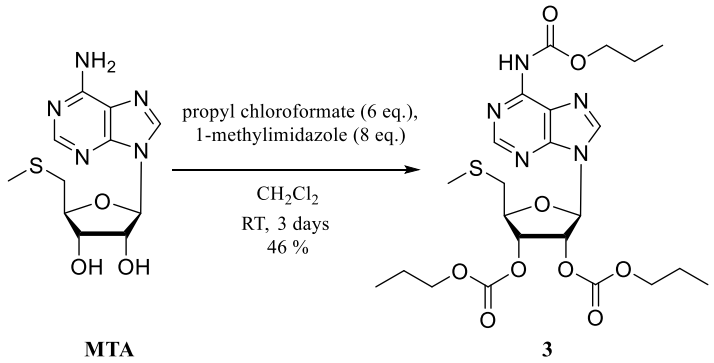
1-Methylimidazole (441.8 mg, 5.38 mmol, 8 eq.) was added to a suspension of 5'-methylthioadenosine (MTA) (200 mg, 0.67 mmol) in 20 ml dichloromethane and cooled to 0 °C in an ice bath. Ethyl chloroformate (438 mg, 4.03 mmol, 6 eq.) was then added dropwise at 0 °C and stirred over 5 mins. The reaction mixture was allowed to warm to room temperature and stirred for 3 days at room temperature in a water bath. The mixture was diluted with dichloromethane (10 ml) and washed successively with saturated $NaHCO_3$(10 ml) and brine (10 ml). The organic layer was dried over Na_2SO_4, filtered and evaporated to give crude product which was purified using column chromatography in 10 % methanol in dichloromethane to give an oily white compound **2** (197 mg, 57%). ^{1}H NMR (400 MHz, Methanol-d_4) δ (ppm): 8.60 (s, 1H), 8.47 (s, 1H), 6.29 (d, J = 5.3 Hz, 1H), 6.04 (t, J = 5.4 Hz, 1H), 5.60 (t, J = 5.1 Hz, 1H), 4.41 (q, J = 5.7 Hz, 1H), 4.28 (q, J = 7.1 Hz, 2H), 4.23 – 4.17 (m, 2H), 4.10 (dd, J = 14.7, 7.2 Hz, 2H), 2.99 (dd, J = 14.4, 5.8 Hz, 1H), 2.93 (dd, J = 14.3, 6.2 Hz, 1H), 2.06 (s, 3H), 1.32 (t, J = 7.1 Hz, 3H), 1.28 (t, J = 7.1 Hz, 3H), 1.19 (t, J = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, Methanol-d_4) δ (ppm): 153.44, 153.12, 151.53, 151.37, 150.38, 149.26, 142.21, 121.75, 85.69, 81.12, 74.73, 74.65, 63.99, 63.82, 60.94, 34.77, 14.43, 12.65, 12.45, 12.32; LCMS (ESI$^+$): 514.47 $[M + H]^+$.

***N*-(Ethyloxycarbonyl)-MTA (LH1207)**



0.1 N NaOH (2.9 ml, 0.29 mmol) was added to a solution of compound **2** (150 mg, 0.29 mmol) in 5 ml ethanol and stirred at room temperature for 12 hours. 1 N HCl was added to the reaction mixture until the pH was neutral. Dichloromethane (10 ml) was then added to the reaction mixture and extracted with brine (5 ml). The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated. The crude product was purified using column chromatography in 3% methanol in dichloromethane to give a white solid **LH1207** (62 mg, 57%). ^{1}H NMR (400 MHz, Methanol-d_4) δ (ppm): 8.46 (s, 1H), 8.25 (s, 1H), 6.05 (d, J = 4.5 Hz, 1H), 4.75 (t, J = 4.6 Hz, 1H), 4.41 – 4.37 (m, 1H), 4.30 (q, J = 4.9 Hz, 1H), 4.18 (q, J = 7.0 Hz, 2H), 2.86 (dd, J = 13.8, 5.3 Hz, 1H), 2.78 (dd, J = 14.1, 5.3 Hz, 1H), 2.05 (s, 3H), 1.23 (t, J = 7.1 Hz, 3H);^{13}C NMR (100 MHz, Methanol-d_4) δ (ppm): 152.17, 151.72, 151.13, 149.65, 142.59, 122.29, 88.88, 84.14, 73.31, 72.55, 61.49, 35.98, 15.03, 13.23; HRMS (ESI$^+$) m/z calcd for $C_{14}H_{20}N_5O_5S$ $[M + H]^+$: 370.1180. Found: 370.1193.

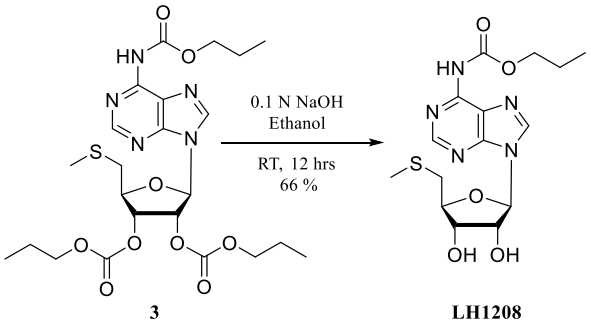
***N*-(Propoxycarbonyl)-2',3'-bis(propylcarbonate)-MTA (3)**



MTA **3**

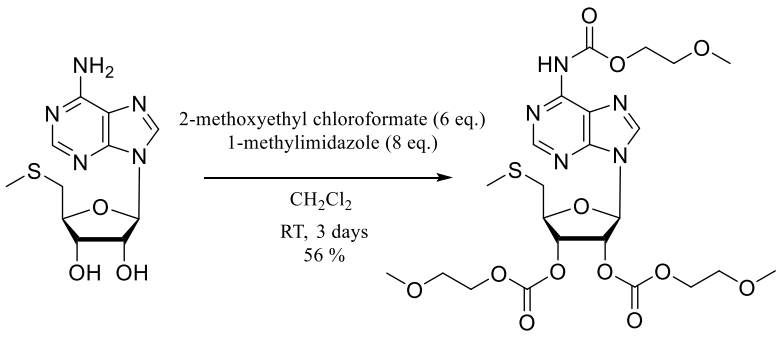
To a suspension of 5'-methylthioadenosine (MTA) (200 mg, 0.67 mmol) in 15 ml dichloromethane, 1-methylimidazole (441.8 mg, 5.38 mmol, 8 eq.) was added and cooled to 0 °C in an ice bath. Propyl chloroformate (492 mg, 4.03 mmol, 6 eq.) was then added dropwise at 0 °C and stirred over 5 mins. The reaction mixture was allowed to warm to room temperature and stirred for 3 days at room temperature in a water bath. The mixture was diluted with dichloromethane (10 ml) and washed successively with saturated $NaHCO_3$(10 ml) and brine (10 ml). The organic layer was dried over Na_2SO_4, filtered and evaporated to give crude product which was purified using column chromatography in 10 % methanol in dichloromethane to give an oily white compound **3** (175 mg, 46%). ^{1}H NMR (400 MHz, Methanol-d_4) δ (ppm): 9.16 (s, 1H), 8.77 (s, 1H), 8.20 (s, 1H), 6.21 (d, J = 5.3 Hz, 1H), 6.05 (t, J = 5.4 Hz, 1H), 5.59 (t, J = 5.0 Hz, 1H), 4.49 (q, J = 5.3 Hz, 1H), 4.23 (t, J = 6.7 Hz, 2H), 4.17 – 4.01 (m, 4H), 3.02 (dd, J = 14.3, 5.3 Hz, 1H), 2.94 (dd, J = 14.3, 6.1 Hz, 1H), 2.12 (s, 3H), 1.76 – 1.62 (m, 6H), 0.99 – 0.88 (m, 9H); ^{13}C NMR (100 MHz, Methanol-d_4) δ (ppm): 152.17, 151.72, 151.13, 149.65, 142.59, 122.29, 88.88, 84.14, 73.31, 72.55, 61.49, 35.98, 15.03, 13.23; LCMS (ESI$^+$): 556.62 $[M+H]^+$.

***N*-(Propoxycarbonyl)-MTA (LH1208)**



To a solution of compound **3** (100 mg, 0.18 mmol) in 5 ml ethanol, 0.1 M NaOH (1.8 ml, 0.18 mmol) was added and stirred at room temperature for 12 hours. 1 N HCl was added to the reaction mixture until the pH was neutral. Dichloromethane (10 ml) was then added to the reaction mixture and extracted with brine (5 ml). The organic layer was dried over Na_2SO_4, filtered and solvent was evaporated. The crude product was purified using column chromatography in 3% methanol in dichloromethane to give a white solid **LH1208** (45 mg, 66%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.55 (s, 1H), 8.14 (s, 1H), 5.96 (d, *J* = 5.3 Hz, 1H), 4.69 (t, *J* = 5.3 Hz, 1H), 4.42 – 4.39 (m, 3H), 4.37 – 4.35 (m, 1H), 4.16 (t, *J* = 6.7 Hz, 2H), 2.83 (dd, *J* = 12.9, 5.0 Hz, 1H), 2.78 (dd, *J* = 13.0, 4.6 Hz, 1H), 2.10 (s, 3H), 1.67 (dq, *J* = 14.2, 7.2 Hz, 2H), 0.92 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 152.44, 151.42, 150.62, 149.41, 141.64, 121.94, 89.46, 84.28, 74.74, 73.19, 67.92, 36.84, 22.07, 16.72, 10.28.; HRMS (ESI$^+$) m/z calcd for $C_{15}H_{22}N_5O_5S$ $[M + H]^+$: 384.1336, found: 384.1350.

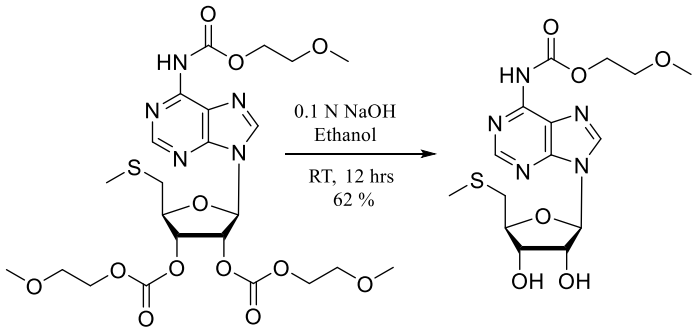
_N_-(2-Methoxyethyoxy)carbonyl-2',3'-bis(2-methoxyethylcarbonate)-MTA (4)



MTA **4**

1-Methylimidazole (441.8 mg, 5.38 mmol, 8 eq.) was added to a suspension of 5'-methylthioadenosine (MTA) (200 mg, 0.67 mmol) in 15 ml dichloromethane and cooled to 0 °C in an ice bath. 2-Methoxy ethyl chloroformate (560 mg, 4.03 mmol, 6 eq.) was then added dropwise at 0 °C and stirred over 5 mins. The reaction mixture was allowed to warm to room temperature and stirred for 3 days at room temperature in a water bath. The mixture was diluted with dichloromethane (10 ml) and washed successively with saturated $NaHCO_3$(10 ml) and brine (10 ml). The organic layer was dried over Na_2SO_4, filtered and evaporated to give crude product which was purified using column chromatography in 10 % methanol in dichloromethane to give an oily white compound **4** (200 mg, 56%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.79 (s, 1H), 8.69 (s, 1H), 8.09 (s, 1H), 6.09 (d, *J* = 5.3 Hz, 1H), 6.00 (t, *J* = 5.3 Hz, 1H), 5.53 (t, *J* = 5.0 Hz, 1H), 4.39 (q, *J* = 5.5 Hz, 2H), 4.24 (q, *J* = 4.5, 4.0 Hz, 3H), 4.16 (t, *J* = 4.6 Hz, 2H), 3.63 – 3.57 (m, 2H), 3.55 (dd, *J* = 6.8, 2.5 Hz, 2H), 3.50 – 3.45 (m, 2H), 3.31 (d, *J* = 6.9 Hz, 6H), 3.25 (s, 3H), 2.93 (dd, *J* = 14.3, 5.4 Hz, 1H), 2.85 (dd, *J* = 14.3, 6.2 Hz, 1H), 2.03 (s, 3H).^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 154.07, 153.75, 153.09, 151.04, 150.92, 149.73, 142.17, 122.75, 86.45, 81.79, 75.54, 75.52, 70.41, 69.97, 69.84, 67.88, 67.75, 64.90, 58.99, 58.93, 36.10, 16.71; LCMS (ESI^+): 604.12 $[M + H]^+$.

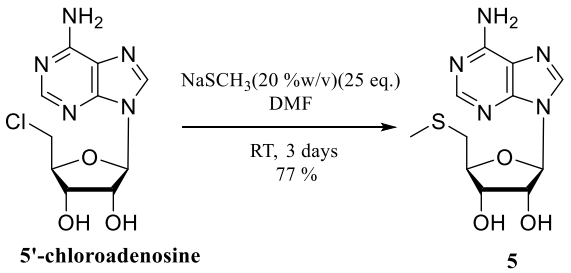
***N*-(2-Methoxyethoxycarbonyl)-MTA (LH1209)**



4 **LH1209**

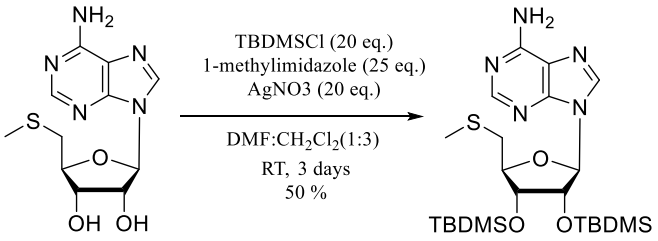
To a solution of compound **4** (170 mg, 0.28 mmol) in 5 ml ethanol, 0.1 M NaOH (2.8 ml, 0.28 mmol, 1 eq.) was added and stirred at room temperature for 12 hours. 1 N HCl was added to the reaction mixture until the pH was neutral. Dichloromethane (10 ml) was then added to the reaction mixture and extracted with brine (5 ml). The organic layer was dried over Na_2SO_4, filtered and solvent was evaporated. The crude product was purified using column chromatography in 3% methanol in dichloromethane to give a white solid **LH1209** (70 mg, 62%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.52 (s, 1H), 8.12 (s, 1H), 5.94 (d, *J* = 5.0 Hz, 1H), 4.69 (t, *J* = 5.2 Hz, 1H), 4.38 – 4.34 (m, 1H), 4.33 – 4.30 (m, 3H), 3.58 (dd, *J* = 8.8, 4.2 Hz, 4H), 3.31 (s, 3H), 2.77 (dq, *J* = 13.9, 6.8, 5.6 Hz, 2H), 2.06 (s, 3H).; ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm):152.42, 150.91, 150.66, 149.39, 141.71, 122.25, 89.87, 84.41,74.65, 73.27, 70.37, 64.93, 58.91, 36.79, 16.64. HRMS (ESI^+) m/z calcd for $C_{15}H_{21}N_5O_6S$ $[M + H]^+$: 400.1681, found: 400.1682.

Methylthioadenosine (5)[28]



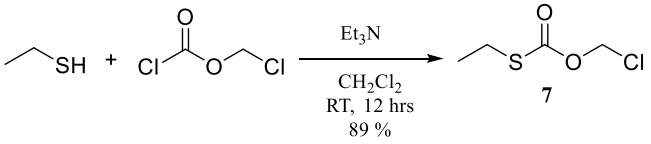
To a solution of 5'-chloro-5'-deoxyadenosine (5 g, 17.5 mmol) in 10 ml DMF, 20% w/v $NaSCH_3$ (30.66 g, 0.43mmol, 25 eq.) was added at RT and stirred in a water bath for 3 days after which, a white solid precipitate was formed. The reaction was monitored by LCMS and after no starting material was observed, the reaction mixture was acidified with conc. HCl (50 ml) until the pH of the reaction mixture was 7. The white solid precipitate formed was triturated with cold water for 1 hour at 0 °C and then filtered. The filtrate was washed several times with cold water and dried under a vacuum for 1 hour. The solids were then dissolved in 80 ml methanol and dried over Na_2SO_4 and filtered. The solvent was evaporated to yield a white flaky solid **5** (4 g, 77%). ^{1}H NMR (400 MHz, Dimethylsulfoxide-d_6) δ (ppm): 8.36 (s, 1H), 8.15 (s, 1H), 5.89 (d, J = 6.0 Hz, 1H), 5.41 (t, J = 6.2 Hz, 1H), 5.32 (t, J = 4.4 Hz, 1H), 4.75 (q, J = 5.3 Hz, 1H), 2.67 (dd, J = 14.4 Hz, 5.5 Hz, 1H), 2.41 (dd, J = 14 Hz, 6.2 Hz, 1H), 2.06 (s, 3H); LCMS (ESI$^+$): 298.27 $[M+H]^+$.

2',3'-bis-*O*-(tert-butyldimethylsilyl)-MTA (6)



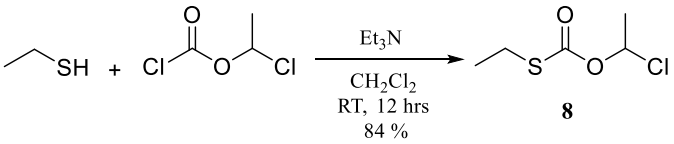
To a solution of MTA (4 g, 13.45 mmol) in 20 ml DMF, $AgNO_3$ (51 g, 269 mmol, 20 eq.) was added and stirred at 0°C for 15 mins to give a turbid yellow solution. *Tert*-butyldimethylsilyl chloride (40.54 g, 269 mmol, 20 eq.) was added followed by the dropwise addition of a solution of 1-methylimidazole (27 g, 336 mmol, 25 eq.) in 60 ml dichloromethane. The reaction was allowed to warm to room temperature and stirred for 3 days. On completion of the reaction as monitored by TLC and LCMS, the reaction mixture was washed twice with 30 ml sat. NH_4Cl solution and 10 ml brine. The organic layer was dried over Na_2SO_4 and filtered. The solvent was evaporated to give a crude oily liquid which was purified by column chromatography in 5% methanol in dichloromethane to give a white flaky solid **6** (3.5 g, 50%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.18 (s, 1H), 7.81 (s, 1H), 5.73 (d, *J* = 5.4 Hz, 1H), 5.53 (s, 2H), 4.94 – 4.90 (m, 1H), 4.16 (t, *J* = 4.2 Hz, 1H), 4.09 (td, *J* = 6.3, 3.3 Hz, 1H), 2.89 (dd, *J* = 14.1, 6.7 Hz, 1H), 2.72 (dd, *J* = 14.1, 6.0 Hz, 1H), 2.00 (s, 3H), 0.80 (s, 9H), 0.64 (s, 9H), -0.01 (d, *J* = 9.1 Hz, 6H), -0.21 (s, 3H), -0.43 (s, 3H).^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 155.47, 152.82, 149.71, 140.63, 120.71, 89.69, 84.51, 74.47, 73.73, 36.60, 25.87, 25.70, 18.07, 17.88, 16.64, -4.39, -4.59, -4.66, -5.10,-5.22, -6.19; LCMS (ESI$^+$): 526.25 $[M + H]^+$.

_O_-(Chloromethyl)_S_-ethyl ester (7)[36]



To a solution of ethyl mercaptan (2.5 g, 38.77 mmol) in 30 ml dichloromethane, chloromethyl chloroformate (5g, 38.77 mmol) was added and stirred at 0 °C for 5 mins. To the ice-cold solution, a solution of triethylamine (3.92 g, 38.77 mmol) in 20 ml dichloromethane was added dropwise over 5 mins. The reaction mixture was stirred at RT for 12 hours after which, the reaction mixture was diluted with 30 ml dichloromethane and extracted thrice with 30 ml water each. The organic layer was dried over Na_2SO_4 and filtered. The solvent was evaporated to give a pale, yellow liquid **7** (5.3 g, 89%) which was used in the next step without purification. ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 5.70 (s, 2H), 2.79 (q, *J* = 7.3 Hz, 2H), 1.27 (t, *J* = 8.2 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 170.27, 70.12, 25.58, 14.69.

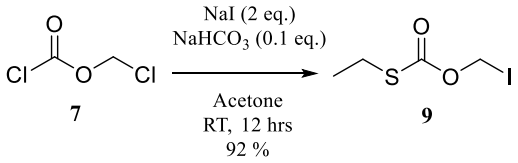
_O_-(Chloroethyl)_S_-ethyl ester (8)[36]



To a solution of ethyl mercaptan (5 g, 80.47 mmol) in 50 ml dichloromethane, 1-chloroethyl chloroformate (11.50g, 80.47 mmol) was added and stirred at 0 °C for 5 mins. To the ice-cold solution, a solution of triethylamine (8.1 g, 80.47 mmol) in 50 ml dichloromethane was added dropwise over 5 mins. The reaction mixture was stirred at RT

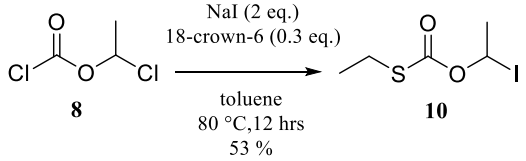
for 12 hours after which the reaction mixture was diluted with 30 ml dichloromethane and extracted thrice with 50 ml water each. The organic layer was dried over Na_2SO_4 and filtered. The solvent was evaporated to give a pale, yellow liquid **8** (11 g, 84%) which was used in the next step without purification. ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 6.51 (q, *J* = 5.2 Hz, 1H), 2.87 (dd, *J* = 3.4 Hz, 5.0 Hz 1H), 2.82 (dd, *J* = 3.3 Hz, 5.0 Hz, 1H), 1.73 (d, *J* = 5.2 Hz, 3H), 1.27 (t, *J* = 8.6 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 169.76, 82.36, 25.46, 25.18, 14.73.

_O_-(Iodomethyl)_S_-ethyl ester(9)[36]



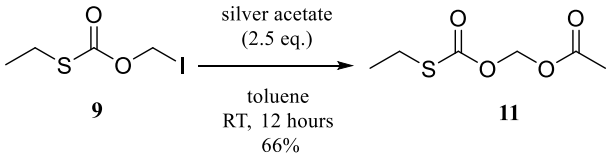
To the above prepared compound **7** (5.3 g, 34.41 mmol) in 30 ml anhydrous acetone, NaI (10.32 g, 68.83 mmol, 2 eq.) and $NaHCO_3$ (290 mg,3.45 mmol, 0.1 eq.) were added simultaneously at RT and the reaction was stirred for 18 hours. The acetone was then removed under a vacuum to give a dark brown colored liquid with NaCl as the byproduct. Diethyl ether was then added to the reaction mixture and the solid NaCl was filtered. The ether was removed using a vacuum to give a pale brown colored liquid **9** (7.8 g, 92% crude) that was used in the next step without purification. ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm):5.92 (s, 1H), 2.85 (q, *J* = 7.5 H, 2H), 1.27 (t, *J* = 7.5 Hz); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 170.05, 70.12, 28.62, 14.72.

***O*-(Iodoethyl)*S*-ethyl ester (10)**[36]



To the above prepared compound **8** (11 g, 65.22 mmol) in 70 ml anhydrous toluene, NaI (19.56 g, 130.45 mmol, 2 eq.) and 18-crown-6 (5.2 g, 19.5 6 mmol, 0.3 eq.) were added simultaneously at RT and the reaction was stirred at 80 °C for 18 hours. The reaction mixture was diluted with 50 ml ethyl acetate and washed thrice with 50 ml 20% $Na_2S_2O_3$ solution each to remove the iodine generated during the reaction. The organic fractions were collected and dried over Na_2SO_4 and filtered. The solvents were evaporated under reduced pressure to give a tan colored liquid **10** (8.6 g, 53% crude) that was used in the next step without purification. ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 6.73 (q, *J* = 7.2 Hz, 1H), 2.73 (q, *J* = 7.1 Hz, 2H), 2.04 (d, *J*= 6.5 Hz, 3H),1.16 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 171.86, 49.57, 30.00, 25.48, 14.74.

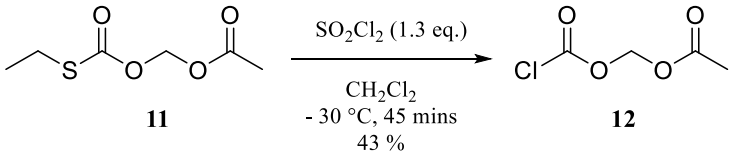
***O*-(Acetyloxy)methyl *S*-ethyl ester (11)**[36]



To a solution of compound **9** (3 g, 15.8 mmol) in 60 ml anhydrous toluene, silver acetate (6.6 g**,** 39.7 mmol, 2.5 eq.) was added and the reaction was allowed to stir at RT overnight. With the addition of silver acetate, the heterogenous mixture turned pale yellow indicating rapid interconversion of silver acetate to silver iodide. After 12 hours, the toluene was

evaporated under a vacuum and the mixture was diluted with 60 ml diethyl ether. It was then stirred at RT for 10 mins after which, it was filtered under a vacuum. The filtrate was collected and solvent was evaporated under pressure to give a slightly yellow oily liquid. The crude product was purified using column chromatography in 5% ethyl acetate in hexane over 30 mins to afford compound **11** as a colorless liquid (1.8 g, 66%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 5.81 (s, 2H), 2.92 (q, *J* = 7.3 Hz, 2H), 2.14 (s, 3H), 1.34 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 170.59, 169.26, 80.15, 25.43, 20.69, 14.54.

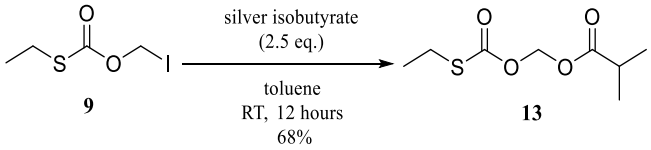
(Acetyloxy)methyl chloroformate (12)[36]



Compound **11** (1.8 g,10 mmol) was cooled to -30 °C in a dry ice-acetonitrile cooling bath and stirred for 5 mins. A solution of sulfuryl chloride (1.7 g, 13 mmol, 1.3 eq.) in 5 ml dichloromethane was cooled to -30 °C and it was then added dropwise over 10 mins using a canula under N_2 atmosphere to compound **11**. The reaction was stirred at that temperature for 45 mins. The completion of the reaction was monitored by NMR at intervals of 15 mins. On completion of the reaction, the byproduct, sulfenyl chloride, and excess sulfuryl chloride were distilled out under a vacuum. The crude product was then purified using vacuum distillation and fractions were collected at a temperature range starting from 40-70 °C. The fraction passing over 55 °C was found to contain compound **12** as a pale, yellow

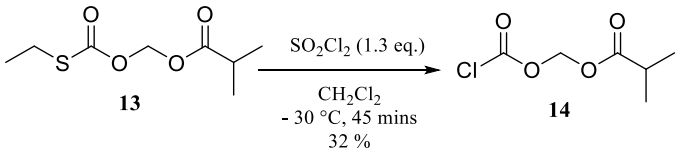
liquid (0.65 g, 43%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 5.84 (s, 2H), 2.21 (s, 1H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 168.98, 150.16, 83.53, 20.64.

_O_-(2-Methylpropanoyloxy)methyl _S_-ethyl ester (13)[36]



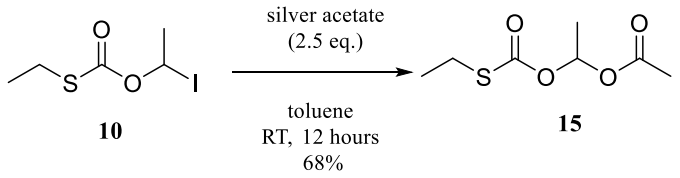
To a solution of compound **9** (3 g, 15.8 mmol) in 60 ml anhydrous toluene, silver isobutyrate (7.8 g 39.5 mmol, 2.5 eq.) was added and the reaction was stirred at RT overnight. With the addition of silver isobutyrate, the heterogenous mixture turned grey indicating rapid interconversion of silver isobutyrate to silver iodide. After 12 hours, the toluene was evaporated under a vacuum and the mixture was diluted with 60 ml diethyl ether. It was then stirred at RT for 10 mins after which, it was filtered under a vacuum. The filtrate was collected, and the solvent was evaporated under reduced pressure to give a pale yellow oily liquid. The crude product was purified using column chromatography in 5% ethyl acetate in hexane over 30 mins to afford compound **13** as a colorless liquid (2.2 g, 68 %). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 5.85 (s, 2H), 2.92 (q, *J* = 7.1 Hz, 2H), 2.63 (m, 1H), 1.34 (t, *J* = 8.3 Hz, 3H), 1.20 (d, *J* = 7.4 Hz, 6H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 175.36, 170.73, 80.15, 33.82, 25.76, 18.8, 15.04.

[(2-Methylpropanoyl)oxy]methyl chloroformate (14)[36]



Compound **13** (2.2 g, 10.67 mmol) was cooled to -30 °C in a dry ice-acetonitrile cooling bath and stirred for 5 mins. A solution of sulfuryl chloride (1.8 g, 13.87 mmol, 1.3 eq.) in 5 ml dichloromethane was added dropwise to compound **13** and stirred at -30 °C over 10 mins using a canula under N_2 atmosphere. The reaction was stirred at that temperature for 45 mins. The completion of the reaction was monitored by NMR at intervals of 15 mins. On completion of the reaction, the byproduct, sulfenyl chloride, and excess sulfuryl chloride were distilled out under a vacuum. The crude product was then purified using vacuum distillation and fractions were collected at a temperature range starting from 40-80 °C. The fraction passing over 65 °C was found to contain compound **14** as a pale, yellow liquid (0.42 g, 32%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 5.82 (s, 2H), 2.67 (m, 1H), 1.23 (d, *J* = 8.5 Hz, 6H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 175.17, 150.48, 83.24, 33.36,18.65.

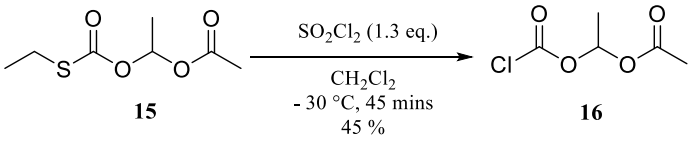
***O*-(Acetyloxy)ethyl *S*-ethyl ester (15)**[36]



To a solution of compound **10** (4 g, 15.37 mmol) in 60 ml anhydrous toluene, silver acetate (6.4 g, 38.44 mmol, 2.5 eq.) was added and the reaction was stirred at RT overnight. With

the addition of silver acetate, the heterogenous mixture turned grey indicating rapid interconversion of silver acetate to silver iodide. After 12 hours, the toluene was evaporated under a vacuum and the mixture was diluted with 60 ml diethyl ether. It was then stirred at RT for 10 mins after which, it was filtered under a vacuum. The filtrate was collected, and the solvent was evaporated under pressure to give a slightly yellow oily liquid. The crude was purified using column chromatography in 5% ethyl acetate in hexane to obtain a colorless liquid **15** (2 g, 68%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 6.87 (q, *J* = 5.4 Hz, 1H), 2.82 (m, 2H), 2.01 (s, 3H), 1.44 (d, *J* = 6.7 Hz, 3H), 1.25 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 169.61, 168.74, 89.89, 25.30, 20.79, 19.58, 14.72.

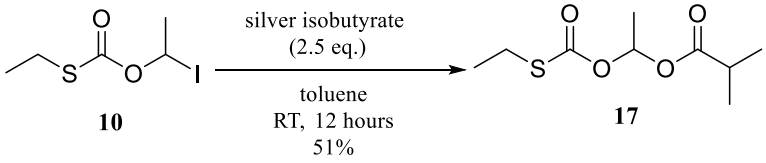
(Acetyloxy)ethyl chloroformate (16)[36]



Compound **15** (2 g,10 mmol) was cooled to -30 °C in a dry ice-acetonitrile cooling bath and stirred for 5 mins. A solution of sulfuryl chloride (1.8 g, 13.52 mmol, 1.3 eq.) in 5 ml dichloromethane was added dropwise to compound **15** and stirred at -30 °C over 10 mins using a canula under N_2 atmosphere. The reaction was stirred at that temperature for 45 mins. The completion of the reaction was monitored by NMR at intervals of 15 mins. On completion of the reaction, the byproduct, sulfenyl chloride, and excess sulfuryl chloride were distilled out under a vacuum. The crude product was then purified using vacuum distillation and fractions were collected at a temperature range starting from 40-80 °C. The

fraction passing over 65 °C was found to contain compound **16** as a pale, yellow liquid (0.72 g, 45%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 6.83 (q, *J* = 5.4 Hz, 1H), 2.02 (s, 3H), 1.14 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 170.45, 151.21, 83.93, 23.74, 15.31.

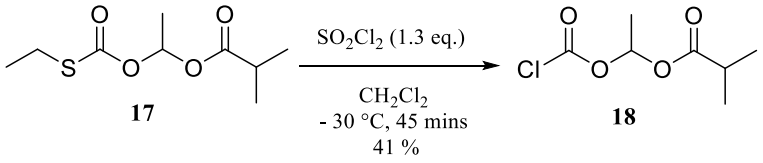
_O_-(2-Methylpropanoyloxy)ethyl _S_-ethyl ester (17)[36]



10 **17**

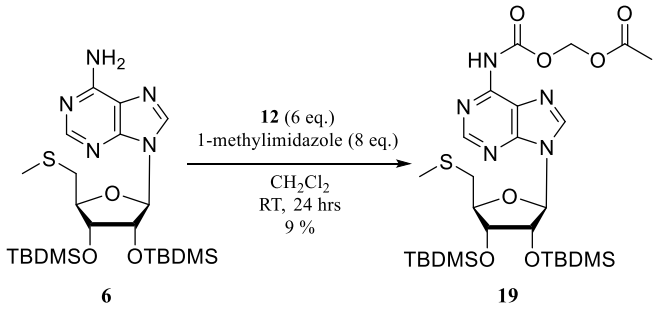
To a solution of compound **10** (4 g, 15.37 mmol) in 60 ml anhydrous toluene, silver isobutyrate (6.4 g, 38.44 mmol, 2.5 eq.) was added and the reaction was allowed to stir at RT overnight. With the addition of silver isobutyrate, the heterogenous mixture turned grey indicating rapid interconversion of silver isobutyrate to silver iodide. After 12 hours the toluene was evaporated under a vacuum and the mixture was diluted with 60 ml diethyl ether. It was then stirred at RT for 10 mins after which, it was filtered under a vacuum. The filtrate was collected, and solvent was evaporated under pressure to give a slightly yellow oily liquid. The crude was purified using column chromatography in 5% ethyl acetate in hexane to obtain a colorless liquid **17** (1.7 g, 51%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 6.87 (q, *J* = 5.4 Hz ,1H), 2.80 (q, *J* = 5.6 Hz, 2H), 2.48 (m, *J* = 7.2 Hz, 1H), 1.43 (d, *J* = 5.5 Hz, 3H), 1.24 (t, *J* = 7.1 Hz, 3H), 1.10 (d, *J* = 1.5 Hz, 3H), 1.09 (d, *J* = 1.2 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 174.88, 169.59, 89.92, 33.81, 25.28, 19.54, 18.65, 18.55.

[(2-Methylpropanoyl)oxy]ethyl chloroformate (18)[36]



Compound **17** (1.7 g,7.7 mmol) was cooled to -30 ℃ in a dry ice-acetonitrile cooling bath and stirred for 5 mins. A solution of sulfuryl chloride (1.3 g, 10.03 mmol, 1.3 eq.) in 5 ml dichloromethane was added dropwise to compound **17** and stirred at -30 ℃ over 10 mins using a canula under N_2 atmosphere. The reaction was stirred at that temperature for 45 mins. The completion of the reaction was monitored by NMR at intervals of 15 mins. On completion of the reaction, the byproduct, sulfenyl chloride, and excess sulfuryl chloride were distilled out under a vacuum. The crude product was then purified using vacuum distillation and fractions were collected at a temperature range starting from 40-80 ℃. The fraction passing over 60 ℃ was found to contain compound **18** as a pale, yellow liquid (0.58 mg, 41%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 6.75 (q, *J* = 5.4 Hz, 1H), 2.52 (m, *J* = 7.1 Hz, 1H), 1.52 (d, *J* = 5.5 Hz, 3H), 1.13 (d, *J* = 3.0 Hz, 3H), 1.11 (d, *J* = 3.2 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 174.60, 148.82, 93.57, 33.72, 19.16, 18.59.

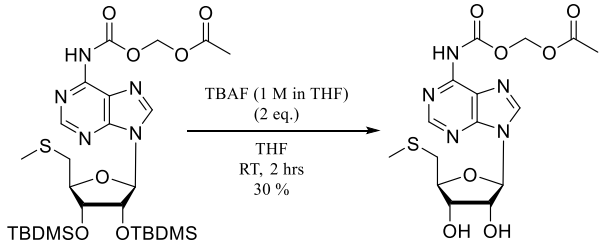
_N_-[(Acetoxy)methyloxy]carbonyl-2',3'-bis-_O_-(_tert_-butyldimethylsilyl)-MTA (19)



To a stirred solution of compound **6** (200 mg, 0.38 mmol) in 3 ml anhydrous dichloromethane, 1-methylimidazole (249 mg, 3.04 mmol, 8 eq.) was added and the solution was stirred at 0 °C for 10 mins. A solution of chloroformate **12 (**347 mg, 2.28 mmol, 6 eq.) in 2 ml dichloromethane was added dropwise to compound **6** in intervals of 2 hours, each time adding 2 eq. at 0 °C. Once the addition of the chloroformate was complete, the reaction mixture was allowed to stir at RT for 24 hours. The progress of the reaction was monitored by TLC and LCMS. On completion of the reaction, the reaction mixture was diluted with 20 ml dichloromethane and the organic layer was washed with brine and saturated NH_4Cl once. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure to give a yellow colored oily crude product. The crude was purified using column chromatography in 5% methanol in dichloromethane to give a colorless white solid **19** (21 mg, 9%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.80 (s, 1H), 8.21 (s, 1H), 8.19 (s, 1H), 5.97 (d, *J* = 5.5 Hz, 1H), 5.93 (s, 2H), 5.09 – 5.05 (m, 1H), 4.33 – 4.30 (m, 1H), 4.28 (dd, *J* = 6.7, 3.1 Hz, 1H), 3.06 (dd, *J* = 14.1, 6.7 Hz, 1H), 2.90 (dd, *J* = 14.0, 5.6 Hz, 1H), 2.18 (s, 3H), 2.17 (s, 3H), 0.97 (s, 9H), 0.80 (s, 9H), 0.17 (d, *J* = 9.6 Hz, 6H), -0.05 (s, 3H), -0.30 (s, 3H) ; ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 173.21, 151.62, 147.52, 145.31,143.68, 140.72, 122.16, 92.46, 88.62, 86.41,

83.07, 70.19, 36.43, 20.63, 16.84, 14.33 -4.21, -4.62, -5.36, -5.42; LCMS (ESI^+): 642.27 $[M + H]^+$.

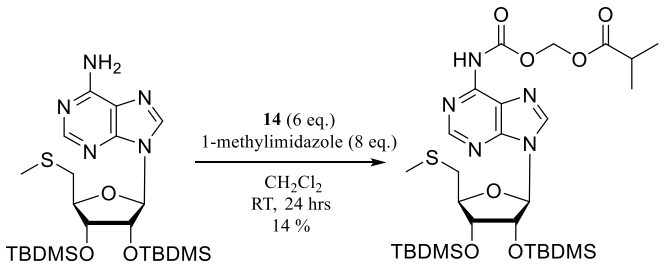
***N*-[(Acetoxy)methyloxy]carbonyl-MTA (LH1210)**



19 **LH1210**

To a solution of compound **19** (21 mg, 0.032 mmol) in anhydrous THF, a 1M solution of TBAF in THF (17 mg, 0.065 mmol, 2 eq.) was added dropwise at 0 ℃ and stirred for 2 hours at RT. The reaction progress was monitored using TLC and LCMS. On disappearance of the starting material, the solvent was evaporated, and the reaction mixture was diluted with 5 ml dichloromethane and washed once with brine. The crude product was purified using 10% methanol in dichloromethane to obtain a sticky white solid **LH1210** (4 mg, 30%). ^{1}H NMR (400 MHz, Methanol-d_4) δ (ppm): 8.71 (s, 1H), 8.34 (bs, 1H), 8.12 (s, 1H), 5.91 (d, J = 5.7 Hz, 1H), 5.84 (s, 2H), 4.74 (t, J = 5.5 Hz, 1H), 4.43 (t, J = 6.0 Hz, 1H), 4.28 (q, J = 6.0 Hz, 1H), 2.84 (dd, J = 14.2 Hz, 5.5 Hz, 1H), 2.72 (dd, J = 14.0 Hz, 5.5 Hz, 1H), 2.16 (s, 3H), 2.08 (s, 3H); ^{13}C NMR (100 MHz, Methanol- d_4) δ (ppm): 171.22, 152.81, 149.63, 147.31, 145.44, 140.72, 122.42, 88.65, 82.53, 76.14, 70.83, 68.11, 35.47, 15.24.

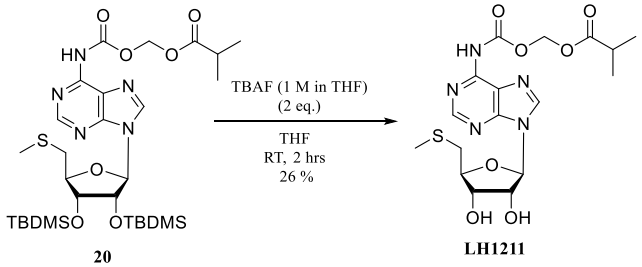
_N_-[(2-Methylpropanoyloxy)methyloxy]carbonyl-2',3'-bis-_O_-(_tert_-butyldimethylsilyl)-MTA (20)



To a solution of compound **6** (200 mg, 0.3 mmol) in 3 ml anhydrous dichloromethane, 1-methylimidazole (249 mg, 3.04 mmol, 8 eq.) was added and the solution was stirred at 0 °C for 10 mins. A solution of chloroformate **14 (**411 mg, 2.28 mmol, 6 eq.) in 2 ml dichloromethane was added dropwise to compound **6** in intervals of 2 hours, each time adding 2 eq. at 0 °C. Once the addition of the chloroformate was complete, the reaction mixture was allowed to stir at RT for 24 hours. The progress of the reaction was monitored by TLC and LCMS. The reaction mixture was diluted with 20 ml dichloromethane and the organic layer was washed with brine and saturated NH_4Cl once. The organic layer was dried over Na_2SO4, filtered and the solvent was evaporated under reduced pressure to give a yellow colored oily crude product. The crude was purified using column chromatography in 5% methanol in dichloromethane to give a colorless white solid **20** (36 mg, 14%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.80 (s, 1H), 8.22 (s, 1H), 8.19 (s, 1H), 5.97 (d, *J* = 5.5 Hz, 1H), 5.94 (s, 2H), 5.07 (d, *J* = 4.3 Hz, 1H), 4.32 (d, *J* = 3.0 Hz, 1H), 4.30 – 4.27 (m, 1H), 3.06 (dd, *J* = 14.0, 6.6 Hz, 1H), 2.90 (dd, *J* = 13.9, 5.5 Hz, 1H), 2.65 (sept, *J* = 7.1 Hz, 1H), 2.18 (s, 3H), 1.23 (d, *J* = 6.9 Hz, 6H) . ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): ^{13}C NMR (100 MHz, Chloroform-*d*) δ 175.29, 152.40, 151.17, 148.58, 143.90,

143.18, 119.53, 89.52, 84.84, 82.31, 75.07, 74.13, 36.62, 34.38, 33.78, 33.69, 18.64, 18.54, 18.20, 18.01, 17.82, 16.68, -4.40, -4.58, -4.63, -5.08; LCMS (ESI^+): 670.28 $[M + H]^+$.

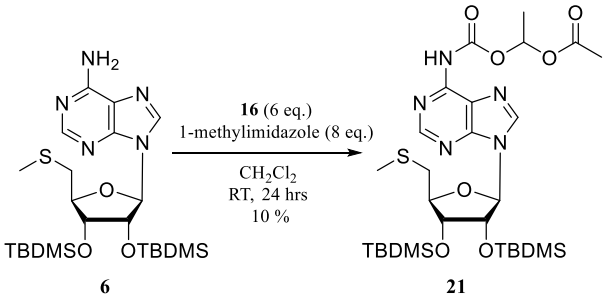
_N_-[(2-Methylpropanoyloxy)methyloxy]carbonyl-MTA(LH1211)



20 **LH1211**

To a solution of compound **20** (36 mg, 0.053 mmol) in anhydrous THF, a 1M solution of TBAF in THF (18.26 mg, 0.069 mmol, 2 eq.) was added dropwise at 0 °C and stirred for 2 hours at RT. The reaction progress was monitored using TLC and LCMS. On the disappearance of the starting material, the solvent was evaporated, and the reaction mixture was diluted with 5 ml dichloromethane and washed once with brine. The crude product was purified in 10% methanol in dichloromethane to obtain a sticky white solid **LH1211** (6 mg, 26%). ^{1}H NMR (400 MHz, Methanol- d_4) δ (ppm): 8.82 (s, 1H), 8.31 (bs, 1H), 8.24 (s, 1H), 6.01 (d, J = 5.7 Hz, 1H), 5.91 (s, 2H), 5.14 (t, J = 5.5 Hz, 1H), 4.47 (t, J = 6 Hz, 1H), 4.31 (q, J = 6.4 Hz, 1H), 3.17 (dd, J = 14.1 Hz, 5.3 Hz, 1H), 2.85 (dd, J = 14.0 Hz, 5.5 Hz, 1H), 2.25 (s, 3H), 1.54 (d, J = 3.0 Hz, 3H), 1.50 (d, J = 3.0 Hz, 3H); ^{13}C NMR (100 MHz, Methanol- d_4) δ (ppm):172.65, 151.82, 148.32, 147.19, 146.32, 144.72, 121.85, 88.46, 84.27, 76.23, 70.84, 67.28, 35.61, 32.48, 17.28, 13.88.

_N_-[(Acetoxy)ethyloxy]carbonyl-2',3'-bis-_O_-(_tert_-butyldimethylsilyl)-MTA (21)

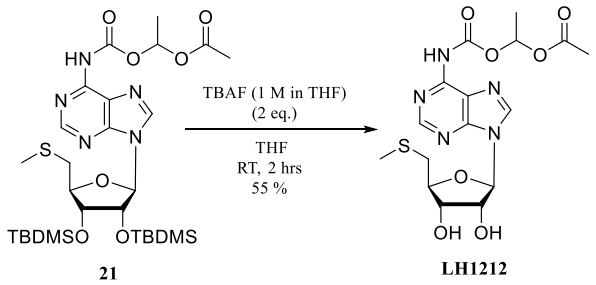


6 **21**

To a solution of compound **6** (200 mg, 0.38 mmol) in 3 ml anhydrous dichloromethane, 1-methylimidazole (249 mg, 3.04 mmol, 8 eq.) was added and the solution was stirred at 0 °C for 10 mins. A solution of chloroformate **16** (379 mg, 2.28 mmol, 6 eq.) in 2 ml dichloromethane was added dropwise to compound **6** in intervals of 2 hours, each time adding 2 eq. at 0 °C. Once the addition of the chloroformate was complete, the reaction mixture was allowed to stir at RT for 24 hours. The progress of the reaction was monitored by TLC and LCMS. The reaction mixture was diluted with 20 ml dichloromethane and the organic layer was washed with brine and saturated NH_4Cl once. The organic layer was dried over Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure to give a yellow colored oily crude product. The crude was purified using column chromatography in 5% methanol in dichloromethane to give a colorless white solid **21** (18 mg, 10%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.10 (s, 1H), 8.04 (bs, 1H), 7.98 (s, 1H), 6.75 (q, *J* = 5.3 Hz, 1H), 6.01 (d, *J* = 5.2 Hz, 1H), 4.36 (t, *J* = 5.5 Hz, 1H), 4.22 (t, *J* = 6.1 Hz, 1H), 4.16 (q, *J* = 6.4 Hz, 1H), 3.12 (dd, *J* = 14.3 Hz, 5.5 Hz, 1H), 2.84 (dd, *J* = 14.2 Hz, 5.5 Hz, 1H), 2.13 (s, 3H), 1.54 (d, *J* = 5.6 Hz, 3H), 1.31 (s, 3H), 1.04 (s, 9H), 0.75 (s, 9H), 0.52 (d, *J* = 7 Hz, 6H), -0.15 (s, 3H), - 0.36 (s, 3H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 170.34, 151.42, 148.62, 147.31, 146.38, 144.92, 122.28, 88.41, 86.27, 82.19, 84.76,

80.11, 35.42, 25.85, 23.64, 18.36, 15.48, 14.27, -4.14, -4.28, -5.36, -5.62; LCMS (ESI^+): 656.23 $[M + H]^+$.

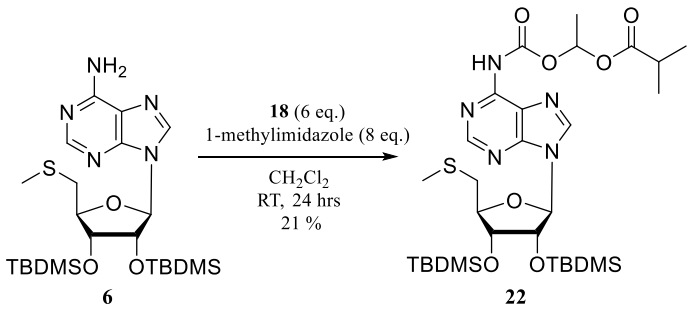
_N_-[(Acetoxy)ethyloxy]carbonyl-MTA (LH1212)



21 **LH1212**

To a solution of compound **21** (18 mg, 0.027 mmol) in anhydrous THF, a 1M solution of TBAF in THF (14.12 mg, 0.054 mmol, 2 eq.) was added dropwise at 0 °C and stirred for 2 hours at RT. The reaction progress was monitored using TLC and LCMS. On the disappearance of the starting material, the solvent was evaporated, and the reaction mixture was diluted with 5 ml dichloromethane and washed once with brine. The crude product was purified in 10% methanol in dichloromethane to obtain a sticky white solid **LH1212** (6 mg, 55%). ^{1}H NMR (400 MHz, Methanol- d_4) δ (ppm): 8.15 (s, 1H), 8.05 (bs, 1H), 7.84 (s, 1H), 6.14 (d, J = 5.7 Hz, 1H), 5.92 (q, J = 6.3 Hz, 1H), 4.32 (t, J = 5.4 Hz, 1H), 4.17 (t, J = 6.3 Hz, 1H), 4.06 (q, J = 6.1 Hz, 1H), 2.84 (dd, J = 14.0 Hz, 5.5 Hz, 1H), 2.65 (dd, J = 14.2 Hz, 5.5 Hz, 1H), 2.06 (s, 3H), 1.43 (d, J = 5.4 Hz, 3H), 1.28 (s, 3H); ^{13}C NMR (100 MHz, Methanol- d_4) δ (ppm): 170.32, 151.41, 148.63, 147.33, 146.28, 144.81, 122.34, 88.36, 86.21, 72.18, 70.38, 35.55, 14.29.

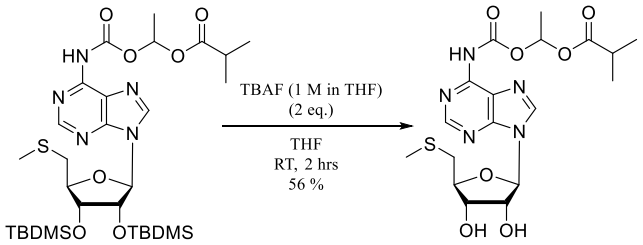
_N_-[(2-Methylpropanoyloxy)ethyloxy]carbonyl-2',3'-bis-_O_-(_tert_-butyldimethylsilyl)-MTA (22)



To a solution of compound **6** (200 mg, 0.38 mmol,) in 3 ml anhydrous dichloromethane, 1-methylimidazole (249 mg, 3.04 mmol, 8 eq.) was added and the solution was stirred at 0 °C for 10 mins. A solution of chloroformate **18** (411 mg, 2.28 mmol, 6 eq.) in 2 ml dichloromethane was added dropwise to compound **6** in intervals of 2 hours, each time adding 2 eq. at 0 °C. Once the addition of the chloroformate was complete, the reaction mixture was allowed to stir at RT for 24 hours. The progress of the reaction was monitored by TLC and LCMS. The reaction mixture was diluted with 20 ml dichloromethane and the organic layer was washed with brine and saturated NH_4Cl once. The organic layer was dried over Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure to give a yellow colored oily crude product. The crude was purified using column chromatography in 5% methanol in dichloromethane to give a colorless white solid **22** (22 mg, 21%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.79 (s, 1H), 8.18 (s, 1H), 6.99 (q, *J* = 5.2 Hz, 1H), 5.96 (d, *J* = 5.5 Hz, 1H), 5.08 (q, *J* = 5.3 Hz, 1H), 4.34 – 4.27 (m, 2H), 3.06 (dd, *J* = 16.7, 6.5 Hz, 1H), 2.90 (dd, *J* = 13.7, 5.5 Hz, 1H), 2.60 (dt, *J* = 14.0, 6.9 Hz, 1H), 2.18 (s, 3H), 1.61 (dd, *J* = 5.3, 1.8 Hz, 3H), 1.24 – 1.19 (m, 6H), 0.97 (s, 9H), 0.80 (s, 9H), 0.16 (d, *J* = 9.5 Hz, 6H), -0.05 (s, 3H), -0.30 (s, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm):

174.56, 150.76, 149.62, 148.27, 146.24, 144.29, 122.38, 92.84, 88.52, 86.42, 77.31, 76.42, 35.47, 33.71, 25.67, 19.27, 18.14, 17.57, 14.82, -4.32, -4.67, -5.21, -5.33; LCMS (ESI^+): 684.35 $[M+H]^+$.

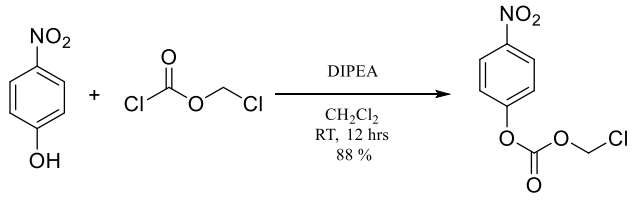
_N_-[(2-Methylpropanoyloxy)ethyloxy]carbonyl-MTA (LH1213)



22 **LH1213**

To a solution of compound **22** (22 mg, 0.032 mmol) in anhydrous THF, a 1M solution of TBAF in THF (16.71 mg, 0.064 mmol, 2 eq.) was added dropwise at 0 °C and stirred for 2 hours at RT. The reaction progress was monitored using TLC and LCMS. On the disappearance of the starting material, the solvent was evaporated, and the reaction mixture was diluted with 5 ml dichloromethane and washed once with brine. The crude product was purified in 10% methanol in dichloromethane to obtain a sticky white solid **LH1213** (8 mg, 56%). ^{1}H NMR (400 MHz, Methanol- d_4) δ (ppm): 8.71 (s, 1H), 8.25 (bs, 1H), 8.02 (s, 1H), 5.96 (d, J = 5.7 Hz, 1H), 5.72 (q, J = 6.4 Hz, 1H), 4.46 (t, J = 5.2 Hz, 1H), 4.32 (t, J = 6.1 Hz, 1H), 4.18 (q, J = 6.0 Hz, 1H), 2.72 (dd, J = 14.1 Hz, 5.5 Hz, 1H), 2.53 (dd, J = 14.2 Hz, 5.5 Hz, 1H), 1.95 (s, 3H), 1.68 (d, J = 5.6 Hz, 3H), 1.24 (d, J = 3.2 Hz, 3H), 1.15 (d, J = 3.1 Hz, 3H); ^{13}C NMR (100 MHz, Methanol- d_4) δ (ppm): 174.31, 150.14, 149.35, 148.22, 147.85, 146.73, 122.17, 88.31, 86.42, 71.22, 70.54, 35.61, 33.29, 19.72, 18.34, 15.44.

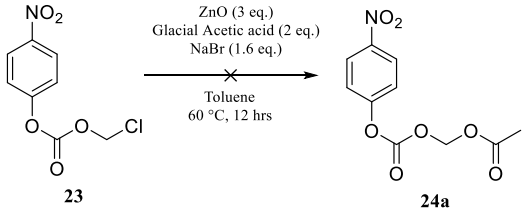
Chloromethyl 4-nitrophenyl ester (23)[37]



23

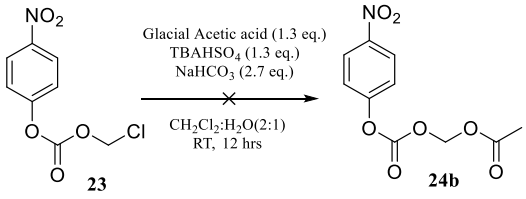
A solution of 4-nitrophenol (600 mg, 4.3 mmol) in 2 ml dichloromethane was cooled in an ice bath and N,N-Diisopropylethylamine (557 mg, 4.3 mmol) was added dropwise at 0 °C. A solution of chloromethyl chloroformate (556 mg, 4.3 mmol,) in 8 ml dichloromethane was added to the solution and the reaction mixture was stirred overnight at RT. The progress of the reaction was monitored by TLC. On completion of the reaction, the reaction mixture was diluted with 70 ml dichloromethane and extracted with 30 ml water. The organic layer was then washed once with 25 ml sat. $NaHCO_3$ and 30 ml brine. The dichloromethane layer was collected, dried over Na_2SO_4 and filtered. The solvent evaporated to give compound **23** (882 mg, 88%) as a white solid that was used in the next step without further purification. ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.26 (dd, *J* = 14.3 Hz, 9.1 Hz, 2H), 7.40 (dd, *J* = 28.1 Hz, 9.4 Hz, 2H), 5.8 (s, 2H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm):154.84, 145.92, 125.55, 125.45, 121.66, 72.69.

(Acetyloxy)methyl 4-nitrophenyl carbonate (24a)[37]



A solution of ZnO (90.67 mg, 1.11 mmol, 3 eq.) and glacial acetic acid (44 mg, 0.74 mmol, 2 eq.) was stirred in toluene at 105 °C for 1 hour. The temperature was lowered to 60 °C and compound **23** (86 mg, 0.37 mmol) was added to the solution, followed by the addition of NaBr (61.12 mg, 0.59 mmol, 1.6 eq.). The reaction mixture was further stirred at that temperature for 12 hours and the water generated, during the reaction, was removed using a Dean Stark apparatus. The progress of the reaction was monitored by TLC. The desired product was not obtained through this route and the starting material was recovered.

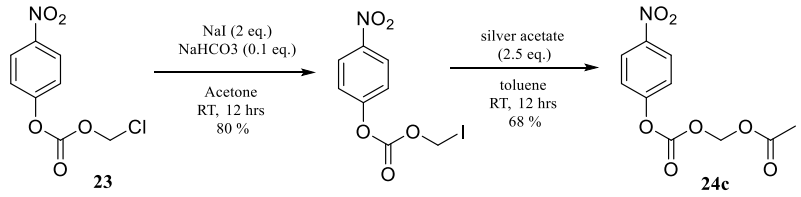
(Acetyloxy)methyl 4-nitrophenyl carbonate (24b)[37]



To the solution of glacial acetic acid (3.36 mg, 0.05 mmol, 1.3 eq.) and $TBAHSO_4$ (20 mg, 0.05 mmol, 1.3 eq.), $NaHCO_3$ (9.8 mg, 0.11 mmol, 2.7 eq.) in dichloromethane: H_2O (2:1) was added and stirred at RT for 2 hours. To the above solution, compound **23** (10 mg, 0.04 mmol) was added at 0 °C and the reaction mixture was stirred at RT for 18 hours. The

progress of the reaction was monitored by TLC. The desired product was not obtained through this route and starting material was recovered.

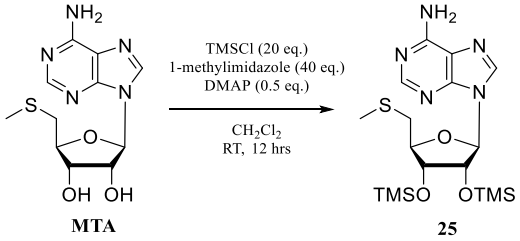
(Acetyloxy)methyl 4-nitrophenyl carbonate (24c)[37]



To a solution of compound **23** (500 mg, 2.15 mmol) in acetone, NaI (648 mg, 4.31 mmol, 2 eq.) and $NaHCO_3$ (18.13 mg, 0.21 mmol, 0.1 eq.) were added and the reaction mixture was stirred overnight at RT. The progress of the reaction was monitored by TLC. On completion of the reaction, the acetone was evaporated under reduced pressure, 10 ml diethyl ether was added to the residue and the mixture was filtered. The filtrate was evaporated under reduced pressure and the residue was diluted with 30 ml dichloromethane and washed twice with 10 ml 20% $Na_2S_2O_3$ and 10 ml brine. The organic layer was collected, dried over Na_2SO_4 and the solvent was then evaporated under a vacuum. The crude product was purified using 5% ethyl acetate in hexane to give a white solid (698 mg, 80%). The solid obtained was reacted with silver acetate (721 mg, 4.32 mmol, 2 eq.) in toluene at RT for 12 hours. On completion of the reaction, the toluene was evaporated, diethyl ether was added, and the solution was then filtered under a vacuum. The filtrate was evaporated to give a crude product which was further purified in 10% ethyl acetate in hexane to give compound **24c** as a white solid (164 mg, 68%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.21 (dd, *J* = 14.2 Hz, 9.1 Hz, 2H), 7.22 (dd, *J* = 28.0 Hz, 9.2 Hz,

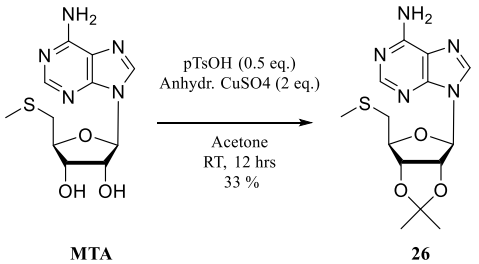
2H), 6.2 (s, 2H), 2.04 (s, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm):168.33, 155.38, 153.82, 145.35, 125.19, 122.42, 76.62, 21.08.

2',3'-bis-*O*-(Trimethylsilyl)-MTA (25)



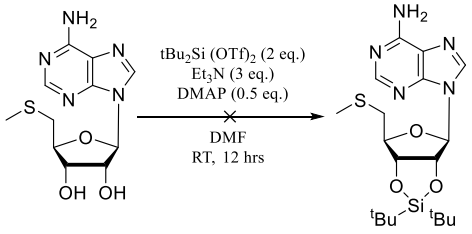
To a solution of MTA (50 mg, 0.16 mmol) in anhydrous dichloromethane, 1-methylimidazole (552.10 mg, 6.7 mmol, 40 eq.) and DMAP (10.27 mg, 0.08 mmol, 0.5 eq.) were added and allowed to cool to 0 °C in an ice bath. To the above solution, a solution of trimethylsilyl chloride (366 mg, 3.36 mmol, 20 eq.) in dichloromethane was added and stirred at RT. The reaction progress was monitored by LCMS. Presence of starting material was detected after 24 hours along with the presence of compound **25.** LCMS (ESI$^+$): 442.50 $[M+H]^+$.

2',3'-bis-*O*-(Isopropylidene)-MTA (26)



To a solution of MTA (30 mg, 0.1 mmol) in 5 ml acetone, p-toluenesulfonic acid (9.6 mg, 0.05 mmol, 0.5 eq.) was added followed by the addition of anhydrous $CuSO_4$ (32 mg, 0.2 mmol, 2 eq.) and the reaction mixture was stirred at RT for 24 hours. The reaction mixture was diluted with 20 ml dichloromethane and washed with 10 ml sat. $NaHCO_3$. The organic layer was dried over Na_2SO_4 and filtered. The solvent was evaporated to give a crude product which was further purified in 10% methanol in dichloromethane to give compound **26** as a white solid (9 mg, 33%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.42 (s, 1H), 7.86 (s, 1H), 6.02 (d, *J* = 5.4 Hz, 1H), 5.47 (t, *J* = 5.5 Hz, 1H), 5.01 (t, *J* = 5.2 Hz, 1H), 4.48 (q, *J* = 5.7 Hz, 1H), 2.76 (dd, *J* = 14.2 Hz, 5.5 Hz, 1H), 2.62 (dd, *J* = 14.0 Hz, 6.0 Hz, 1H), 2.01 (s, 1H), 1.54 (s, 1H), 1.32 (s, 1H) ; LCMS (ESI$^+$): 338.11 $[M+H]^+$.

2',3'-bis-*O*-(di-*tert*-butylsilylene)-MTA (27)

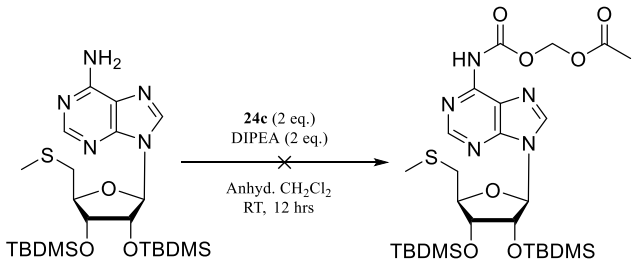


MTA **27**

To a solution of MTA (8 mg, 0.02 mmol) in 0.5 ml DMF, triethylamine (6 mg, 0.06 mmol, 3 eq.) was added at 0 °C and then, di-*tert*-butylsilylbis(trifluoromethanesulfonate) (23 mg, 0.04 mmol, 2 eq.) was added dropwise. The reaction mixture was stirred at RT overnight. DMAP (2 mg, 0.01 mmol, 0.5 eq.) was added as a catalyst. The reaction progress was

monitored by TLC and LCMS, however, only the presence of starting material was observed.

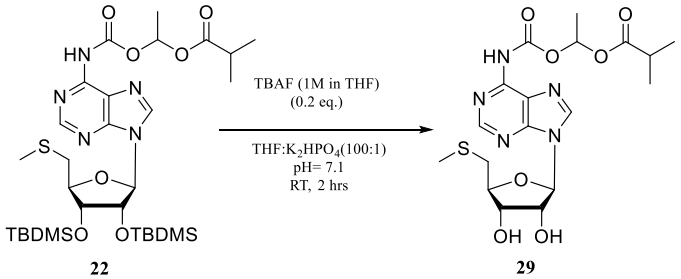
***N*-[(Acetoxy)methyloxy]carbonyl-2',3'-bis-*O*-(*tert*-butyldimethylsilyl)-MTA (28)**



6 **28**

To a solution of compound **6** (100 mg, 0.19 mmol) in 5 ml dichloromethane, DIPEA (50 mg, 0.38 mmol, 2 eq.) was added and stirred at RT for 5 mins. A solution of compound **24c** (96 mg, 0.38 mmol, 2 eq.) in 1 ml dichloromethane was added dropwise to the above solution and stirred for 12 hours at RT. The reaction progress was monitored by TLC and LC-MS. After 48 hours, no conversion was seen.

***N*-[(2-Methylpropanoyloxy)ethyloxy]carbonyl-MTA (29)**

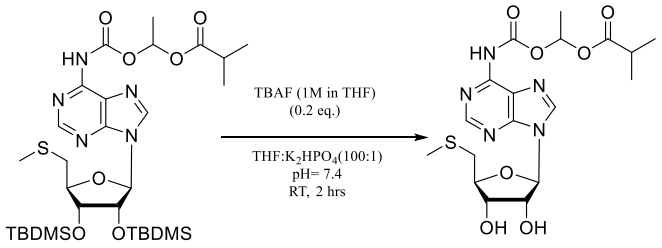


22 **29**

To a solution of compound **22** (2 mg, 0.0029 mmol) in 0.524 ml of THF, 5.84 µl (0.2 eq.) of a 0.1 M TBAF buffered solution prepared by diluting 1M TBAF in THF 10 times with 0.1 M K_2HPO_4 buffer (pH=7.1) was added. The reaction mixture was monitored by

LCMS for progress of reaction for 2 hours at RT and the formation of MTA (LCMS (ESI^+): 298.32 $[M+H]^+$) was observed in less than 1 hour.

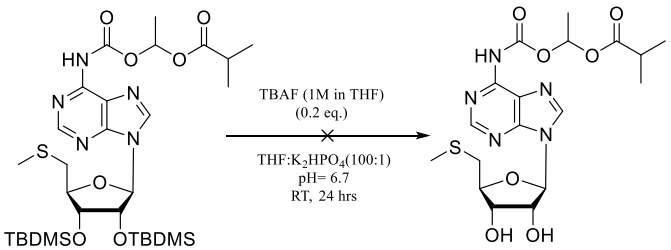
***N*-[(2-Methylpropanoyloxy)ethyloxy]carbonyl-MTA (30)**



22 **30**

To a solution of compound **22** (2 mg, 0.0029 mmol) in 0.524 ml of THF, 5.84 µl (0.2 eq.) of a 0.1 M TBAF buffered solution prepared by diluting 1M TBAF in THF 10 times with 0.1 M K_2HPO_4 buffer (pH=7.4) was added. The reaction mixture was monitored by LCMS for progress of reaction for 2 hours at RT and the formation of MTA (LCMS (ESI^+): 298.32 $[M+H]^+$) was observed in less than 1 hour.

***N*-[(2-Methylpropanoyloxy)ethyloxy]carbonyl-MTA (31)**

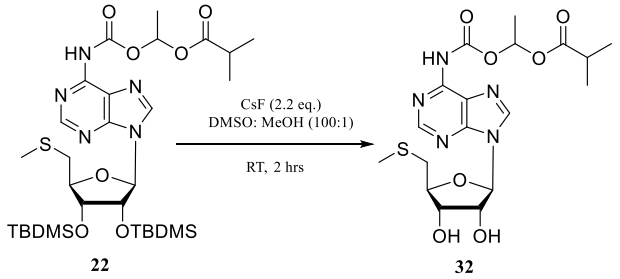


22 **31**

To a solution of compound **22** (2 mg, 0.0029 mmol) in 0.524 ml of THF,5.84 µl (0.2 eq.) of a 0.1 M TBAF buffered solution prepared by diluting 1M TBAF in THF 10 times with 0.1 M K_2HPO_4 buffer (pH=6.7) was added. The reaction mixture was monitored by

LCMS for progress of reaction for 24 hours at RT. Presence of mono-deprotected form of compound **22** (LCMS (ESI^+): 570.41 $[M+H]^+$) was observed.

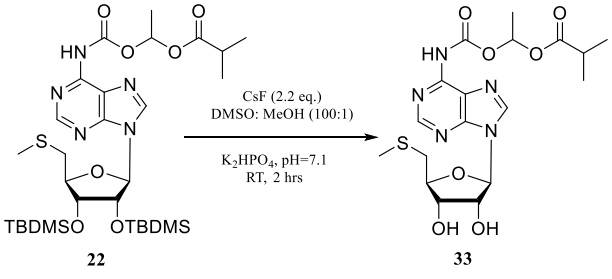
***N*-[(2-Methylpropanoyloxy)ethyloxy]carbonyl-MTA (32)**



22 32

To a solution of compound **24** (2 mg, 0.0029 mmol) in 0.58 ml DMSO, 8.75 µl of a 0.67 M CsF solution prepared in DMSO:MeOH=1: 2 was added. The progress of the reaction was monitored by LCMS and stirred at RT for 2 hours and the presence of MTA (LCMS (ESI^+): 298.32 $[M+H]^+$) was detected in less than 1 hour.

***N*-[(2-Methylpropanoyloxy)ethyloxy]carbonyl-MTA (33)**



22 33

To a solution of compound **22** (2 mg, 0.0029 mmol) in 0.58 ml DMSO, 8.75 µl of a 0.67 M CsF solution prepared in DMSO:MeOH=1:2 was added. To the above solution, 5.8 µl of phosphate buffer, pH =7.1 was added. The progress of the reaction was monitored by

LCMS. No byproduct MTA was detected with complete conversion to desired product in 2 hours. LCMS (ESI^+): 456.13 $[M+H]^+$.

Preparation of 200 ml 50 mM Phosphate buffer containing 1 mM EDTA

NaH_2PO_4 (1.38 g, 10 mM) and EDTA (74.4 mg, 0.1 mM) were dissolved in 180 ml deionized water. The pH was first adjusted to 7.4 with 5 N NaOH and then with 1N NaOH. The volume was adjusted to 200 ml and the buffer was filtered and stored at 4 °C.

Stability Assay of MTA prodrugs in Phosphate Buffer

The stock solution of the prodrug (10 mM in DMSO) was diluted 10-fold to give a 1 mM working solution in 10% DMSO. 100 µl of the 1 mM solution was added to 875 µl of 50 mM phosphate buffer containing 1 mm EDTA, pH 7.4 and incubated at 37 °C for 5 minutes in an eppendorf vial. The vial was then centrifuged at a speed of 9 x 1000g for 5 minutes and the supernatant was split into 3 fractions of 292.5 µl, one for stability in phosphate buffer, one for activation in human liver microsomes and one for activation in mouse liver microsomes.

The fraction for analyzing stability was mixed with 7.5 µl of deionized water and incubated at 37 °C for 3 days. Time points were collected after every 0 min, 3 min, 5 min, 10 min, 15 min, 30 min, 45 min, 1hr, 3 hr, 5 hr, 8 hr, 10 hr, 12 hr, 24 hr, 48 hr, 72 hr. At each time point, 20 µl aliquots were taken and diluted with 40 µl cold acetonitrile and centrifuged at 9 x 1000g for 5 mins. 40 µl of the supernatant were taken and diluted with 40 µl deionized

water and frozen before injecting on the HPLC. The samples were analyzed using a Perkin Elmer Series 2000 HPLC with a Phenomenex Kinetix C18 (50 x 4.6 mm) column and a gradient of 10 - 90% acetonitrile containing 0.1% TFA in 10 min at a flowrate of 0.6 mL/min was used with detection wavelength of 220 and 254 nm.

In-vitro Activation Assay in Human and Mouse Liver Microsomes[38]

The mouse and human liver microsomes were obtained from Xeno Tech with a protein concentration of 20 mg/ml and stored in eppendorf vials at – 70 °C. Stock solution of 1 mM compound in 1 ml DMSO was diluted 10-fold to give a 1 mM solution in 10% DMSO. 100 µl of the 1 mM solution was added to 875 µl of 50 mM phosphate buffer containing 1 mm EDTA, pH 7.4 and incubated at 37 °C for 5 minutes in an eppendorf vial. The vial was then centrifuged at a speed of 9 x 1000g for 5 minutes and the supernatant was split into 3 fractions of 292.5 µl, one for stability in phosphate buffer, one for activation in human liver microsomes and one for activation in mouse liver microsomes. The fraction for analyzing activation was mixed with 7.5 µl of either the human or mouse liver microsomes and incubated at 37 °C for 3 days. Time points were collected after every 0 min, 3 min, 5 min, 10 min, 15 min, 30 min, 45 min, 1hr, 3 hr, 5 hr, 8 hr, 10 hr, 12 hr, 24 hr, 48 hr, 72 hr. At each time point, 20 µl aliquots were taken and quenched with 40 µl cold acetonitrile and stored on ice for 30 mins before centrifugation at 9 x 1000g for 5 mins. 40 µl of the supernatant were taken and diluted with 40 µl deionized water and frozen before injecting on the HPLC. The samples were analyzed using a Perkin Elmer Series 2000 HPLC with a Phenomenex Kinetix C18 (50 x 4.6 mm) column and a gradient of 10 - 90% acetonitrile

containing 0.1% TFA in 10 min at a flowrate of 0.6 mL/min was used with a detection wavelength of 220 and 254 nm. The half-life was calculated from the first order rate equation of the conversion of prodrug to MTA.

In-vitro Oxidative Activation Assay in presence of NADPH

Stock solution (10 mM in DMSO) was diluted 10-fold to give a solution of 1 mM in 10% DMSO. 100 µl of the 1 mM solution was added to 875 µl of 50 mM phosphate buffer containing 1 mm EDTA, pH 7.4 and incubated at 37 °C for 5 minutes in an eppendorf vial. The vial was then centrifuged at a speed of 9 x 1000g for 5 minutes and the supernatant was split into 2 fractions, each of 292.5 µl, one for oxidative activation in mouse liver microsomes and one for human liver microsomes. Each of the fractions was mixed with 20 µl of NADPH and either 20 µl of mouse liver microsomes or human liver microsomes and incubated at 37 °C for 3 days. Time points were collected after every 0 min, 3 min, 5 min, 10 min, 15 min, 30 min, 45 min, 1hr, 3 hr, 5 hr, 8 hr, 10 hr, 12 hr, 24 hr, 48 hr, 72 hr. At each time point, 20 µl aliquots were taken and quenched with 40 µl cold acetonitrile and stored on ice for 30 mins before centrifugation at 9 x 1000g for 5 mins. 40 µl of the supernatant were taken and diluted with 40 µl deionized water and frozen before injecting on the HPLC. The samples were analyzed using a Perkin Elmer Series 2000 HPLC with a Phenomenex Kinetix C18 (50 x 4.6 mm) column and a gradient of 10 - 90% acetonitrile containing 0.1% TFA in 10 min at a flowrate of 0.6 mL/min was used with a detection wavelength of 220 and 254 nm. The half-life was calculated from the first order rate equation of the conversion of prodrug to MTA.

In-vitro Activation Assay in 50 % Human Serum

Stock solution of compound (10 mM in DMSO) was diluted 10-fold to give a 1 mM solution in 10 % DMSO. 100 µl of the above solution was added to 400 µl of 50 mM phosphate buffer, vortexed and incubated at 37 °C for 5 mins. It was then centrifuged at 9 x 1000g for 5 mins and the supernatant was split into 2 fractions of 230 µl each, one for control in H_2O and other for activation in Human Serum. The control fraction was mixed with 230 µl of water, while the other was mixed with 230 µl of Human Serum. Both fractions were incubated at 37 °C for 2 days. Time points were calculated after every 0 min, 3 min, 5 min, 10 min, 15 min, 30 min, 45 min, 1hr, 3 hr, 5 hr, 8 hr, 10 hr, 12 hr, 24 hr, 48 hr. For every time point, 20 µl of aliquots were taken and diluted with 40 µl cold Acetonitrile:Methanol=2:1, vortexed and stored on ice for 30 mins. The samples were then centrifuged at 9 x 1000g for 5 mins and the supernatant was collected. 40 µl of the supernatant were diluted with 40 µl of deionized water and stored at – 30 °C before injection on the HPLC. The samples were analyzed using a Perkin Elmer Series 2000 HPLC with a Phenomenex Kinetix C18 (50x4.6mm) column and a gradient of 10 - 90% acetonitrile containing 0.1% TFA in 10 min at a flowrate of 0.6 mL/min was used with a detection wavelength of 220 and 254 nm. The half-life was calculated from the first order rate equation of the conversion of prodrug to MTA.

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