SYNTHESIS AND EVALUATION OF L-CYSTINE CRYSTALLIZATION INHIBITORS AND PRODRUGS FOR CYSTINURIA

by

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ABSTRACT OF THE DISSERTATION

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Cystinuria is a lifelong and recurrent stone disease characterized by renal L-cystine calculi due to defective renal and intestinal reabsorption of L-cystine and dibasic amino acids. Cystinuria is caused by defects in one or both *SLC3A1* and *SLC7A9* genes located on chromosomes 2p and 19q, respectively. L-Cystine crystallizes in a zwitterionic form with stable crystals and low solubility due to the hydrogen-bond networks within the solid. The presence of four terminal groups, two amino and two carboxylic groups, with the ability to form hydrogen-bond networks makes L-cystine more unique than other amino acids or standard dipeptides. The medical management of the disease focuses mainly on lowering the concentration of L-cystine in urine or increasing L-cystine solubility in urine. Hyperdiuresis to decrease the concentration of L-cystine in urine and alkalinization are used to improve L-cystine solubility and if these techniques fail the next step in the treatment algorithm is introduction of thiol drugs. Drugs like D-penicillamine and tiopronin react with L-cystine to form more soluble mixed disulfides, but they are poorly tolerated due to their numerous adverse side effects. A new

alternative approach to the prevention of L-cystine kidney stones through molecular mimicry was suggested recently by Ward and colleagues. L-CDME was able to reduce the mass yield of crystallization and maintain a metastable supersaturated L-cystine concentration which is sufficient for preventing stone formation. However, the in vivo stability of L-CDME is a real concern for it to be used as a therapeutic since esters are liable to esterase enzyme cleavage and its metabolite is L-cystine which would worsen the disease. Our group converted the ester to the amide which is more stable in vivo that lead to better inhibition with enhanced stability profile. The diamide analogues were more active and more stable according to our crystallization inhibition assay, real-time in situ atomic force microscopy (AFM) and in vitro chemical stability study. Research in this dissertation focused on the structure-activity relationship (SAR) of the most active analogues. Our results showed the importance of keeping the core structure of L-cystine intact in order to maintain the strong binding affinity to the L-cystine crystal and inhibit the crystallization of L-cystine. Our research led to compound 8-L-cystinyl bis(1,8-diazaspiro[4.5]decane) (**56**) with an EC_{50} of 25.1 nM which is 1.7 fold more potent than LH708 (**9**), L-cystine bis(N′-methylpiperazide), in our crystallization inhibition assay. The bioavailability of LH708 (**9**) in *SLC3A1* knockout mice was found to be 18%. To improve the oral bioavailability of LH708, several prodrugs with better lipophilicity were designed, synthesized, and evaluated for their stability and activation.

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DEDICATION

To my parents, my husband and my kids

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ABBREVIATIONS

Ac	Acetyl
ACN	Acetonitrile
AFM	Atomic force microscopy
AGT1	Aspartate/glutamate transporter 1
AOP	Tris(dimethylamino)(3H-1,2,3-triazolo[4,5-b]pyridin-3-yloxy)phosphorus hexafluorophosphate
BBMV	Brush-border membrane vesicle
Boc	*t*-Butoxycarbonyl
BOP	Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
Bu	Butyl
Cbz	Benzoxycarbonyl
Cys	Cysteine
DCC	N,N'-Dicyclohexylcarbodiimid
DCM	Dichloromethane
DIPEA or DIEA	Diisopropylethylamine
DFT	Density-functional theory
DMAP	4-Dimethylaminopyridine
DMF	*N, N* - Dimethylformamide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA	Ethylenediaminetetraacetic acid
EtOAc	Ethyl acetate
FCC	Flash column chromatography
Fmoc	9-Fluorenylmethoxycarbonyl
GGA	Generalized-gradient approximation
HATU	Hexafluorophosphate azabenzotriazole tetramethyl uranium
HBTU	Hexafluorophosphate benzotriazole tetramethyl uranium
HILIC	Hydrophilic interaction liquid chromatography
HMPA	Hexamethyl-phosphoramide
HOBt	1-Hydroxybenzotriazole
IBCF	Isobutyl chloroformate
IS	Internal standard
IV	Intravenous
LA	Lipoic acid
L-CDME	L-Cystine dimethyl-ester
L-CME	L-Cystine methyl-ester
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MBD	Many-body dispersion
MeOH	Methanol
MPG	Alpha- mercaptopropionylglycine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBC	*N*-Boc-cysteine
NHS	*N*-Hydroxysuccinimide
NMM	*N*-Methylmorpholine
NMP	*N*-Methylpyrolidone
OPA	*O*-Phthaldialdehyde
PCNL	Percutaneous nephrolithotomy
PyAOP	((7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate)
PyBOP	(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
Ser	Serine
SNP	Sodium nitroprusside
SWL	Shockwave lithotripsy
TBTA	*tert*-Butyl 2,2,2-trichloroacetimidate
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin-layer chromatography

CHAPTER ONE

INTRODUCTION

Cystinuria is an inherited aminoaciduria characterized by renal L-cystine calculi due to defective renal and intestinal reabsorption of L-cystine, a dimer of the amino acid cysteine linked via a disulfide bond, and dibasic amino acids (lysine, ornithine and arginine).[1, 2] The defective transport of dibasic amino acids and L-cystine across the apical membranes of proximal renal tubular and jejunal epithelial cells is the main characteristic of the disorder.[3] Due to the low solubility of L-cystine in urine it precipitates leading to L-cystine crystals that could grow to form larger renal stones which produce obstruction, infection, and renal insufficiency.[2, 3] At physiological pH L-cystine has a solubility of 250-500 mg/L and the usual range of daily L-cystine excretion in cystinuria patients is 600-1400 mg.[4] Cystinuria is the only disease amongst the heterogeneous group of kidney stone diseases that is completely caused by genetic mutations.[5] Cystinuria has a worldwide prevalence ranging from 1:2000 at the Mediterranean East Coast to 1:100000 in Sweden with an average of 1 in 7000 neonates which represents approximately 1% and 3-10% of adult and pediatric stone disease, respectively.[3, 5-7] Although there is smaller prevalence of cystinuria than other kinds of kidney stones, L-cystine stones are larger, recur more frequently, and are more likely to cause chronic kidney disease.[8] Indeed unlike other common kidney stones, L-cystine stones may fill the renal collecting system and the L-cystine crystallites can scrape the urinary tract causing pain and bleeding.[9] Patients with cystinuria are susceptible to reduced kidney function and chronic kidney damage because of the high rate of stone

recurrence and the need for multiple procedures or surgical intervention due to the fact that L-cystine stones are dense, large and mostly resistant to extracorporeal shockwave lithotripsy (SWL).[6, 10] As many as 70% of patients could have renal insufficiency and <5% could get end-stage renal disease.[11] Cystinuria is a lifelong and recurrent stone disease which is challenging to manage due to the lack of current therapy that reverses the underlying derangement of dibasic amino acid transport and the necessity of repeated urological interventions.[11] A number of factors can contribute to the stone formation in cystinuria patients which could be summarized as: environmental factors such as dietary intake of fluids, salt and protein and the individual's genetic background which appear clear with the substantial variability existed in the onset of lithiasis among cystinuria patients.[12] Despite new knowledge on the molecular-genetic basis of the disease, the pharmacotherapeutic options for treatment have not improved in decades. Both the evaluation and the treatment of cystinuric patients continue to be a major challenge for medical care providers.[6, 13]

1.1 Amino Acid Transporter

1.1.1 Genetics

The first identified mutation in a cystinuria patient in 1994 lead to the involvement of the *SLC3A1* gene in the etiology of the disease. Five years later, a mutation in the *SLC7A9* gene was discovered.[5] Cystinuria was classified based on the excretion of L-cystine and dibasic amino acids in obligate heterozygotes that was criticized and Della Strologo and colleagues started a new classification based on the affected genes *SLC3A1* and *SLC7A9*.[6] Based on the excretion cystinuria was classified into three types: I, II and III, where type I excrete L-cystine at normal level while type II and III have elevated excretion.[5, 6] Type

II and III were characterized later on as non-type I since the lack of correlation between the elevated excretion and the mutation in type II/III cystinuria.[5, 14] The new classification based on the affected gene classifies cystinuria into two types: type I, caused by mutations in *SLC3A1* gene, and non-type I, mainly by *SLC7A9* gene with predomination of type I cystinuria with >60% of the cases of the disease.[3] The disease's mode of transmission is different for each type; autosomal recessive mode for type I cystinuria and autosomal dominant with incomplete penetrance for non-type I cystinuria.[14] Cystinuria is caused by defects in one or both *SLC3A1* and *SLC7A9* genes located on chromosomes 2p and 19q, respectively. *SLC3A1* encodes the heavy subunit rBAT of the renal amino acid transporter needed to localize the transporter to the plasma membrane. *SLC7A9* encodes the light subunit $b^{0,+}$AT that controls the catalytic transporting component. The two subunits rBAT and $b^{0,+}$AT are linked by a disulfide bridge to form the transporter (Figure 1).[11, 12, 14] The Human Genome Mutation Database recorded 160 different mutations in the *SLC3A1* gene and 116 in the *SLC7A9* gene.[6, 15] Reabsorption of >90% of L-cystine occur in segments S1-S2 of the proximal tubule where rBAT/ $b^{0,+}$AT is highly expressed. rBAT is a type II membrane N-glycoprotein (~80-90 kDa) with a single transmembrane (TM) domain, intracellular N-terminus and large extracellular C-terminus (~50-60 kDa), while $b^{0,+}$AT is highly hydrophobic and not glycosylated (~50 kDa) with 12-TM-domain.[12] The in vivo direct evidence for the complex was only proved recently when Chillaron and collegues used antibodies directed against rBAT and $b^{0,+}$AT. They demonstrated that rBAT- $b^{0,+}$AT heterodimerizes in the kidney brush-border membrane and quantification of the interaction shows that rBAT is the only $b^{0,+}$AT associated heavy subunit.[16]

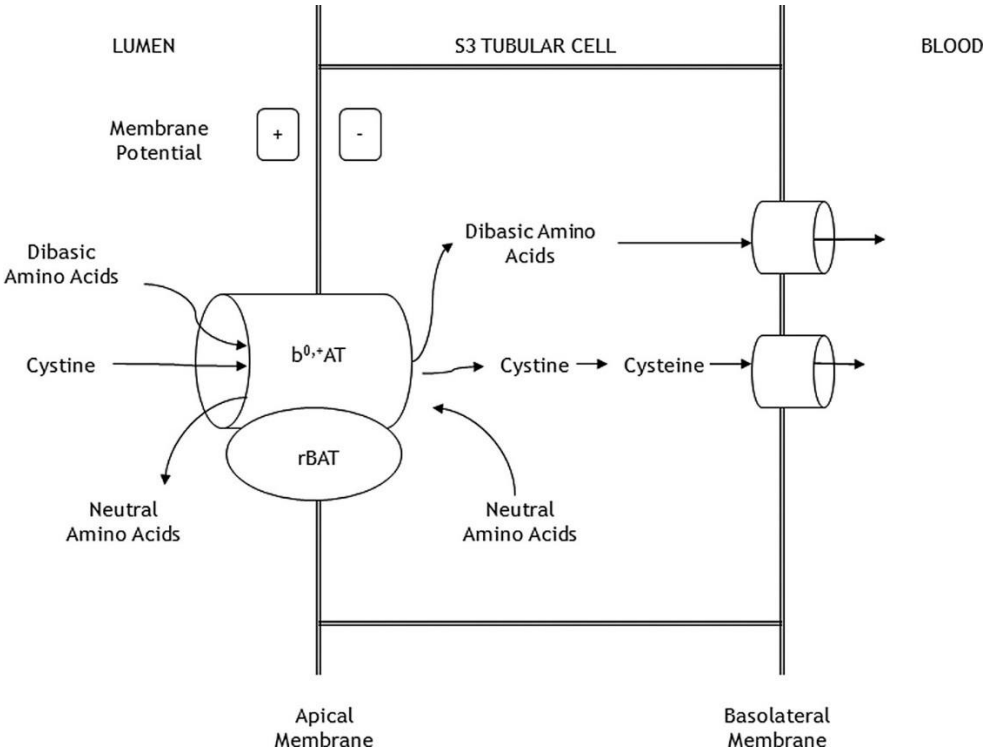


Figure 1. Heteromeric Amino Acid Transporter for L-Cystine and Dibasic Amino Acid Transport in the Proximal Tubule.[17] (Reproduced with permission from Elsevier).

The involvement of the rBAT gene in cystinuria confirmed the significance of this gene in the reabsorption of L-cystine and dibasic amino acids in kidney and intestine.[1] rBAT and $b^{0,+}$AT are both expressed along the proximal tubule, but with opposite expression gradients where rBAT expression increases from S1 to S3 and the reverse holds for $b^{0,+}$AT.[16] The discrepancy in the expression between rBAT and $b^{0,+}$AT in the proximal tubules suggests that there is another rBAT associated light chain likely from the same family as $b^{0,+}$AT as data shows that they share the same electrophoretic mobility.[16, 18, 19] Also, unknown subunit heterodimer of rBAT was suggested upon the comparison of homozygous $b^{0,+}$AT knockout (*SLC7A9-/- SLC3A1+/+*) and rBAT mutant (*SLC7A9+/+ SLC3A1-/-*) mice where $b^{0,+}$ system transport activity was lost completely or was

approximately 10% conserved, respectively.[16, 20, 21] Indeed, a heterodimer containing rBAT has been observed in a $b^{0,+}$AT-deficient mice and when $b^{0,+}$AT is defective in mice only small fractional excretion of L-cystine was observed suggesting that L-cystine transporters other than $b^{0,+}$AT possess high reserve capacity to compensate largely the loss of $b^{0,+}$AT function.[18] Kanai and colleagues[18] determined that aspartate/glutamate transporter 1 (AGT1) is the transporter corresponding to the missing partner of rBAT in the S3 segment of the proximal tubules. AGT1 is an Na+-independent acidic amino acid transporter expressed specifically in the kidney at the apical membrane of the S3 segment. The proof of the presence of the dimer was supported by an in situ proximity ligation assay and further was confirmed by the observation that AGT1 disappeared from the brush-border membrane vesicle (BBMV) in mutant mice lacking rBAT expression. Furthermore, they generated new anti-AGT1 antibodies which revealed that rBAT is a heavy chain of AGT1 and a transport assay of the AGT1-rBAT heterodimer reconstituted into proteoliposomes which revealed that it transports L-cystine as well as aspartate and glutamate.[18]

1.1.2 Transporters

The heteromeric amino acid transporter (HAT) family forms heterodimers composed of a 12 membrane spanning light chain (SLC7) and a single membrane spanning heavy chain (SLC3).[16, 18] The light chain catalyzes transport functions while the heavy chain is vital for plasma membrane localization and stabilization of the light chain. These chains are covalently bound via a disulfide bridge and until recently only two heavy chains have been identified SLC3A1/rBAT and SLC3A2/4F2hc/CD98hc.[18] Amino acid transporters participate in interorgan amino acid nutrition by their transport across the plasma

membrane which mediate and regulate the flow of these ionic nutrients into the cells. Specific proteins that recognize, bind and transport these amino acids are responsible for their transfer across the hydrophobic domain of the plasma membrane.[1] Special amino acid transport systems located in the apical membrane in the intestine are responsible for the absorption of amino acids in the small intestine. The absorbed amino acids are released into the portal vein by a set of different basolateral transport systems to be transported to the liver. There, they would be used in the liver or delivered systematically to other tissues as nutrients. In addition, in the kidney the epithelial cells reabsorb valuable nutrients in the urine where more than 95% of amino acids are cleared in the S1-S3 segments of the proximal tubule.[22] General properties of mammalian amino acid transport were revealed after the pioneering work of Halvor N. Christensen's group in the early 1960s such as broad substrate specificity where several amino acids share the same transport system and stereospecificity where the L-stereoisomers transport is faster. The isolation of the first brain GABA transporter and the identification of the first cationic amino acid transporter in the early 1990s provided the foundation for the cloning of mammalian amino acid transporter genes.[1] In cystinuria the transporter consists of a heavy subunit of type II membrane glycoprotein (rBAT) and a light subunit which is an unglycosylated membrane protein bearing 12 putative transmembrane domains ($b^{0,+}AT$), as shown in Figure 1. In order to mediate high-affinity transport of L-cystine and dibasic amino acids, the heterodimer travels to the plasma membrane.[16] The transport system $b^{0,+}$ exchange dibasic amino acids and L-cystine for neutral amino acids from the intestinal and renal tubular lumen.[12] The transporter is expressed in the S1-S2 segments of the proximal tubule and the small intestine where it controls the dibasic amino acids and L-

cystine exchange for other neutral amino acids in 1:1 stochiometry.[5] System $b^{0,+}$ is a Na^{+}-independent transporter which is an antiporter coupling the uptake of cationic amino acids and L-cystine to the efflux of neutral amino acids.[22] The driving forces of the exchange are the high intracellular concentration of neutral amino acids, the electric potential across the plasma membrane and the intracellular reduction of L-cystine to cysteine.[12] The activity of system $b^{0,+}$ is achieved by the heterodimeric transporter rBAT/ $b^{0,+}$AT and mutations of either of them would lead to cystinuria, but with different phenotypes.[22] rBAT/ $b^{0,+}$AT accounts for >90% of renal L-cystine absorption as it represents the main mechanism of tubular L-cystine transport, so mutations in the system could lead to strong trafficking effects or loss of function.[5, 12] The defect in the amino acid transport leads to high concentrations of L-cystine and other dibasic amino acids in urine leading to precipitation of L-cystine due to its reduced solubility at physiological pH that could result in recurrent L-cystine renal calculi, obstructive uropathy, hypertension, infection and rarely renal failure.[14] Even though patients with cystinuria lose cysteine, lysine, and arginine, they do not experience malnutrition due to the apical absorption of dipeptides and tripeptides from dietary protein by the intestinal apical transporter solute carrier family 15 member (Figure 2).[12]

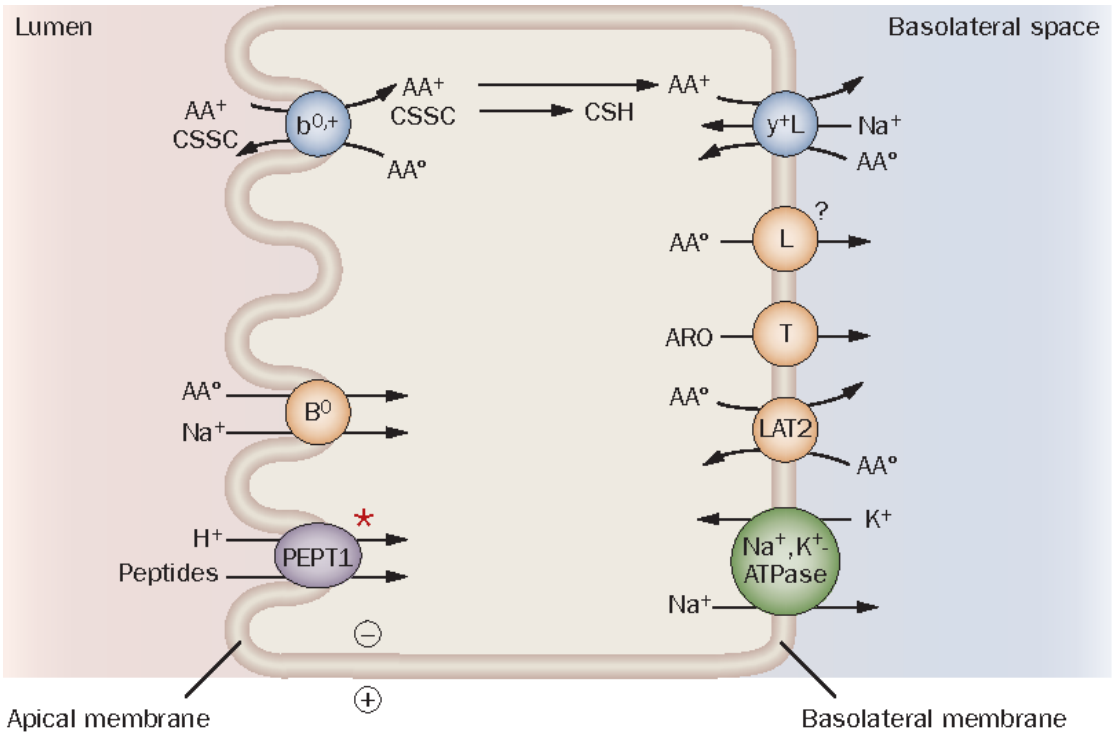


Figure 2. Uptake of Dipeptides and Tripeptides Through the Apical H+-dependent PEPT1 in the Small Intestine. [12] (Reproduced with permission from Springer Nature).

1.1.3 Mouse Models

A first murine model for type I cystinuria was described in 2003 with mutation in *SLC3A1* resulting in a single amino acid substitution in the extracellular domain of rBAT. The mouse model is suitable to study the pathophysiology as well as the evaluation of therapeutic and metaphylactic approaches as it mimics the etiology and clinical manifestations of human cystinuria type I.[23] Also a mouse model for cystinuria developed by homologous recombination generates mice with a disruption of *SLC7A9* in a mixed genetic background. Expression of $b^{0,+}$AT protein is completely abolished in the kidney of *SLC7A9*−/− mice which presents a dramatic hyperexcretion of L-cystine and dibasic

amino acids, while *SLC7A9+/−* mice show moderate but significant hyperexcretion of these amino acids.[24]

1.2 L-Cystine Crystal

1.2.1 Morphology

Cystinuria has characteristic crystals which are usually hexagonal, translucent and white and they are visible in 17 to 25% of patients' urine samples.[5] Since it is a sulfur-containing amino acid, the urine may have a characteristic odor of rotten eggs especially after laser fragmentation of ureteric calculi.[11] The stones are pale yellow flecked with shiny crystallites.[9, 11] The nature of the L-cystine stones make fragmentation by extracoporal lithothripsy difficult with the need of percutaneous nephrostomy placement and removal for larger stones.[5, 6]

1.2.2 Bonding Networks

The two known polymorphic forms of L-cystine crystals are the tetragonal phase (P41)[25] and the hexagonal phase (P6122)[26] where both crystallize with the molecule in its zwitterionic form.[27] The structure of hexagonal L-cystine has been solved by three-dimensional methods with the hydrogen bond networks linking the molecules together, in the late 1950s by Oughton and Harrison.[26] The realization that L-cystine crystallize in a zwitterionic form with stable crystals and low solubility has been correlated to the hydrogen-bond networks within the solid. What makes L-cystine unique compared to other amino acids or standard dipeptides is the presence of four terminal groups, two amino and two carboxylic groups, with the ability to form hydrogen-bond networks.[28] The L-cystine crystal is formed of hydrogen-bonded layers with principal intermolecular

interactions take the form of NH···O hydrogen bonds, formed between the ammonium and carboxylate moieties and unusually short S···S contacts. These contacts are approximately co-linear with the S −S covalent bond with a distance of 3.444 Å which is much shorter than the sum of the van der Waals radii of the S atoms (3.7 Å).[27] Oughton and Harrison[26] provided the bond lengths and angles as well as L-cystine conformation in the crystal. The bond lengths between the atoms in the L-cystine molecule (-S-C1-C2(N)-C3O1O2)$_2$ are 1.82 Å for S-C1 bond, 1.51 Å for C1-C2, and 2.03 Å for S-S. Their data shows the disposition of tile H atoms about the N atom, as three hydrogen atoms arranged approximately tetrahedrally around each N atom, and the nearly equal lengths of the C3-O1 and C3-O2 bonds which clearly indicate a zwitterion form. Even more they found an interesting feature of the structure which is the close van der Waals contacts between each sulphur atom and its non-bonded neighboring atoms as 3.47 Å for S···S, 3.67 Å for S···C1 and 3.21 Å for S···N (intramolecular).[26] Recently, Galvan and colleagues[28] studied the intermolecular interactions that govern the stability of the L-cystine crystal by using density-functional theory (DFT) with the generalized-gradient approximation (GGA) and including many-body dispersion (MBD) interactions. These terminal groups in zwitterionic crystals obtain charged $-COO^-$ and $-NH3^+$ which can form head-to-tail chains (Figure 3, panel 1) or ring-shape conformation sequence (Figure 3, panels 2 and 3) linked via the N−H···O hydrogen bonds. In addition, L-cystine crystal has also an added intermolecular S···S close contact (Figure 3, panel 4) which has the ability to form chains in a plane perpendicular to the C axis. The head-to-tail interactions, as shown in Figure 3, panel 1, tetramer has nine hydrogen bonds and one S···S interaction, while the ring-shape

tetramer, as in Figure 3, panels 2 and 3, has ten hydrogen bonds and two S⋯S interactions.[28]

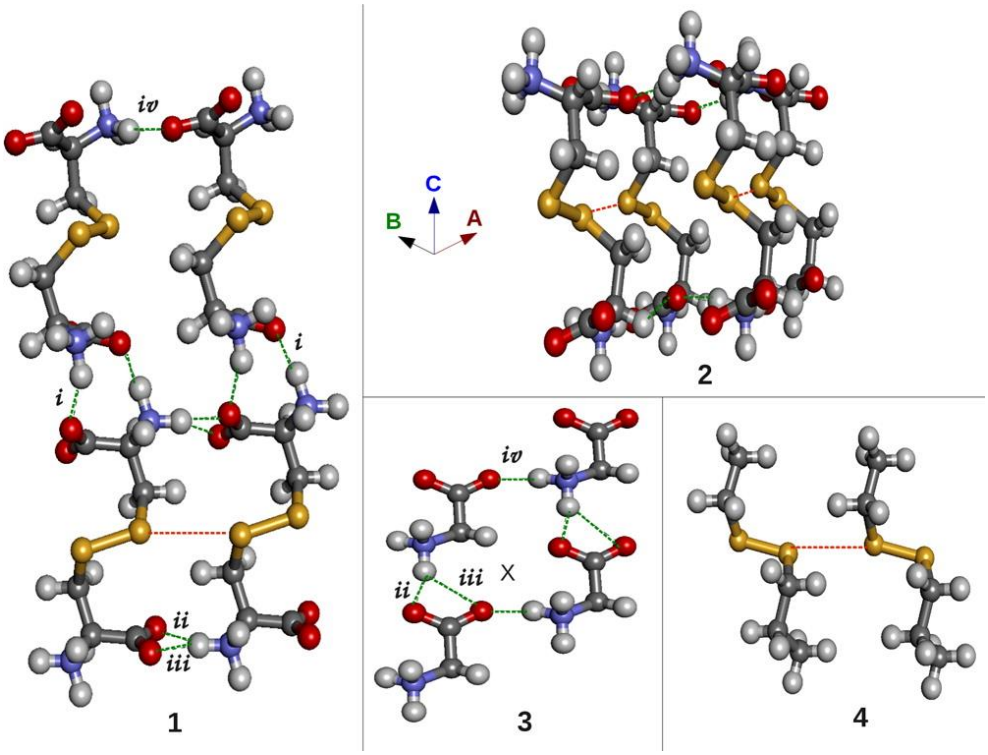


Figure 3. Hydrogen Bond Networks and S⋯S Interactions Present in the L-Cystine Crystal.[28] (Reproduced with permission from American Chemical Society).

1.3 Medical Management

1.3.1 Pharmaceutical

The low solubility of L-cystine in urine leads to kidney stones and hence the therapy is focused on lowering the urine L-cystine concentration or increasing L-cystine solubility in urine.[2] The medical management of the disease starts with hydration and urinary alkalinization mainly besides a diet low in methionine and sodium.[11] The goal of hyperdiuresis is to reach a urine volumes of 3 L/d which require ingesting 4-4.5 L of

water per day.[11] Only then if that proved to be ineffective a thiol drugs will be prescribed because of the concern about untoward side effects.[2] In addition, the serious damages caused by the disease to the kidneys and the surrounding organs and the episodic stone symptoms which requires frequent stone removal make the causative therapy a crucial matter.[5] Currently the treatment of cystinuria is focused on reducing the L-cystine concentration or reacting L-cystine to form more soluble disulfides.[2, 5]

1.3.1.1 Urine Alkalinization

The goal of urine alkalinization is to achieve a level of urine pH above 7-7.5 which requires significant and frequent doses of alkali that is difficult for patients to comply.[6] The urinary pH is important since the solubility of L-cystine at physiological pH of 7 is approximately 250 mg/L while it double and triple at pH 7.5 and 8, respectively. As described before a urine pH of >7.5 may encourage the dissolution of formed calculi, but the pH should be monitored carefully since urine pH of >7.5 can lead to other kind of calculi like calcium phosphate.[11] Indeed, increasing urinary pH to above 7 using alkali therapy is safe unless idiopathic hypercalciuria occurs.[9] Sodium bicarbonate was the first line alkalinizing agent, but it was found that the high sodium load may increase L-cystine excretion besides the increased risk of hypertension following long-term therapy. Now a days potassium citrate is the first line alkalinizing agent, but caution should be taken in renal impairment cases.[11]

1.3.1.2 Drug Therapy

If the attempts to solubilize L-cystine with hydration and alkalinization failed then drugs such as D-penicillamine, tiopronin, and captopril could be used. The common

pharmacotherapeutic treatment for cystinuria includes thiol drugs such as penicillamine or tiopronin, but high percentage of patients experience side effects accompanying the consumption of the drugs as 84% and 75%, respectively.[9, 11] These thiol compounds react chemically with L-cystine by a thiol exchange reaction to form a thiol-cysteine mixed disulfide (Figure 4) which is 50-200 times more soluble than L-cystine and could be safely excreted in urine.[2, 11] This means that they must be given at high doses since decomposition of one molecule of L-cystine requires consumption of two molecules of the drug.[8] The need for high doses is hard to achieve since these compounds are not specific, so adverse reactions are common.[8, 11] The adverse effects of D-penicillamine include rash, arthralgia, leukopenia, gastrointestinal intolerance and nephritic syndrome and also vitamin B-6 (pyridoxine) deficiency is expected with long-term therapy. In addition, it is recommended that patients on D-penicillamine therapy undergo regular monitoring of white blood cell count, haemoglobin and platelet levels and urinalysis.[11] In general thiol drugs have numerous side effects, within the usual range of therapeutic dosage, including foul odor, nausea, fever, fatigue, skin rash, premature skin aging, proteinuria and hypersensitivity.[6, 9] Recently the effect of alkalinization on the ability of thiol drugs to undergo disulfide exchange with L-cystine was studied in vitro and it shows that the reaction occurs more rapidly at high pH and suggested a goal of urine pH of above 7.5.[2] Reportedly patients do not comply to therapy because of side effects or cost and besides a typical medical regimen may include up to 10 tablets or more daily and this poor compliance leads to high incidence of surgical intervention.[29]

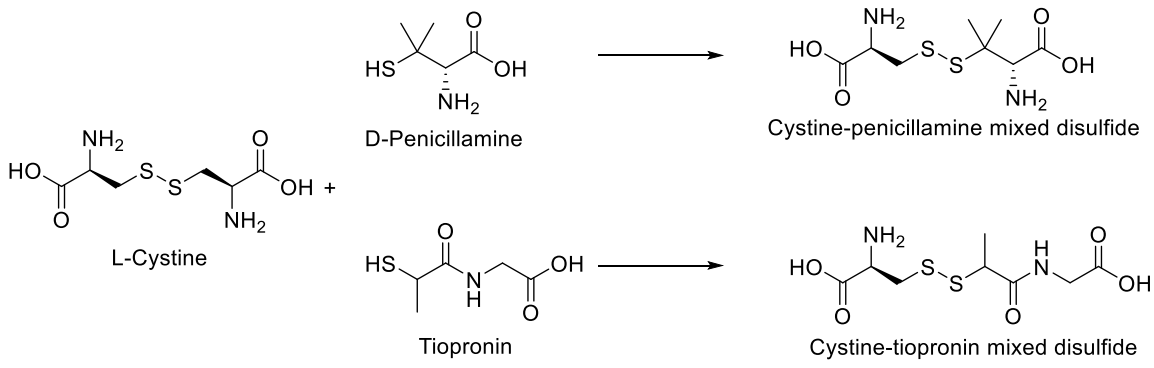


Figure 4. Thiol Exchange Reaction with L-Cystine to Form a Thiol-cysteine Mixed Disulfide.

1.3.1.3 Recent Advances

Recently, Ward and colleagues[30] suggested a new alternative approach to the prevention of L-cystine kidney stones through molecular mimicry which shows promising results. The inhibition achieved through binding of molecular imposters that bind to L-cystine crystal and block the attachment of incoming L-cystine solute molecules which result in a substantial reduction in the overall crystal yield.[31, 32] The two inhibitors are L-cystine dimethyl-ester (L-CDME) and L-cystine methyl-ester (L-CME) where the carboxylate groups in L-cystine are replaced by methyl-ester groups or only one methyl-ester group, respectively. These molecules will obtain specific binding at the L-cystine crystal surfaces that was revealed by in situ real-time atomic force microscopy (AFM) and parallel bulk crystallization studies. In addition the inhibitors were able to reduce the mass yield of crystallization and maintain a metastable supersaturated L-cystine concentration which is sufficient for preventing stone formation.[30] Also they studied the effect of L-CDME (Figure 5C) addition on the crystal growth hillocks which results in roughened step edges at 56 uM (Figure 5B) compared to L-cystine as shown in Figure

5A.[33] In vivo testing further confirm the efficacy of these molecular imposters where *SLC3A1* knockout mice fed daily with 10 mg/kg for 4 weeks resulted in decrease in stone size and total stone burden.[34]

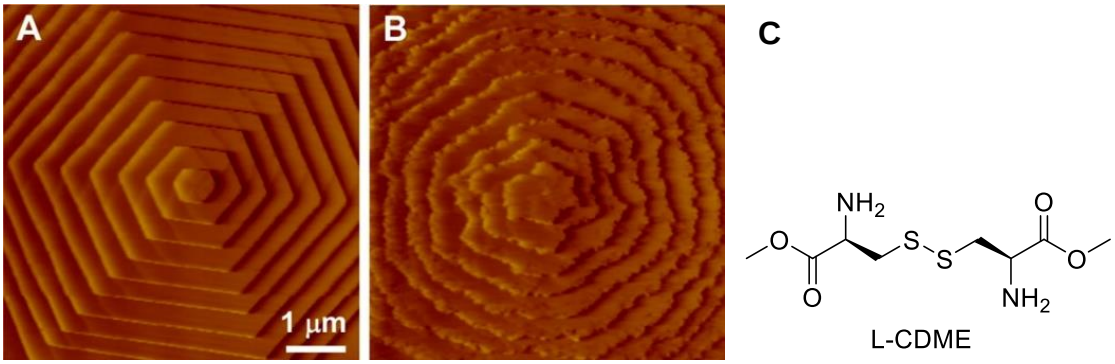


Figure 5. L-Cystine Growth Hillocks for Impurity Free Solution (A) Compared to Solution Containing L-CDME (B), L-CDME Chemical Structure (C).[33] (Reproduced with permission from American Chemical Society).

Zee and colleagues[10] used the *SLC3A1−/−* mouse model, which develops L-cystine urolithiasis, to identify compounds that inhibit stone formation by comparison with drugs currently used in clinics or under investigation. They treated mice with tiopronin, L-CDME, sulforaphane and α-lipoic acid (α-LA) and compared them to mice on a regular diet. Their results showed that only α-LA gave a lower growth rates for stone formation while all other drugs showed no significant difference to untreated mice. The effect of 0.5% supplemented diet with α-LA, equivalent to a 2700 mg/d human dose, was reversed upon withdrawal of the drug which suggest that continuous treatment is necessary. Unfortunately, lower doses of 0.1% and 0.25% α-LA in mouse diet were significantly less effective. On the other hand, in vitro L-cystine precipitation analysis of α-LA in *SLC3A1−/−* mice urine had no detectable effect on L-cystine solubility which means that

the effect seen with the in vivo studies was likely for α-LA-derived metabolites. More studies will be required to determine the active metabolite(s) and the mechanism behind increasing L-cystine solubility.[10]

Fu and colleagues[8] performed in vitro L-cystine growth inhibition assay where crystallization mass yield of L-cystine from a 3 mM saturated solution without additives was set to 100 wt%. They then calculated the mass yield in the presence of 20 mg/L of thiol drugs used in clinics for cystinuria like: tiopronin, D-penicillamine and captopril and as known with their mild effect they only decreased the mass yield of L-cystine by about 7 wt%, while N-acetyl-L-cysteine (a thiol drug used commonly as an expectorant) gave about 35 wt% while more than 80 wt% was obtained for L-CDME. A supernatant of a prepared mixture of N-acetyl-L-cysteine with an excess of L-cystine to give the unsymmetrical disulfide N-acetyl-L-cystine gave similar effect to the mass yield as the corresponding thiol. These results showed the significance of molecular mimics mechanism for the inhibition of L-cystine crystal growth in comparison to the mechanism of the current medications through the reaction with L-cystine generating more soluble asymmetric disulfides.[8] Even though N-acetyl-L-cysteine can form more soluble cysteine disulfide than L-cystine, it should disqualify as a treatment since it could lead to more urinary L-cystine in vivo.[9]

1.3.2 Urological Intervention

1.3.2.1 Noninvasive

Shockwave lithotripsy (SWL) is a noninvasive stone fragmentation technology used since the 1980.[35, 36] Mostly extracorporeal shock wave lithotripsy failed for patients with

cystinuria due to the increased resistance of L-cystine stones to fragmentation.[37] The chemical structure of L-cystine and the physio-chemical properties of L-cystine stones are thought to contribute to decrease SWL fragility.[38] Percutaneous nephrolithotomy (PCNL) is considered for large L-cystine calculi, complex stones or SWL resistant stones. The drawbacks of PCNL could be summarized as the high rates of retreatment and recurrence after treatment.[11]

1.3.2.2 Open Surgery

In cases where pharmaceutical treatment and fragmentation attempts have failed surgical intervention will be required to break up and remove stones and it could even require nephrectomy in some cases.[37] The open surgical stone removal approach should be avoided for cystinuric patients as the recurrence nature of the stone formation of the disease so a minimally invasive approach should be considered.[39] Studies showed that the expected rate of the 5-year stone recurrence after surgical intervention is 73%.[11]

1.4 Our Group Research

Even though L-CDME shows good in vitro inhibition, its in vivo stability remains a real concern not only because it would not give the desirable concentration if taken orally but also mainly because its metabolite is L-cystine which would worsen the disease. In fact it is known that our bodies are full of esterases which are enzymes that have the ability to hydrolyze an ester to give the corresponding carboxylic acid.[40] In addition toxicity is another concern as L-CDME has been used to produce an animal model of cystinosis which is a disorder where intracellular lysosomal L-cystine accumulation occurs.[41] To

solve the problem of the stability our group converted the ester to the better in vivo stable amide (Figure 6) leading to similar or better inhibition with enhanced stability profile.[42]

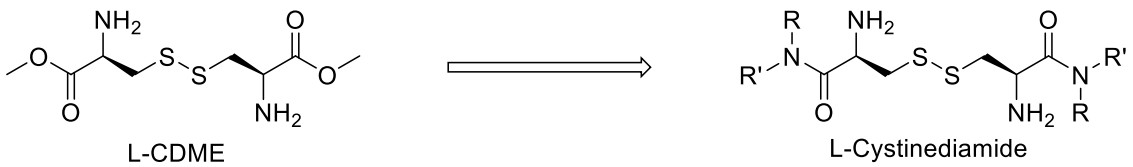


Figure 6. Chemical Structure of L-CDME and Our New L-Cystine Diamides.

Initial research on this project was done by Dr. Yanhui Yang, who began by investigating the effects of replacing the methyl-ester on L-CDME with different amide substituents and he studied the added effect of the methylated amine as well. The synthesis of these analogues could be found in our recent publication.[43]

All diamide analogues showed better or similar profile for increasing the aqueous solubility of L-cystine and thus inhibiting L-cystine crystallization compared to L-CDME (Figure 7 and Table 1). Five analogues (**5**,**7**-**10**) were shown to have better inhibition ability than L-CDME and were carried out for dose-response characterization (Figure 8). Among the five analogues the L-cystine bis(N-methyl piperazide) (LH708, **9**) and bismorpholide (LH707, **8**) were the most potent with EC_{2X} of 0.26 and 0.86 µM, respectively versus 6.37 µM for L-CDME. The EC_{2X} is calculated as the concentration required to double the apparent aqueous solubility of L-cystine.

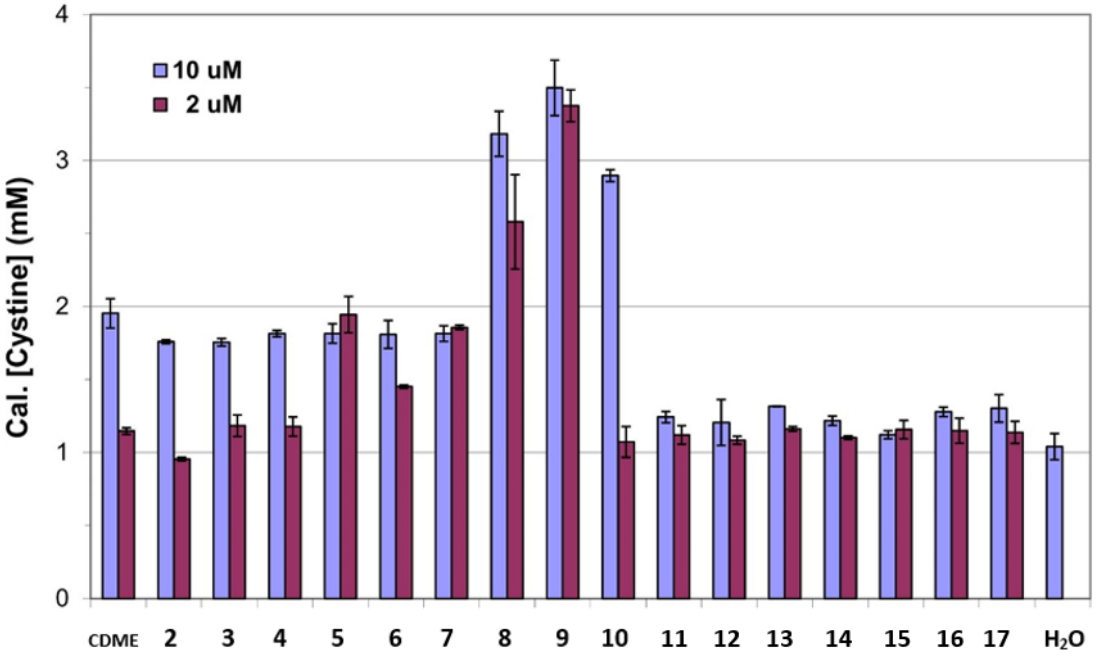
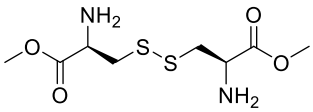
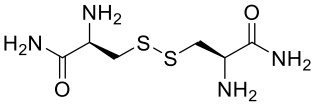
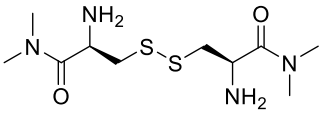
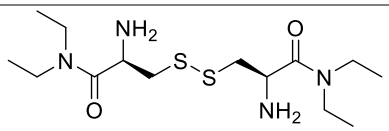
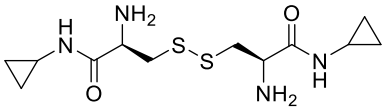
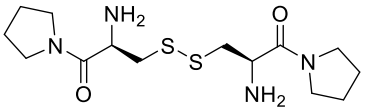
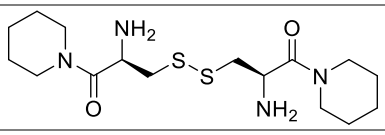
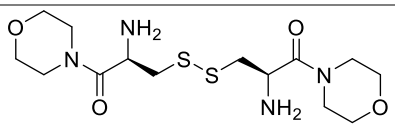
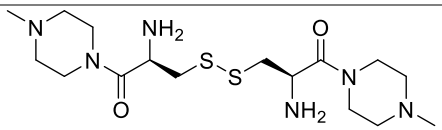
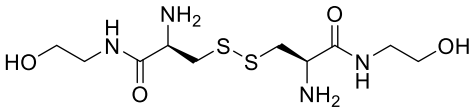


Figure 7. Aqueous Solubility of L-Cystine in Presence of L-Cystine Diamides **2-10** and **11-17** in Comparison to L-CDME and Water Controls.[43]

Table 1. Aqueous Solubility of L-Cystine in Presence of L-Cystine Diamides and Binding Energies to L-Cystine Crystal Surface.

	Structure	**Aqueous Solubility of L-Cystine**		**EC_{2X} (µM)**	**$E_{Binding}$ (kcal/ mol)**
		2 uM	**10 uM**		
1 (L-CDME)		1.14	1.95	6.37	-316
2 (LH701)		0.95	1.75	--	-287
3 (LH702)		1.18	1.75	--	-296
4 (LH703)		1.17	1.81	--	-288

5 (LH704)		1.94	1.81	3.53	-292
6 (LH705)		1.45	1.80	--	-302
7 (LH706)		1.85	1.81	1.59	-307
8 (LH707)		2.57	3.18	0.86	-324
9 (LH708)		3.37	3.49	0.26	-429
10 (LH709)		1.07	2.89	2.02	-307

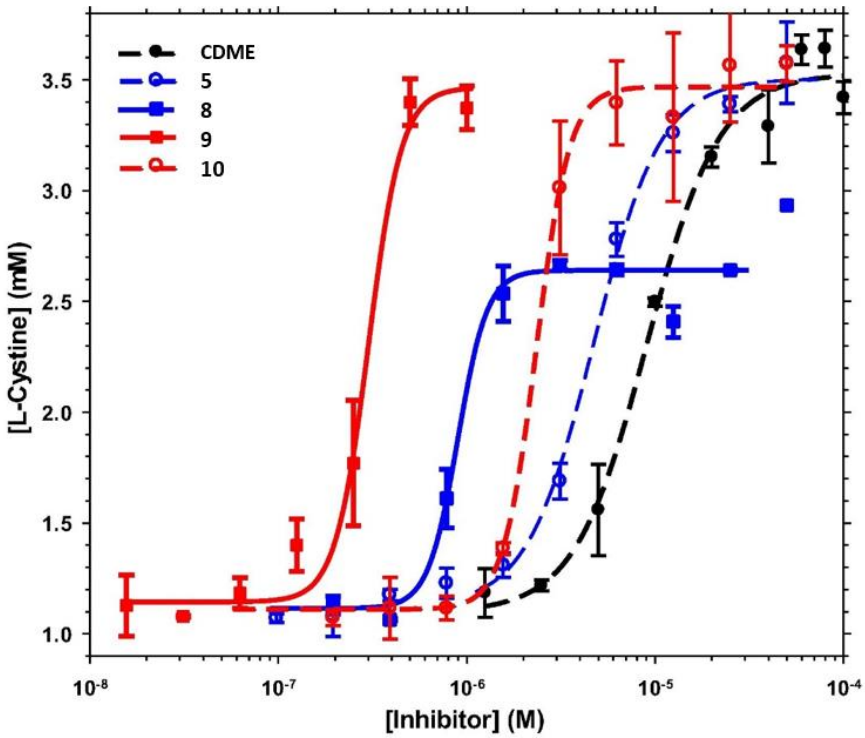


Figure 8. Effect of L-CDME, **5**, **8**, **9** and **10** on the aqueous concentration of L-cystine.[43]

Methylated amine analogues were also synthesized using the procedure published recently.[43]

As illustrated in Figure 7 and Table 2 all the methylated diamide analogues lack the inhibition ability apparently due to the hindrance of the hydrogen bonding ability to L-cystine as shown before in the L-cystine crystal binding studies where it shows that each amino group could form 2 to 3 hydrogen bonds and even a small group as the methyl could hinder the binding. This means that we should keep the amino groups free for new analogues.

The binding energies of the L-cystine diamides and the N,N'-dimethyl L-cystine diamides onto the {100} surface of L-cystine crystal, which had been identified as the fast growing faces, are listed in Tables 1 and 2. BIOVIA's Materials Studio software suite was used for the crystal morphology calculation and various adsorption/ docking experiments that were performed. The binding energy of L-cystine molecule onto the {100} surface of L-cystine crystal was used as a reference and was calculated to be -85.8 kcal/mol. All the L-cystine diamides and L-CDME showed greater binding energies than the reference L-cystine. In agreement with the result of our in vitro crystallization inhibition assay compound **9** has a surface binding energy 43% greater in magnitude than L-CDME with a large proportion (42%) of binding energy coming from the two N-methylpiperazine groups. Figure 9 (A-C) shows the structure configuration of L-cystine, L-CDME and compound **9** when absorbed onto the {100} surface of L-cystine crystal. The demethylated L-cystine diamides (**11**-**17**) on the other hand have noticeably lower binding energies than their unmethylated analogues which is also consistent with our experimental data. A comparison of structural configurations of **7** and **15**, respectively, is

illustrated in Figure 9 (D and E) when the respective molecules are adsorbed onto the {100} surface of L-cystine crystal. The unmethylated analogue **7** formed a total of three hydrogen bonds (indicated by blue dashed lines) between compound **7** and three different L-cystine molecules, while in the methylated analogue **15** the ammonium moieties were shielded by the methyl groups and could only form one hydrogen bond with the carboxylate moiety in the substrate, which explains the drop in the binding energy.

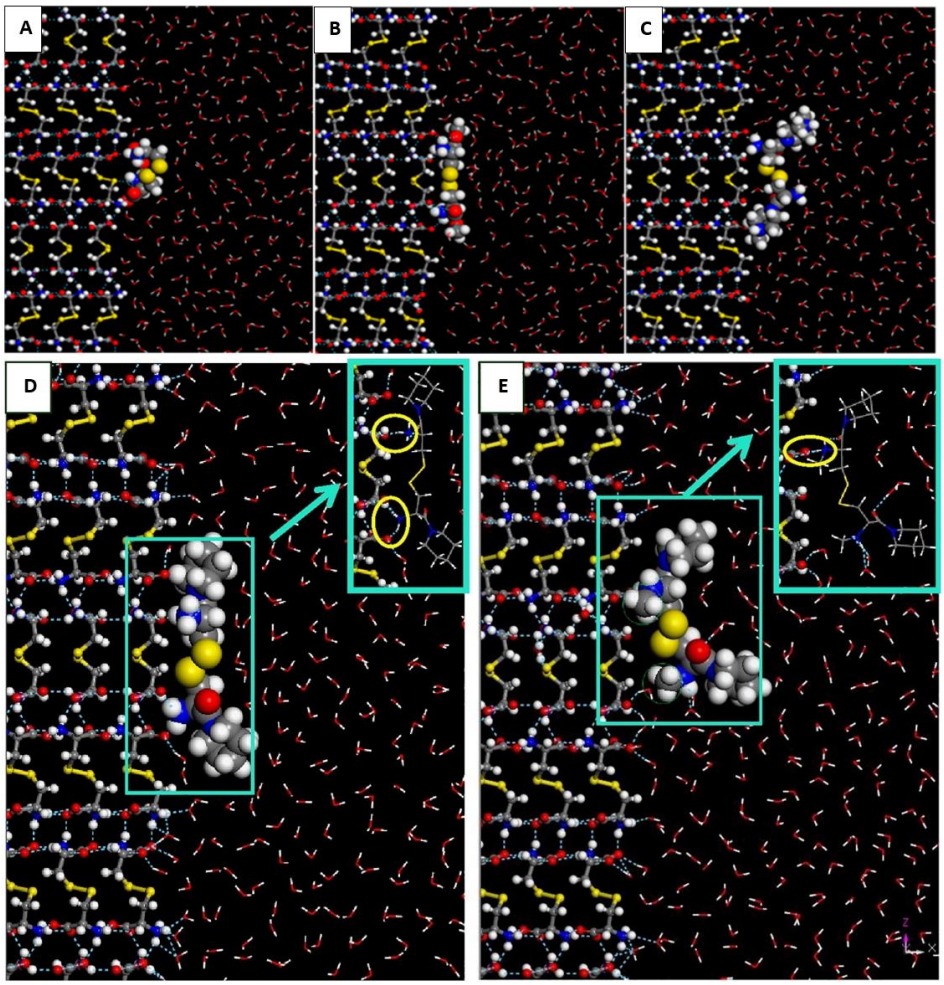
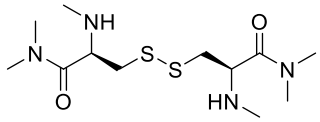
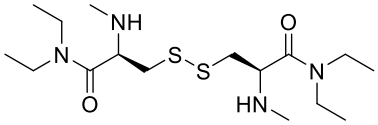
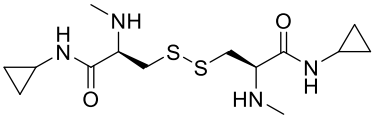
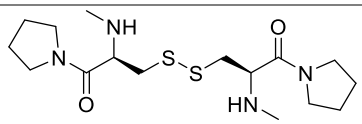
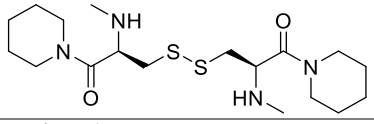
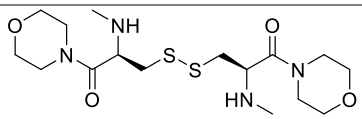
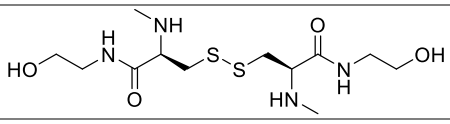


Figure 9. Structure Configurations of L-Cystine (A), L-CDME (B) and **9** (C), **7** (D) and **15** (E) Adsorbed Onto the {100} Surface of L-Cystine Crystal (in Ball-n-stick Representation).[43, 44]

Table 2. Aqueous Solubility of L-Cystine in Presence of N,N'-Dimethyl L-Cystine Diamides and Binding Energies to L-Cystine Crystal Surface.

	Structure	**Aqueous Solubility of L-Cystine**		**$E_{Binding}$ (kcal/mol)**
		2 uM	**10 uM**	
11 (LH710)		1.11	1.24	-264
12 (LH711)		1.08	1.20	-235
13 (LH712)		1.16	1.31	-260
14 (LH713)		1.10	1.21	-247
15 (LH714)		1.15	1.12	-234
16 (LH715)		1.14	1.27	-267
17 (LH716)		1.13	1.30	-273

1.5 Summary

Cystinuria is an inherited disease characterized by defective renal and intestinal reabsorption of L-cystine and dibasic amino acids (lysine, ornithine and arginine). The defect in the reabsorption leads to L-cystine crystallization and formation of renal L-cystine stones because of its limited solubility in urine while the aforementioned dibasic amino acids get excreted from the body with no precipitation. Cystinuria is a rare disease with a worldwide prevalence of 1 in 7000 neonates, but require attention since in

comparison to other stone disease L-cystine stones are larger, recur more frequently, and are more likely to cause chronic kidney disease. Even more, the pharmacotherapeutic options for treatment have not improved in decades despite progress made in the molecular genetics of the disease.

Cystinuria is the only disease amongst the heterogeneous group of kidney stone diseases that is completely caused by genetic mutations in two genes *SLC3A1* and *SLC7A9* genes located on chromosomes 2p and 19q, respectively. Based on the affected gene, cystinuria is classified into two types: type I, caused by mutations in *SLC3A1* gene, and non-type I, mainly by *SLC7A9* gene with predomination of type I cystinuria with >60% of the cases of the disease. *SLC3A1* encodes the heavy subunit rBAT while *SLC7A9* encodes the light subunit $b^{0,+}$AT of the renal amino acid transporter which is defected in cystinuria and the two subunits are linked by a disulfide bridge.

L-cystine crystallizes out in two polymorphic forms: the tetragonal phase (P41) and the hexagonal phase (P6122) where both crystallize with the molecule in its zwitterionic form. The presence of four terminal groups, two amino and two carboxylic groups, with the ability to form hydrogen-bond networks with principal intermolecular interactions take the form of NH⋯O hydrogen bonds, formed between the ammonium and carboxylate moieties and an unusually short S⋯S contacts gives L-cystine uniquely stable crystals with low solubility.

The treatment of cystinuria focuses mainly on lowering the concentration of L-cystine in urine or increasing L-cystine solubility in urine. The medical management of the disease starts with hydration by ingesting 4-4.5 L of water per day and urinary alkalinization to achieve a level of urine pH above 7-7.5, besides a diet low in methionine and sodium. If

hydration and alkalinization failed, then L-cystine binding thiols could be used which react chemically with L-cystine to form a thiol-cysteine mixed disulfide which has elevated solubility compared to L-cystine. These thiol drugs include D-penicillamine, tiopronin, and captopril. The problem with these drugs is their limited therapeutic effect besides numerous side effects, within the usual range of therapeutic dosage, including foul odor, nausea, fever, fatigue, skin rash, premature skin aging, proteinuria and hypersensitivity. Recently, a new alternative approach to the prevention of L-cystine kidney stones through molecular mimicry was suggested and it shows promising results. L-CDME obtains specific binding at the L-cystine crystal surfaces that was revealed by in situ real-time atomic force microscopy (AFM) and parallel bulk crystallization studies. The molecule was able to reduce the mass yield of crystallization and maintain a metastable supersaturated L-cystine concentration which is sufficient for preventing stone formation.

In cases where pharmaceutical treatment has failed noninvasive stone fragmentation technology like SWL could be used, but it has a high failure rate for cystinuric patients due to physio-chemical properties of L-cystine stones. If the SWL failed, then PCNL could be considered although it has a high rates of retreatment and recurrence after treatment. The final approach upon the failure of all medical interventions would be the open surgical stone removal which should be avoided as possible since the expected rate of the 5-year stone recurrence after surgical intervention is 73%.

The expected instability of L-CDME encouraged our group to convert the esters in the compound to the better in vivo stable amide. All diamide analogues showed better or similar profile for increasing the aqueous solubility of L-cystine and thus inhibiting L-

cystine crystallization compared to L-CDME. On the other hand, all the methylated diamide analogues lack the inhibition ability due to apparently the hindrance of the hydrogen bonding ability to L-cystine which means that the amino groups should be kept free for new analogues.

CHAPTER TWO

IMPROVING L-CDME STABILITY AND ACTIVITY

2.1 Research Objectives

With the promising results obtained with the diamides analogues of L-cystine we wanted to explore the structure activity relationship of the most active analogues. The first series showed the advantage of having alkyl substitution on the amine that forms the amide bond. Indeed, better inhibition ability was obtained with bulkier alkyl groups which is probably due to the better blockage of new L-cystine attachments. The aforementioned analogues also showed improved inhibition ability with hetero atoms added to the alkyl substitution especially the basic amino group as seen with compound **9**. On the other hand, no substitution are tolerable on the amino groups even as small as a methyl group as seen with analogues **11-17**.

Even though the in vivo stability issue present in L-CDME was resolved, there is still a concern of the in vivo stability of these compounds represented in the disulfide bond. Disulfide bonds are reactive in vivo where they can undergo bimolecular nucleophilic substitution reaction with free thiol resulting in thiol-disulfide exchange.[45] Accordingly the diamide analogues could react with cysteine in vivo to give the corresponding cysteine mixed disulfide. We wanted to explore the effect of replacing the disulfide bond with more stable bonds like thioether or amide.

In addition, we wanted to compare the in vitro stability of the cystine diamides in comparison to the diester (L-CDME) to prove the proposed enhanced stability of the

diamide analogues. The chemical stability of each analogue could be studied in buffer to compare their half-life. The disappearance of the compounds could be followed to calculate the half-life.

The site of action for these analogues is the urine and hence it is important to prove their bioavailability especially with their highly polar nature. Oral bioavailability in wild-type versus knockout mice could give us a good idea about the bioavailability of our analogues. LC-MS/MS can be used to quantitate the concentration of the L-cystine diamide in biological sample.

The high polarity of the L-cystine diamides could lead to low bioavailability in vivo which can be compensated through prodrugs masking the free amino groups. Prodrugs then would be tested for their activation in serum and different liver microsomes. The disappearance of the prodrug could be followed to calculate the half-life.

2.2 Effect on the Metastable Supersaturation Range of L-Cystine

The effect of the different designed analogues on the aqueous concentration of L-cystine was used to determine their inhibition ability. This would be done in a supersaturated solution of L-cystine in Millipore water prepared according to the literature method.[30] The first testing start by analyzing the concentration of L-cystine in solution after three days of incubation with two different concentrations (2 and 10 µM) and only if it shows inhibition a dose response curve would be performed to obtain the EC_{2X} or EC_{50}. The EC_{2X} is calculated as the concentration required to double L-cystine concentration in solution without observable crystallization which was used earlier instead of EC_{50} in the project since there was unexplainable inconsistent maximum of plateau reached with

different inhibitors which was thought to be related to the ability of each inhibitor. Figure 8 shows the inconsistency of the plateau reached with high concentrations of compounds L-CDME, **5**, **8**, **9** and **10** which ranged between about 2.5-3.5 mM while the super saturated solution prepared should be of about 3 mM (2.92 mM). Since the assay for these analogues was done once only it was hard to reach a conclusion about the reason for the inconsistency. It was found latter on after optimizing the preparation of the super saturated solution that the reason for the low plateau was actually due to the crystallization of the super saturated solution before it was used in the assay.

As expected preparing a super saturated solution is challenging especially when the solution need to be stable for about an hour for sample dilutions. First we thought that after forming the super saturated solution by reflux the solution should be kept stable with no stirring or any disturbance through the slow cooling process, but the solution would crystallize to the aqueous solubility of L-cystine in water which is about 0.7 mM at 25 °C according to the literature.[46] This precipitation is due to the fact that without stirring the solution would lose its uniformity and that would initiate crystallization. Then we proceeded with stirring at low speed during the cooling period and that gave us the super saturated solution which was stable for about 20-30 minutes. The problem then appeared to be with the reproducibility of the EC_{50} of the inhibitors. Even though the maximum plateau was consistent now because the solution was prepared and used at super saturation level still the EC_{50} varied highly. After testing all different parameters in the assay procedure such as refluxing temperature and time, vial used, Millipore water system, cooling time, speed of stirring during cooling, it was found that the reason of the inconstancy was due to the preparation of the super saturated solution again. Finally, we

found out that using medium stirring for the cooling process lead to a stable super saturated solution for more than an hour with a reproducible EC_{50}. Also it is worth mentioning that for heating we used oil bath and when the oil level set exactly at the level of the solution inside the flask a ring of precipitate forms during the cooling process due to evaporation at temperatures lower than reflux temperature with no condensation so the oil level should be kept slightly under the solution level.

The effect on the metastable supersaturation of L-cystine would be measured after incubating different concentrations of test compounds in the L-cystine super saturated solution, prepared as described before, for 72 h at 25 °C. The measurement of the L-cystine concentration was then obtained using either a fluorescence-based assay or a colorimetric assay as shown in Figure 10. In the fluorescence-based assay, the L-cystine disulfide bond was reduced to L-cysteine and the sulfhydryl group was then alkylated with iodoacetic acid to form S-carboxymethyl L-cysteine. This was followed by derivatization with O-phthaldialdehyde (OPA) and N-Boc-cysteine (NBC) which afforded a blue fluorescence derivative D as shown in Figure 10. On the other hand, in the colorimetric assay the L-cystine disulfide bond was broken using potassium cyanide then sodium nitroprusside was added to give a colored complex (G) as shown in Figure 10.[47] The two assays gave reproducible results. Even though the colorimetric assay is a faster assay with less steps involved it has two disadvantages: (1) the short lived color complex (2 m) which require an automated dispenser for the fast addition of sodium nitroprusside solution, (2) the assay involved the use of very toxic materials especially the sodium nitroprusside.[47]

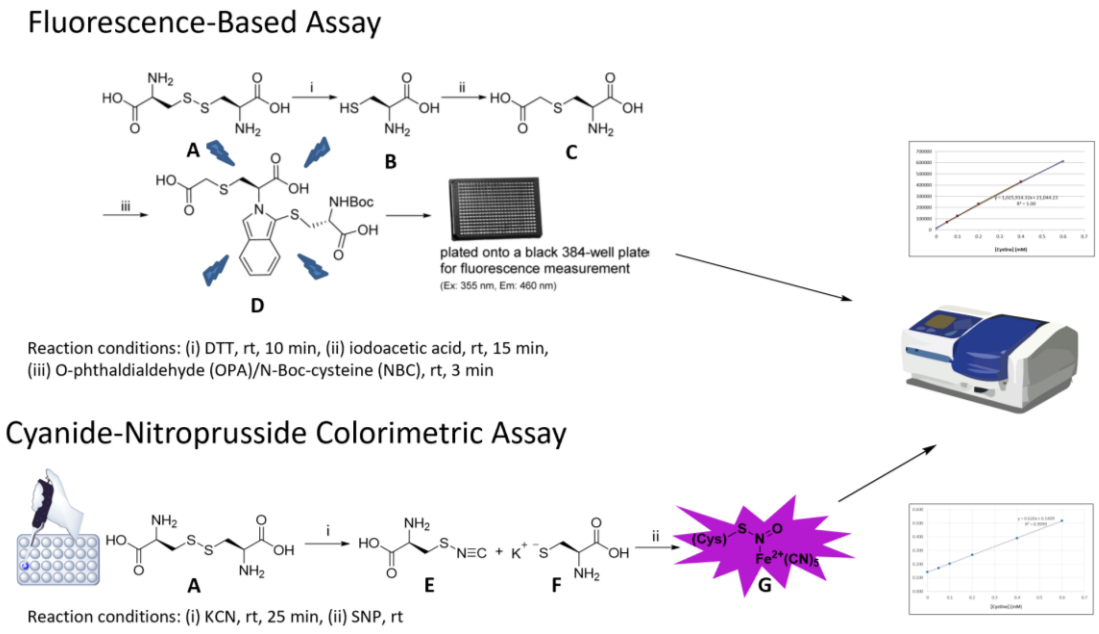


Figure 10. Fluorescence-based and Colorimetric Assay for L-Cystine Concentration Measurement.

The dose response curves for L-CDME, **8** and **9** were recalculated using the optimized procedure and showed lower EC_{50} as shown in Figure 11B and Table 3. The assay was repeated four times at least for each compound and the error bars represent the standard deviation calculated. Figure 11A illustrate the dose response curves for L-CDME, **8** and **9** before optimizing the assay.

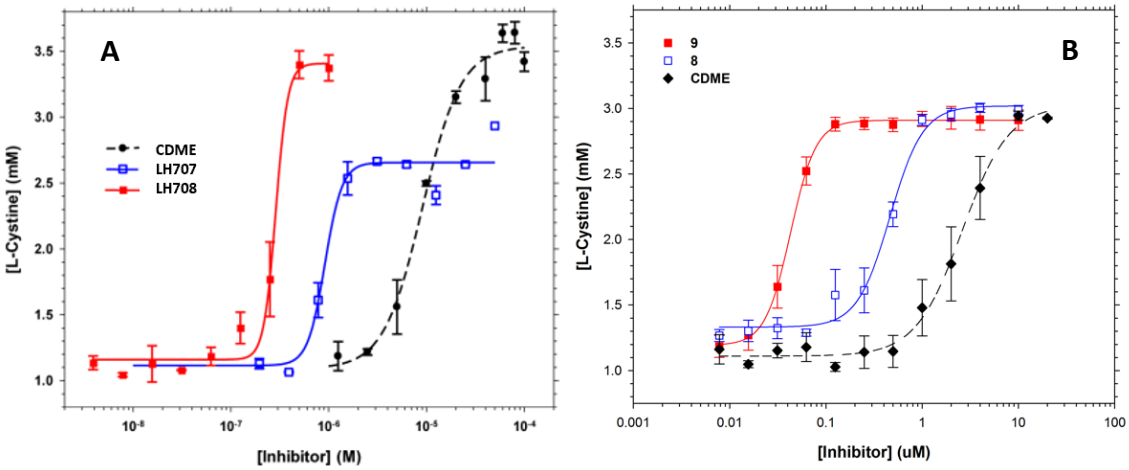
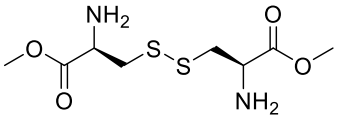
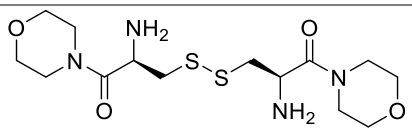
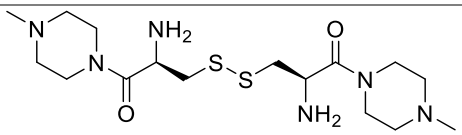


Figure 11. Dose Response Curves for L-CDME, **8** and **9**; Before (A) and After Optimization (B).

Table 3. EC_{50} Values of L-CDME, **8** and **9** After Assay Optimization.

	Structure	EC_{50} (nM)	N
1 (L-CDME)		2621 ± 234	4
8 (LH707)		464.6 ± 39.1	4
9 (LH708)		42.8 ± 0.7	9

N is the number of repeated assay measurements.

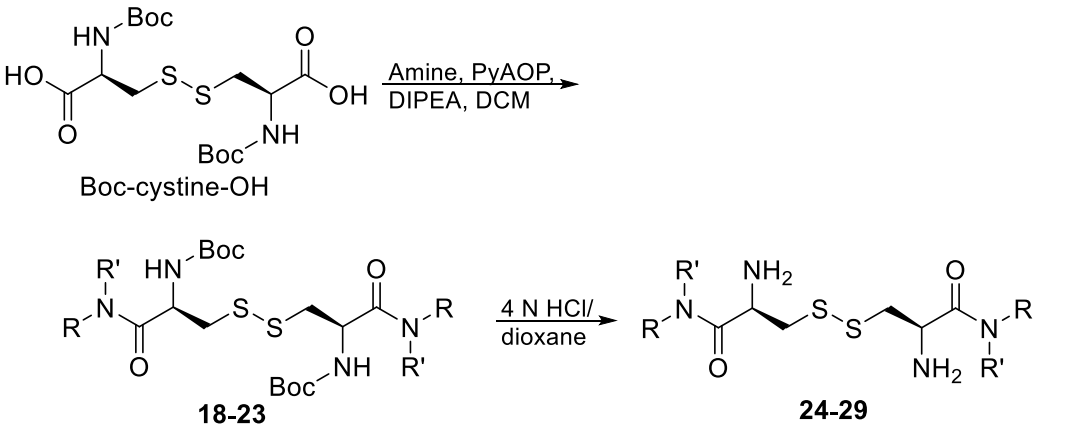
2.3 Synthesis and Evaluation of Different Piperazide L-Cystine Analogues of Compound 9

As shown before the N-methyl piperazide analogue **9** is the most potent inhibitor of L-cystine crystallization in the previous series so we sought to carry on to study the structure activity relationship of this analogue. The N-methyl of the piperazide moiety

was removed or replaced by different alkyl groups as shown in Scheme 1. The piperazide **29** is actually a possible metabolite of N-methyl piperazide analogue in vivo and it showed good inhibition activity in the in vitro L-cystine crystallization inhibition assay as shown in Table 4. From the other alkyl analogues, *tert*-butyl, cyclo-propyl, cyclo-pentyl, and phenyl, the *tert*-butyl and cyclo-pentyl analogues were comparable to the N-methyl piperazide **9** with an EC_{50} of 32 and 39.5 nM, respectively as shown in Table 4 and Figure 12. On the other hand, the phenyl analogue **27** showed a dramatic decrease in the activity due to either the rigid planar structure or more probably the electron withdrawing ability of the ring which decreases the basicity of the amine. Also 3,3-dimethyl-piperazide analogue **28** was tested for L-cystine crystallization inhibition and it showed more than two-fold decrease in the inhibition ability compared to the methyl piperazide **9** and the piperazide analogue **29**.

These analogues were synthesized through in situ peptide coupling starting from the commercially available Boc-L-cystine-OH using PyAOP in presence of the amine and DIPEA as base.[48] The diamide analogues were then treated with 4 N HCl/ dioxane to give the corresponding HCl salts in good yields as shown in scheme 1.[49]

Scheme 1. Synthetic Route for Compounds **24-29**.



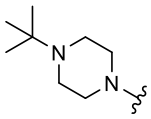
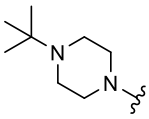
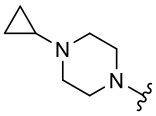
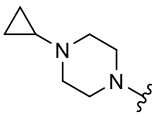
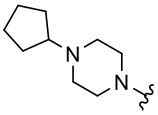
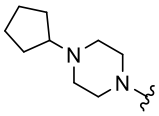
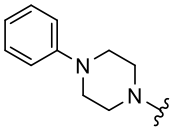
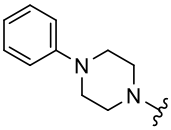
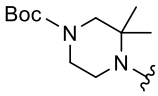
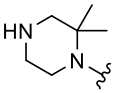
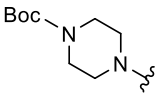
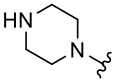
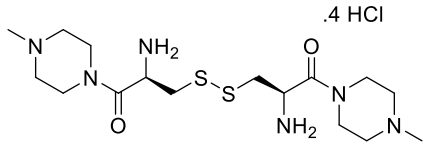
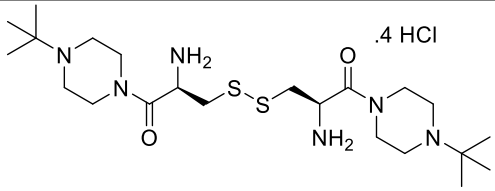
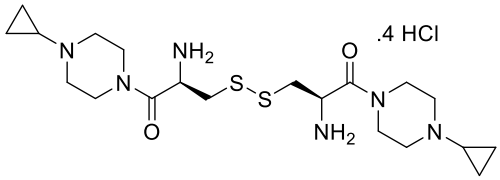
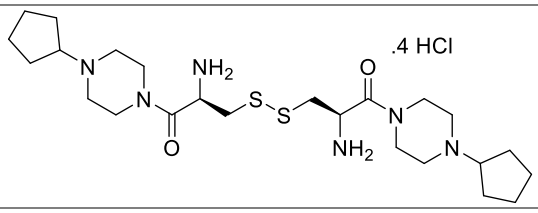
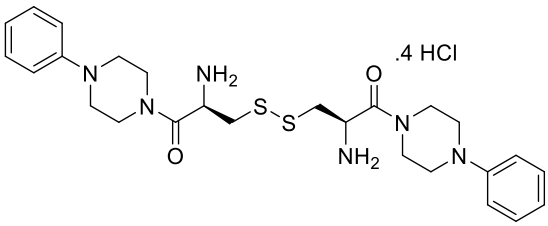
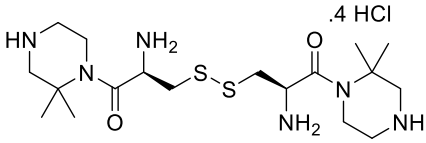
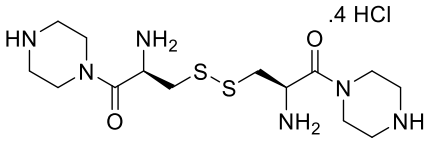
Compound		Yield (%)	Compound		Yield (%)
18		73	**24**		84
19		67	**25**		80
20		75	**26**		76
21		73	**27**		88
22		60	**28**		80
23		60	**29**		85

Table 4. Aqueous Solubility of L-Cystine in Presence of Compounds **24-29**.

	Structure	EC_{50} (nM)	Ratio
9 (LH708)		42.8 ± 0.7	1.00
24 (LH1729)		32.0 ± 2.5	1.34
25 (LH1730)		68.8 ± 4.1	0.62
26 (LH1731)		39.5 ± 1.3	1.08
27 (LH1732)		500.3 ± 26.5	0.09
28 (LH1733)		108.0 ± 3.1	0.40
29 (LH1726)		52.6 ± 0.8	0.81

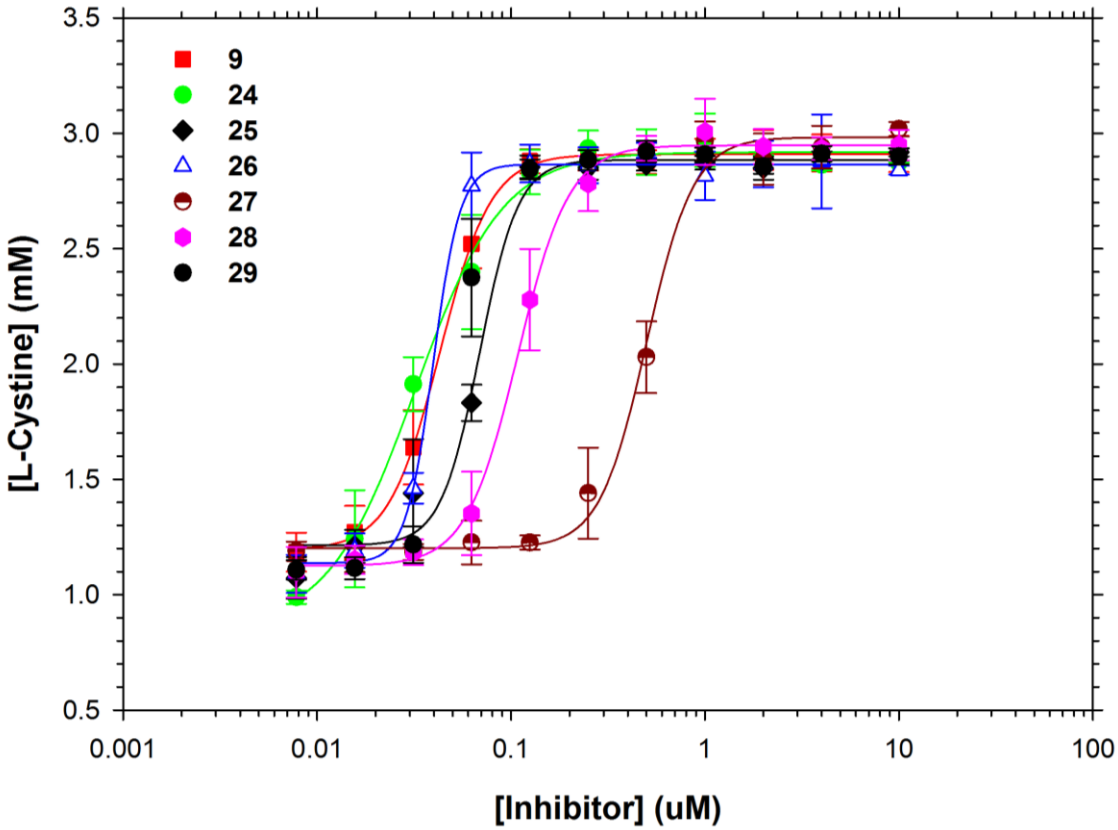
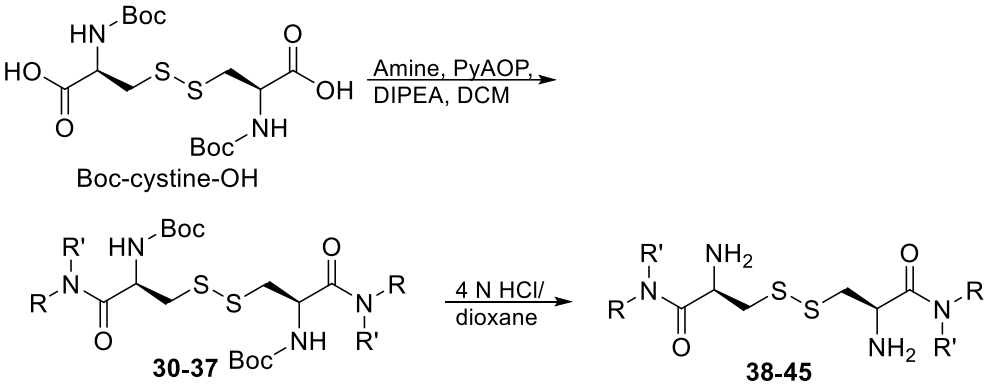
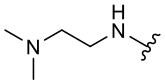
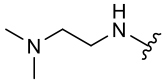
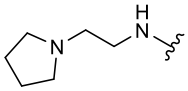
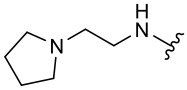
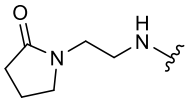
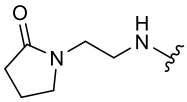
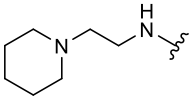
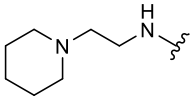
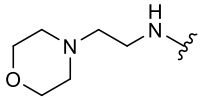
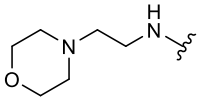
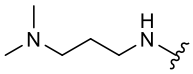
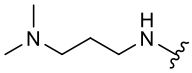
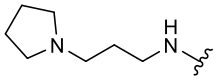
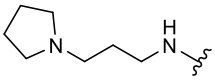
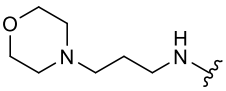
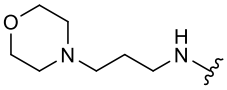


Figure 12. Dose Response Curves for Compounds **24-29** in Comparison to **9**.

2.4 Synthesis and Evaluation of Linear L-Cystine Diamides Analogues of Compound 9

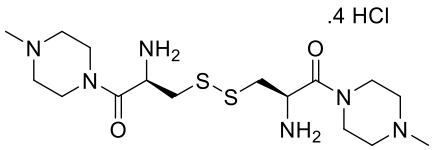
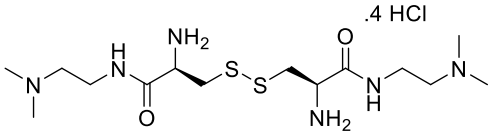
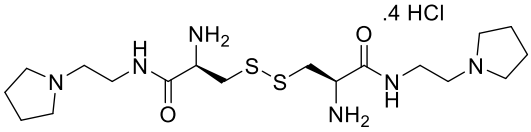
Scheme 2. Synthetic Route for Compounds **38-45**.

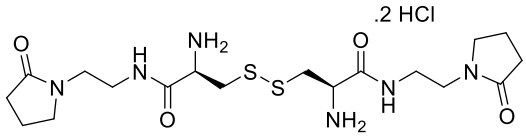
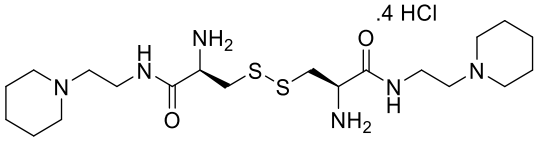
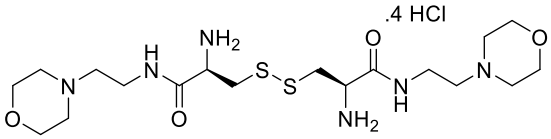
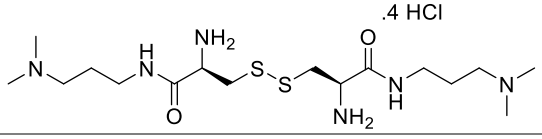
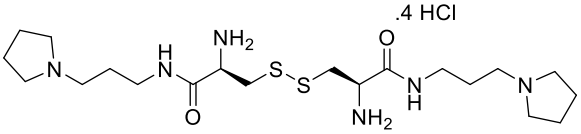
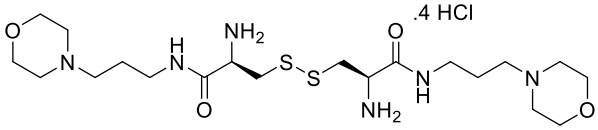


Compound		Yield (%)	Compound		Yield (%)
30		95	**38**		73
31		81	**39**		86
32		81	**40**		99
33		90	**41**		86
34		80	**42**		100
35		58	**43**		68
36		81	**44**		45
37		80	**45**		85

In addition, a new series of linear analogues (Scheme 2) were designed since we thought that more flexible analogues could bind with ease to the L-cystine crystal leading to potent inhibitors. Using N,N-dimethylethylenediamine as a linear analogue for N-methylpiperazine where the difference is actually a methylene group with a much enhanced flexibility achieved through the ring opening. Also the N,N-Dimethyl-1,3-propanediamine was used but both were less active as an inhibitor compared to N-methyl piperazide analogue as shown in Table 5. On the other hand, replacing the dimethylamine in both ethylene analogues **38** with different cyclic analogues like the pyrrolidine, piperidine or morpholine led to better inhibition potency while the opposite holds for the cyclic analogues for the propane **43**. Since these compounds are very polar which could lead to low bioavailability the 2-pyrrolidone analogue **40** was synthesized, but it showed a drastic decrease in the inhibition ability with almost 40 fold decrease in the crystallization inhibition in the L-cystine crystallization assay compared to the pyrrolidine analogue **39**. This again shows the importance of the basic amino group for the inhibitor. The dose response curves of these analogues are illustrated in Figure 13.

Table 5. Aqueous Solubility of L-Cystine in Presence of Compounds **38-45**.

	Structure	EC_{50} (nM)	Ratio
9 (LH708)		42.8 ± 0.7	1.00
38 (LH1734)		163.4 ± 10.8	0.26
39 (LH1735)		116.4 ± 6.1	0.37

40 (LH1736)		4150 ± 541	0.01
41 (LH1737)		142.7 ± 9.8	0.30
42 (LH1738)		79.7 ± 7.7	0.54
43 (LH1739)		114.3 ± 9.4	0.37
44 (LH1740)		163.3 ± 12.5	0.26
45 (LH1741)		160.2 ± 10.6	0.27

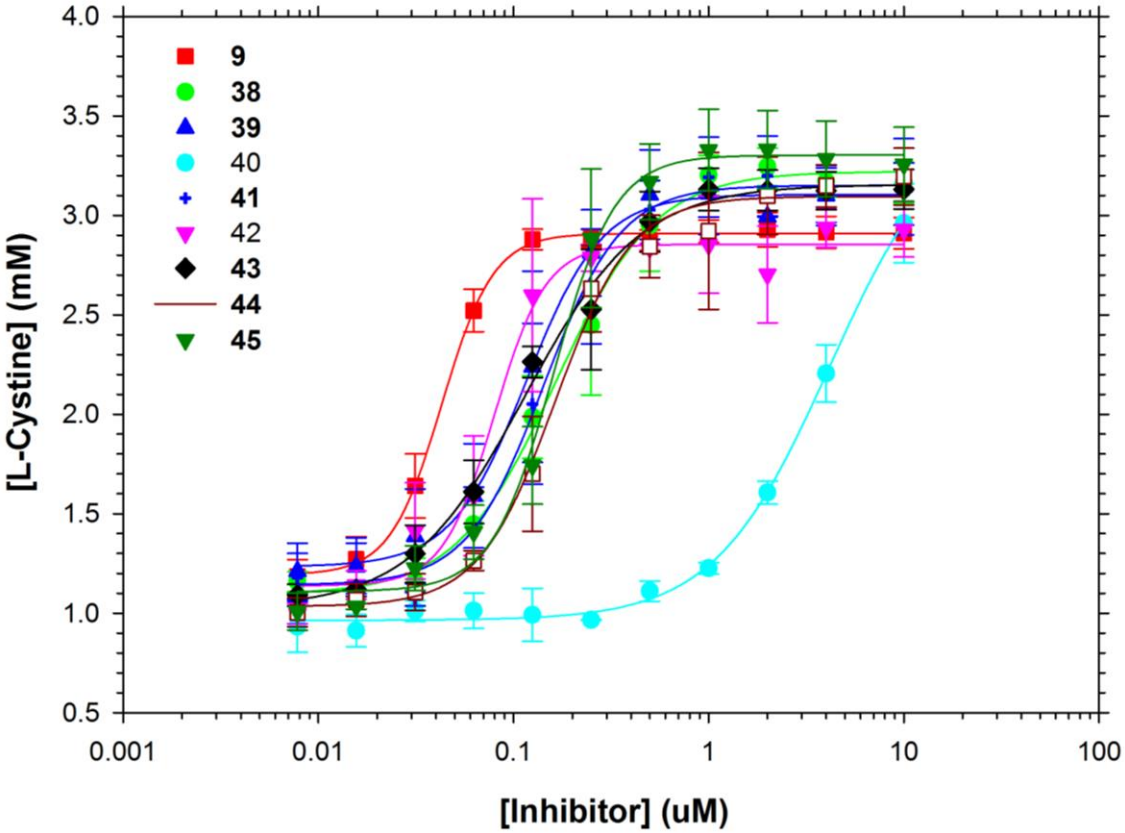


Figure 13. Dose Response Curves for Compounds **38-45** in Comparison to **9**.

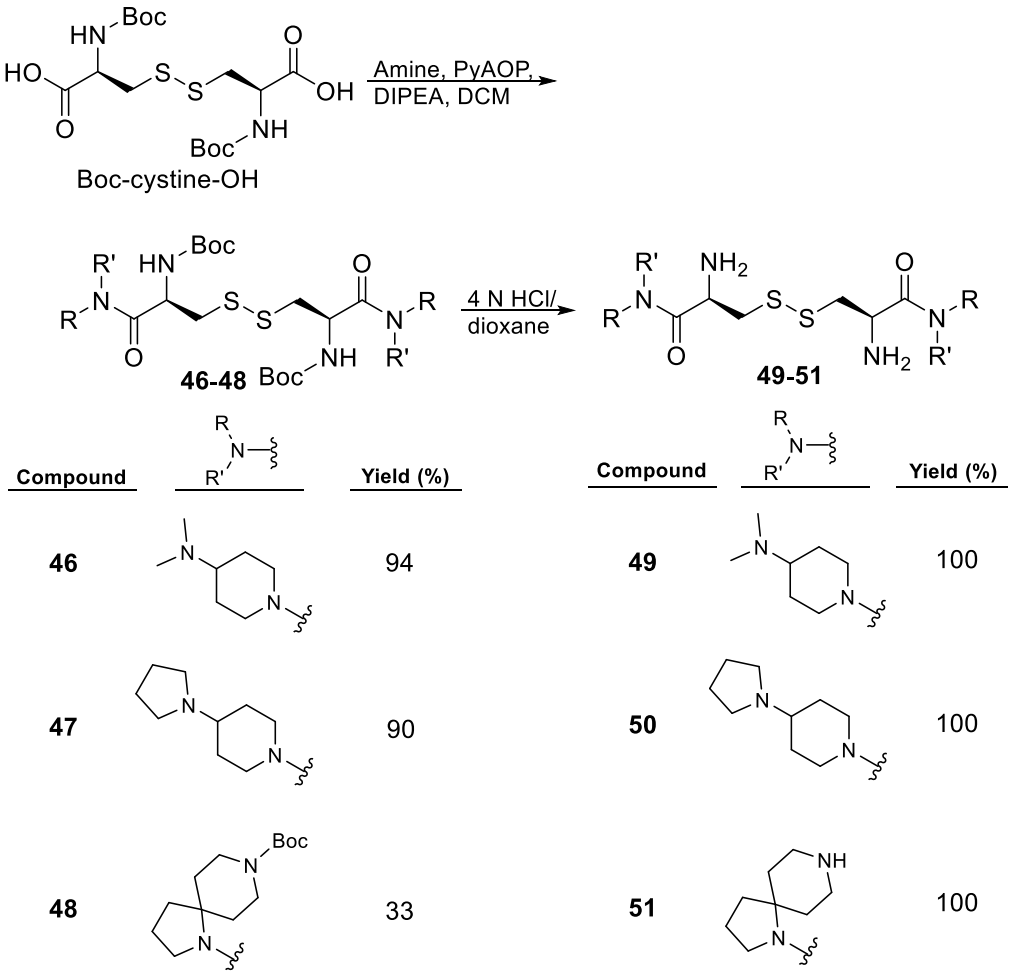
2.5 Synthesis and Evaluation of Piperidine and Spiro Heterocyclic L-Cystine Analogues of Compound 9

Another series of analogues were tested having the amino group out of the ring in comparison to N-methylpiperazine which would test the effect of changing the position of the basic center (amine) while maintaining the six membered ring. 4-(Dimethylamino)-piperidine and 4-(1-pyrrolidinyl)piperidine analogues **49** and **50**, respectively were made. Also the 1,8-diazaspiro[4.5]decane analogues were synthesized with the amide bond formed with the amino group in the five or the six membered rings **51** and **56**, respectively. Both piperidyl analogues **49** and **50** maintained the inhibitory activity as seen with **9** which means that the change in the amine position did not affect the binding

of the inhibitor to the L-cystine crystal. On the other hand, **56** showed almost two-fold enhanced crystallization inhibition compared to **9** while its analogue with the amide bond formed with the amine on the five membered ring **51** showed almost three fold decrease crystallization inhibition compared to **9**, as shown in Table 6. The reduction in the activity seen with **51** could be explained as the effect of the large six membered ring which is pointed to the important hydrogen bonding region between the inhibitor and the L-cystine crystal and thus make the binding harder. The dose response curves of analogues **49**-**51** and **56** are illustrated in Figure 14.

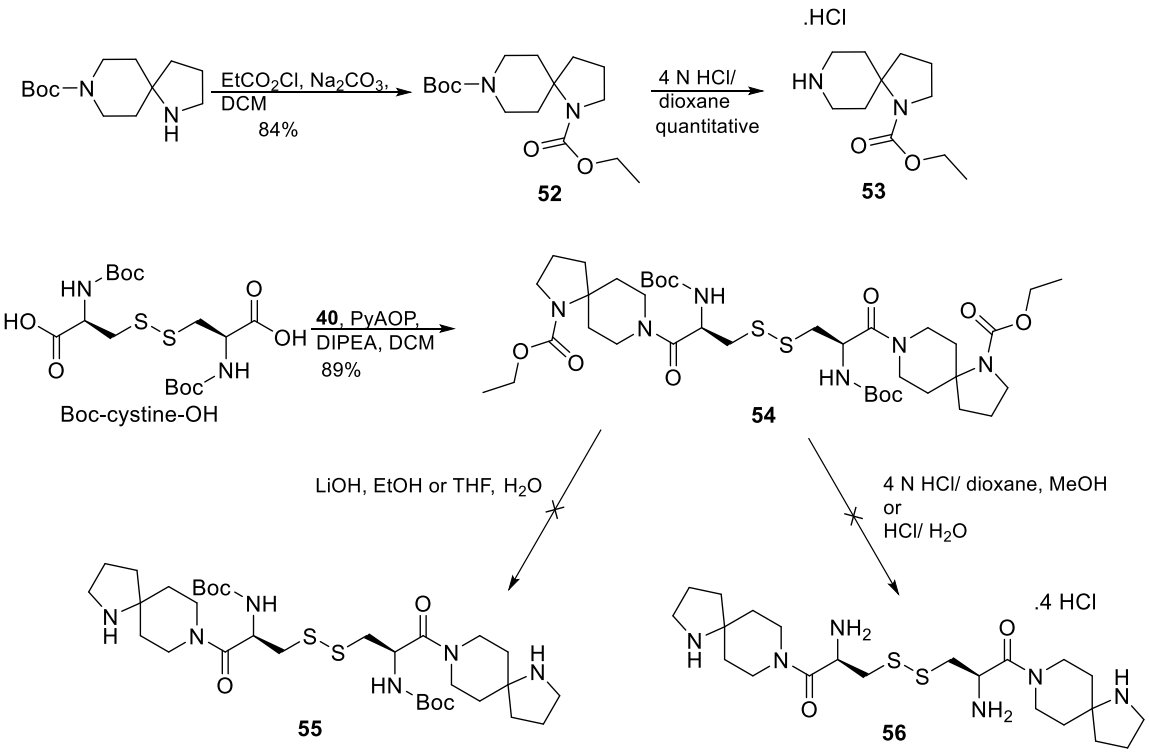
Analogues **49**-**51** were synthesized through in situ peptide coupling starting from the commercially available Boc-L-cystine-OH using PyAOP in presence of the amine and DIPEA as base as shown in scheme 3. The deprotection of the amino groups was achieved with treatment of the analogues with 4 N HCl/ dioxane to give the corresponding HCl salts in good yields as shown in scheme 3.

Scheme 3. Synthetic Route for Compounds **49-51**.



To synthesize compound **56** the starting amine (tert-butyl 1,8-diazaspiro[4.5]decane-8-carboxylate) was needed first to be protected with acid stable protection group before the coupling. Ethyl carbamate was chosen as it has easy synthesis and deprotection conditions. First the starting amine was treated with ethylchloroformate in DCM in the presence of sodium carbonate to give **52** with a good yield.[50] Then the Boc group was deprotected with 4 N HCl/ dioxane to give the corresponding HCl salt **53** in good yield as shown in scheme 4. **54** was synthesized through in situ peptide coupling starting from the commercially available Boc-L-cystine-OH using PyAOP in presence of the amine **53** and

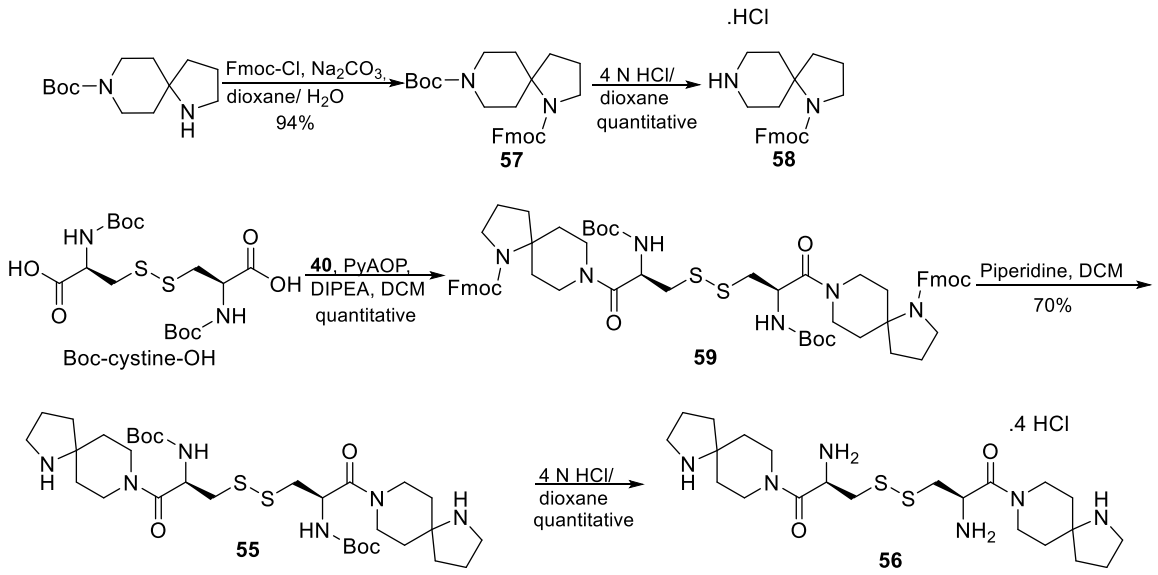
DIPEA as base with 89% yield. Different methods for the deprotection of ethylcarbamate were used in presence of 2 equivalent of base at 0 °C, but the starting material would decompose fast with no desired product achieved. Also total deprotection with HCl using different methods failed to deprotect the ethylcarbamate as shown in Scheme 4.



Scheme 4. Attempts at Obtaining Compound **56**.

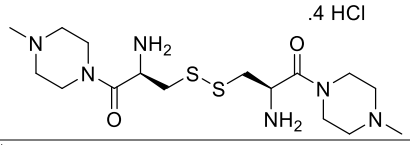
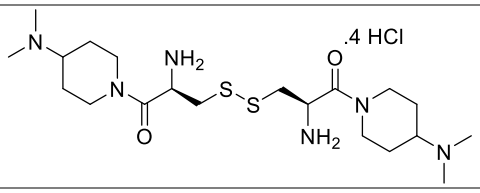
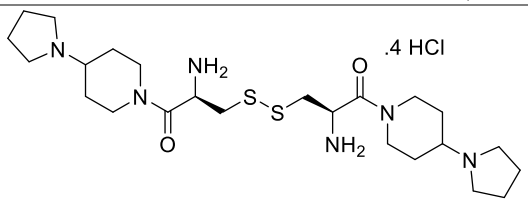
The starting amine (*tert*-butyl 1,8-diazaspiro[4.5]decane-8-carboxylate) was protected then using Fmoc-Cl which is acid stable, and would make the detection of the compounds easier, to give compound **57** in 94% yield. Then the Boc group was deprotected with 4 N HCl/ dioxane to give the corresponding HCl salt **58** in a quantitative yield. As shown in Scheme 5, **59** was synthesized through in situ peptide coupling starting from the commercially available Boc-L-cystine-OH using PyAOP in presence of the amine **58** and DIPEA as base in quantitative yield. Then the Fmoc groups were deprotected using

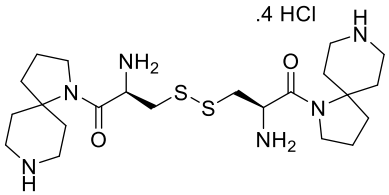
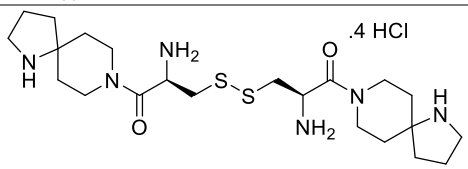
piperidine in DCM to give compound **55** in 70% yield.[51] Finally, deprotection of the Boc protected amino groups was achieved with treatment of the **55** with 4 N HCl/ dioxane to successfully give the corresponding HCl salt **56** in a quantitative yield as shown in Scheme 5.



Scheme 5. Synthetic Route for Compounds **56**.

Table 6. Aqueous Solubility of L-Cystine in Presence of Compounds **49**-**51** and **56**.

	Structure	EC_{50} (nM)	Ratio
9 (LH708)		42.8 ± 0.7	1.00
49 (LH1751)		51.2 ± 3.7	0.84
50 (LH1752)		36.5 ± 1.6	1.17

51 (LH1754)		120.2 ± 13.0	0.36
56 (LH1753)		25.1 ± 2.4	1.70

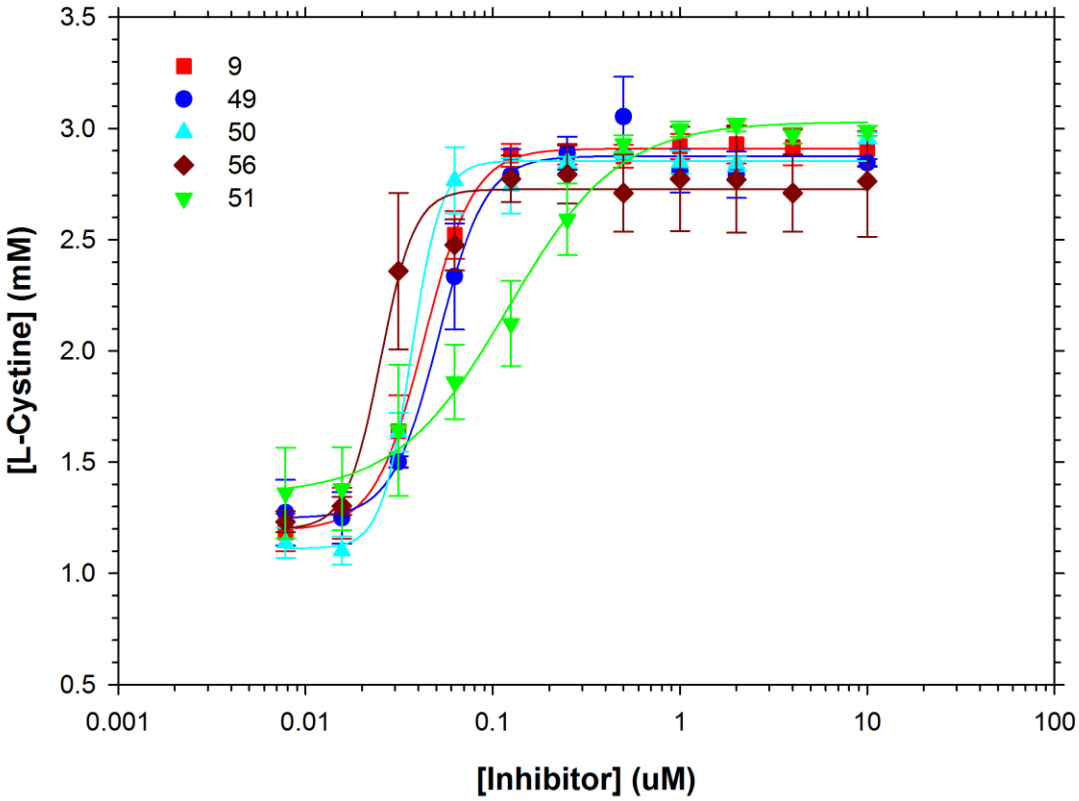
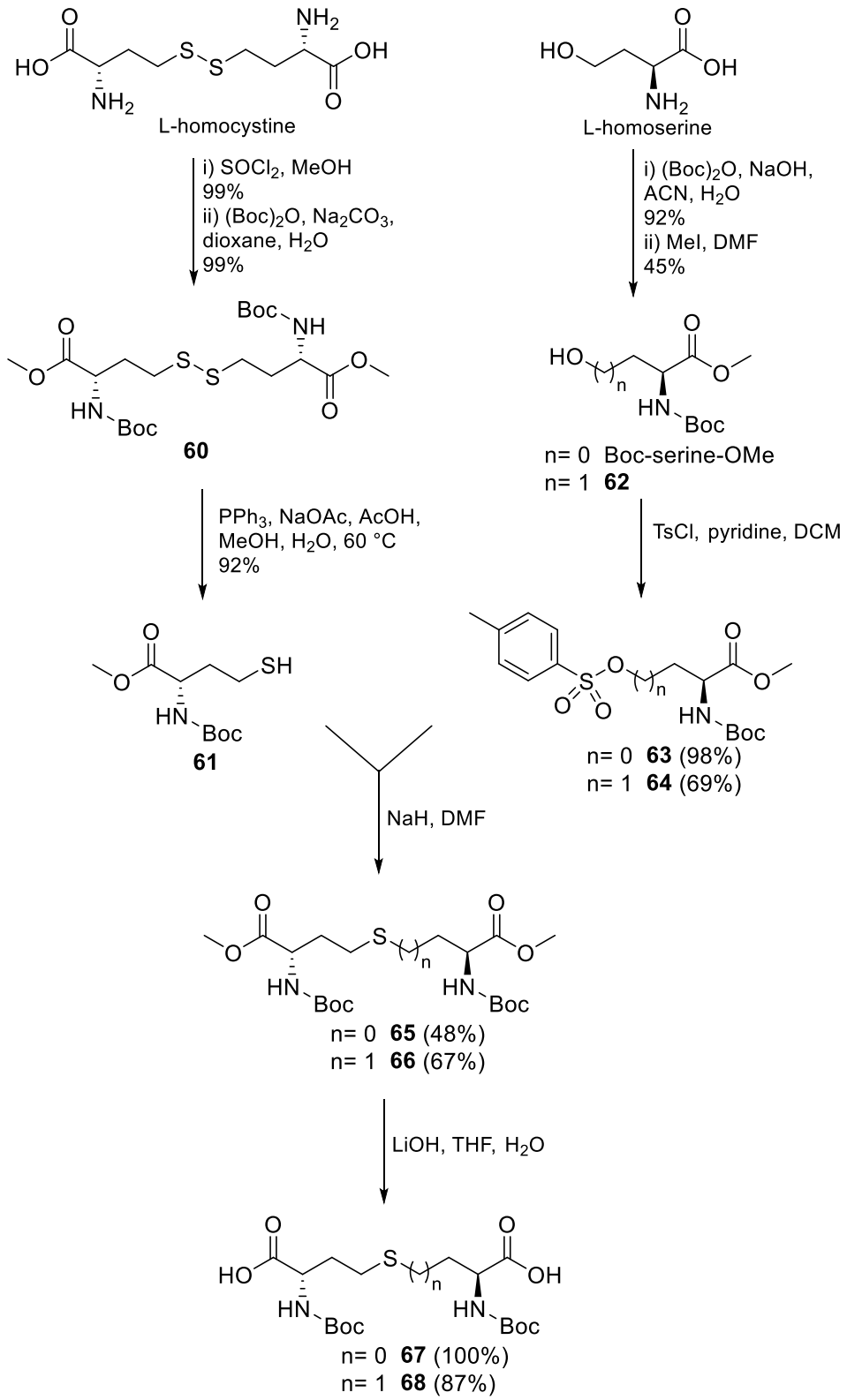


Figure 14. Dose Response Curves for Compounds **49-51**, **56** in Comparison to **9**.

2.6 Synthesis and Evaluation of Thioether and Amide Analogues of Compounds 9 and 38

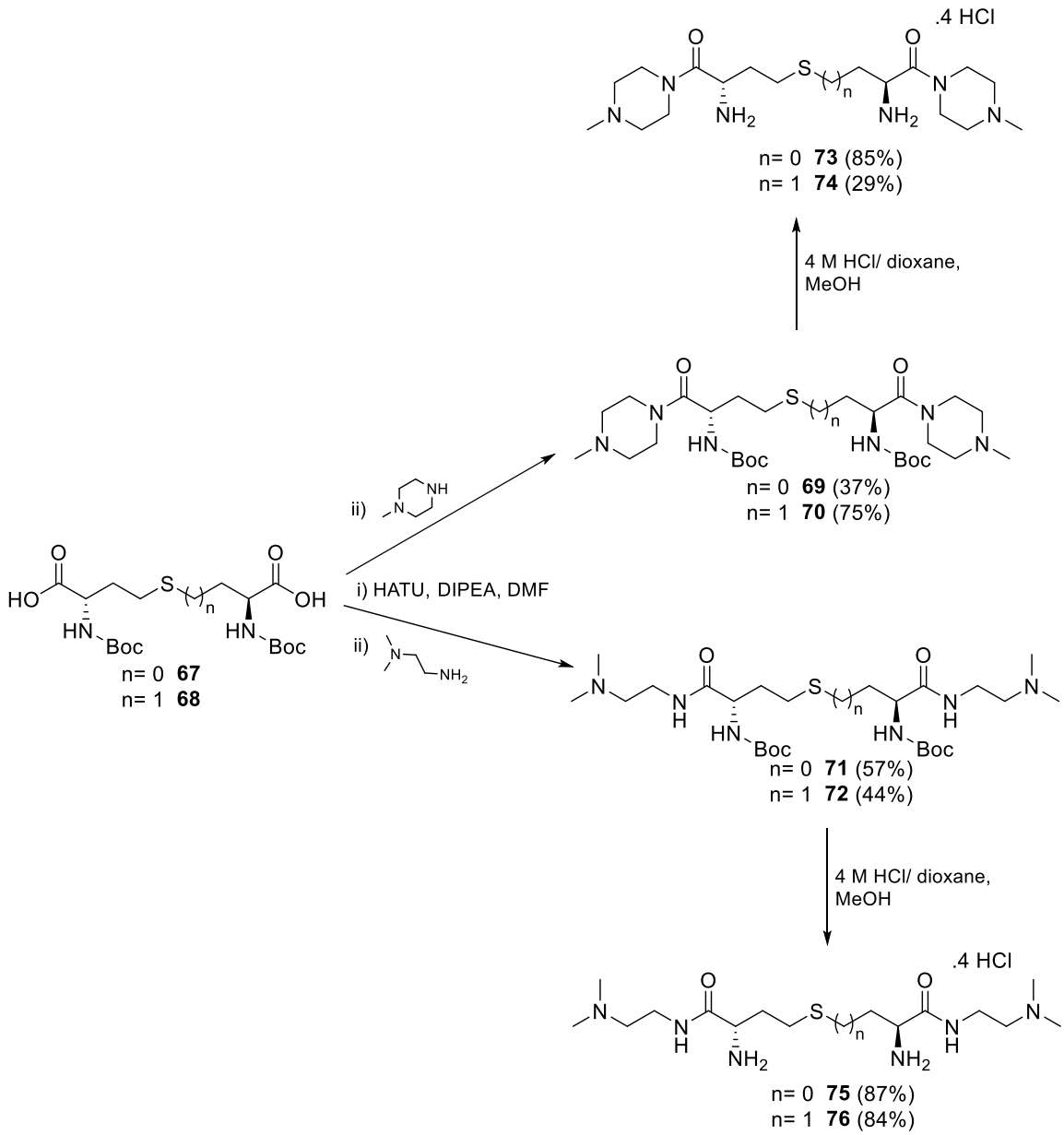
The known instability of the disulfide bond in vivo encouraged us to synthesize new analogues with more stable linkages replacing the central disulfide bridge like thioether

and amide.[52] One of the sulfur atoms was replaced with one or two methylene groups to accommodate the large atomic size of the sulfur as in compounds **73-76**. Also the disulfide was replaced with amide or acetamide which again could replace the large disulfide bridge like in compounds **86**, **88**, **90** and **92**.

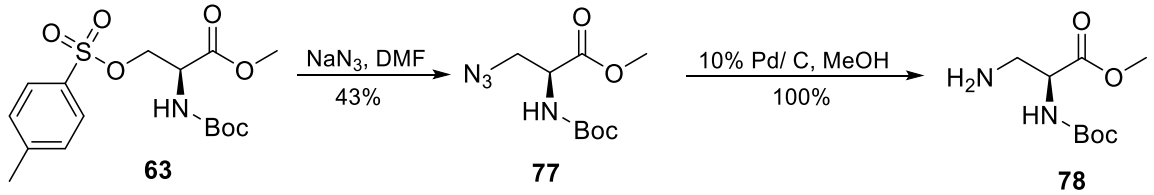


Scheme 6. Synthetic Route for Intermediates **67** and **68**.

The synthesis of the thioether analogues started for the first half with methyl ester protection of L-homocystine using thionyl chloride and methanol followed by amino Boc protection using di-tert-butyl dicarbonate in presence of sodium carbonate to give **60** in 99% yield.[53] This was followed by reduction of the disulfide to obtain thiol **61** using triphenyl phosphine.[54] The preparation of the other half of the thioether molecules started with protected serine or homoserine. L-Homoserine was protected using di-tert-butyl dicarbonate in presence of sodium hydroxide to give the sodium salt in 92% yield. Then the carboxylic acid was protected as methyl ester using methyl iodide in 45% yield.[55] The protected serine and homoserine hydroxyl groups were activated with tosyl chloride in presence of pyridine to give **63** and **64**, respectively in good yields.[56, 57] Reacting the two halves to prepare the thioether was tested with different bases and equivalents. In pyridine and DMF (1:1, v/v) the desired product was formed after stirring overnight at room temperature and at lower concentration the main product seen was the disulfide of the thiol **61**. Also other bases like triethylamine and diisopropylethylamine were tested in different equivalents, but the main product was the disulfide too. The reaction needs either a stronger base to push the reaction to completion fast in order to compete with the oxidation side product or a high temperature should be applied, but studies showed that racemization occurs at high temperature and prolonged exposure to base so we sought to use a strong base with no heating.[58-61] Thioether **65** and **66** were achieved by reacting thiol **61** with tosylate **63** or **64**, respectively in presence of sodium hydride. These molecules were deprotected using lithium hydroxide to give the dicarboxylic acids **67** and **68** as shown in Scheme 6.[62]

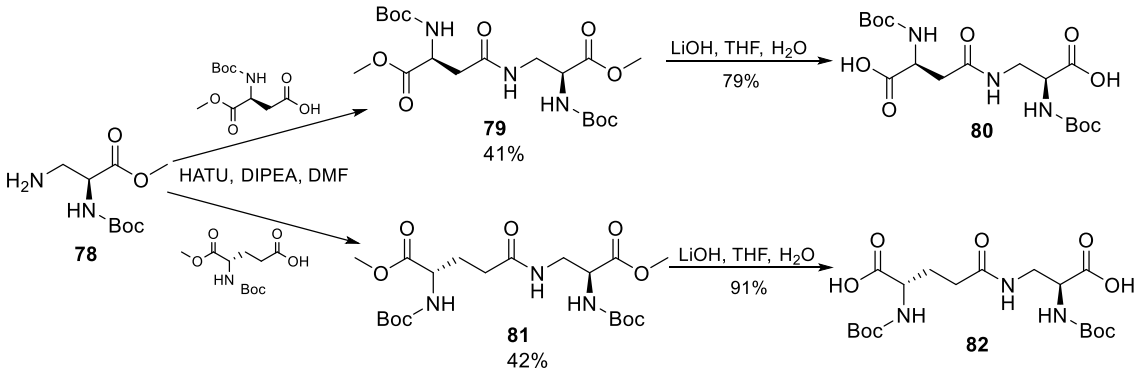


Scheme 7. Synthetic Route for Compounds **73-76**.



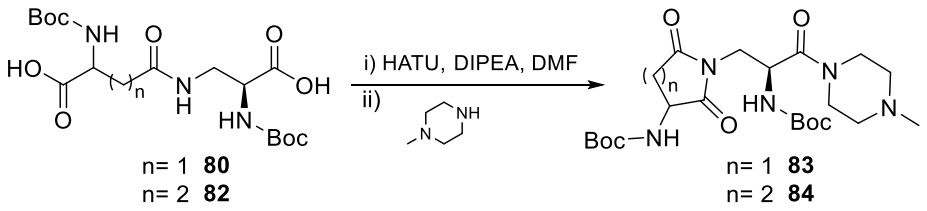
Scheme 8. Synthetic Route for Intermediate **78**.

The dicarboxylic acids **67** and **68** were activated using HATU in presence of DIPEA then N-methyl piperazine or dimethyl ethylenediamine was added to give the diamides analogues **69-70** or **71-72**, respectively.[63] The HCl salts of the diamide thioether analogues **73-74** and **75-76** were obtained using 4 M HCl in dioxane as shown in Scheme 7.



Scheme 9. Synthetic Route for Intermediates **80** and **82**.

The synthesis of the central amide analogues started with hydroxyl group activation using tosyl chloride in presence of pyridine to give **63** as shown in Scheme 6. Then the tosylate was treated with sodium azide to give **77** in 43% yield.[64] As illustrated in Scheme 8, the azide then was reduced to amino group using 10 wt% Palladium on Carbon to give the corresponding amine **78** in 100% yield.[65] Protected L-aspartic or glutamic acids were activated using HATU as a coupling reagent in presence of DIPEA as a base then **78** was added to get the corresponding amides **79** and **81**. This was followed by ester hydrolysis to give the dicarboxylic acids **80** and **82** in good yields as shown in Scheme 9.

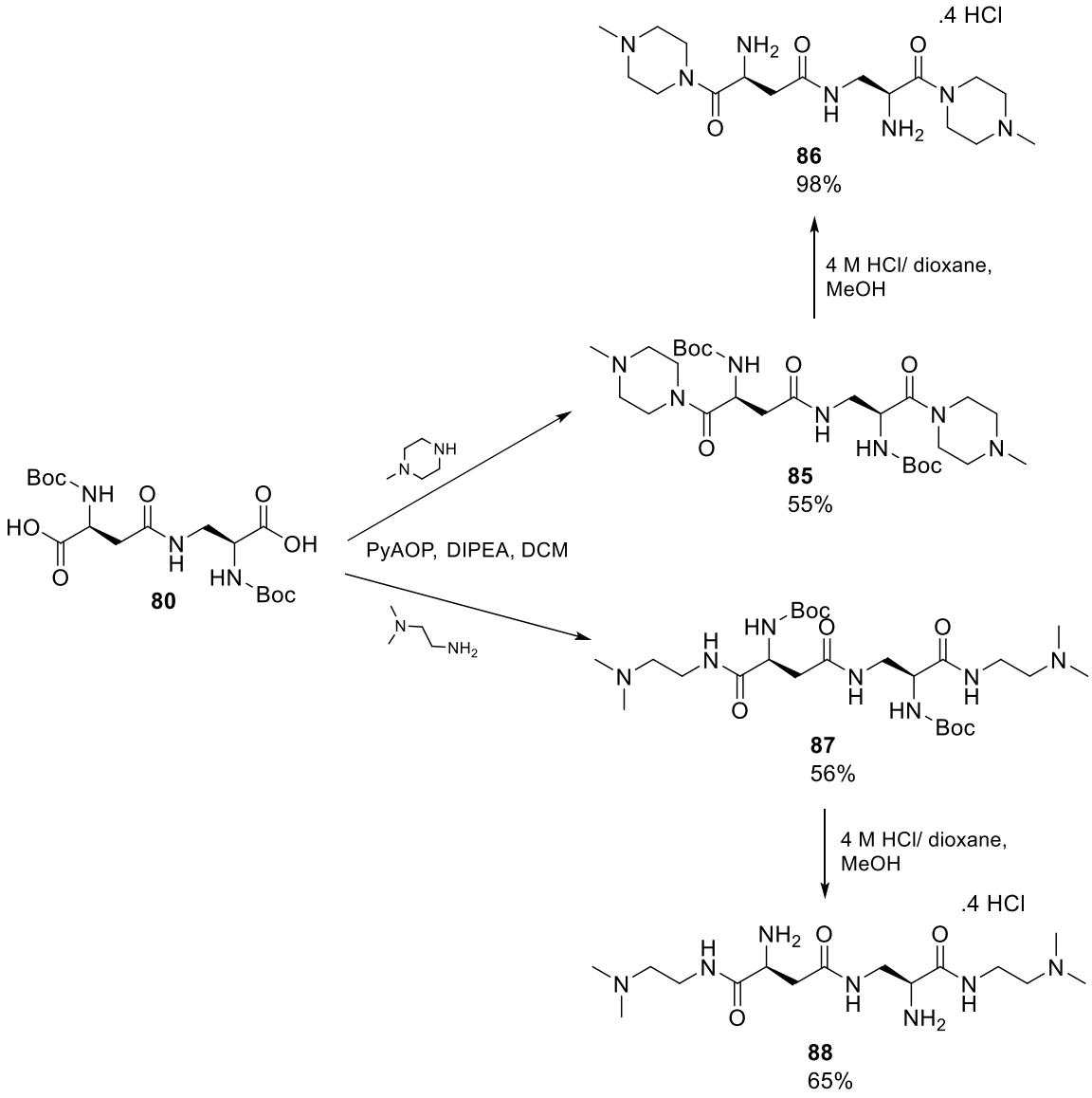


Scheme 10. Attempts at Obtaining Compound **85** and **87**.

The first attempts toward the diamide of compounds **80** and **82** failed using HATU as a coupling reagent. The stepwise reaction with the aminium salts like HATU involves the activation of the carboxyl group to form an activated ester intermediate and after that the amine would be added and the nucleophilic attack on the activated ester will yield the amide bond.[66] In the case of compounds **80** and **82** the presence of the central amide served as a intramolecular nucleophile and formed a cyclized product before the amine addition forming five or six membered rings, respectively. Upon the amine (N-methyl piperazine) addition only one activated ester was available for forming the amide bond and gave compounds **83** and **84**, as shown in Scheme 10.

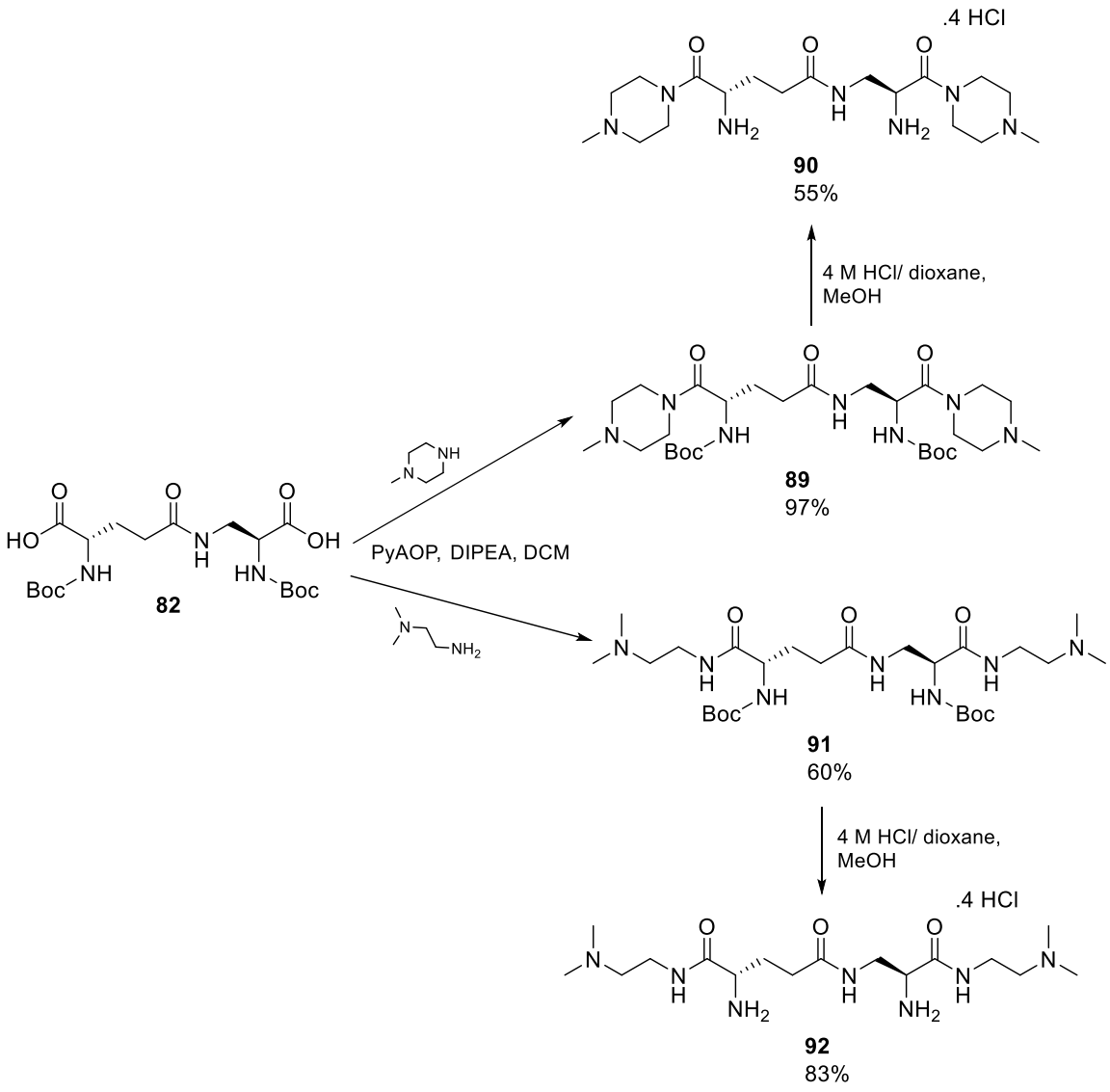
Phosphonium and aminium coupling reagents are commonly used in coupling reactions. The aminium type represented by HBTU and HATU where the difference is the faster reaction with HATU due to the enhanced electron withdrawing effect of the corresponding formed -OAt active ester during the coupling reaction. The coupling reaction should be proceeded in stepwise with the aminium coupling reagents since the amino group could attack the reagent too forming the guanidinium side product. On the other hand, the phosphonium coupling reagents have no guanylation-activity to amino function and so the reaction is performed in situ in presence of the amine. The BOP and AOP reagents provides excellent coupling behavior, but have the severe drawback of the

high toxicity of the formed byproduct during the reaction HMPA (hexamethyl-phosphoramide) due to its carcinogenicity. PyBOP and PyAOP are used as the non-toxic version of the previous analogues while maintaining the same effective coupling properties. As mentioned before for HATU, PyAOP has faster coupling rates compared to PyBOP due to the enhanced electron withdrawing effect of the activated ester formed.[67, 68]



Scheme 11. Synthetic Route for Compounds **86** and **88**.

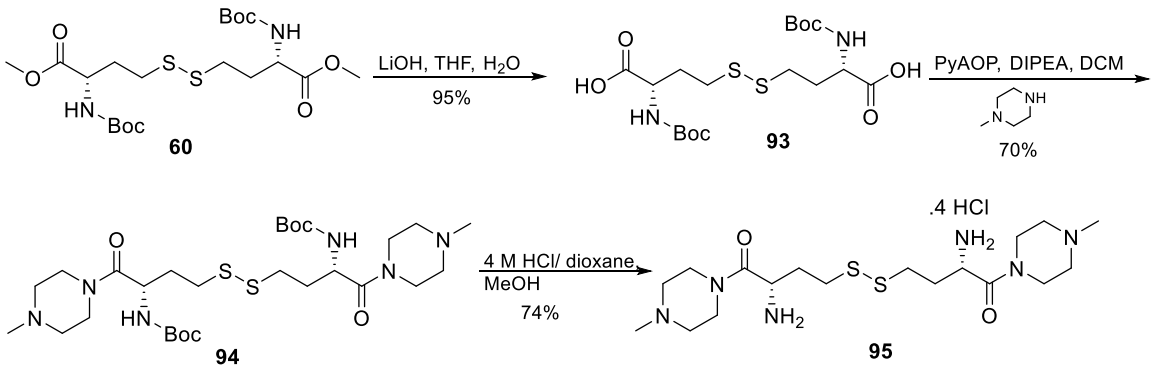
The diamides **85**, **87**, **89** and **91** were obtained successfully using PyAOP in presence of DIPEA starting from the diacids **80** and **82** as shown in Schemes 11 and 12. The diamides were then carried over for the deprotection step using 4 M HCl in dioxane to give the corresponding HCl salts **86**, **88**, **90** and **92**.



Scheme 12. Synthetic Route for Compounds **90** and **92**.

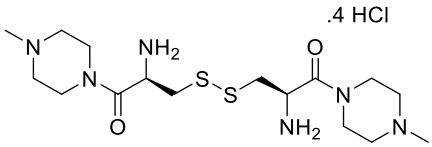
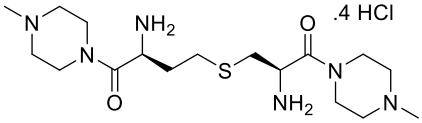
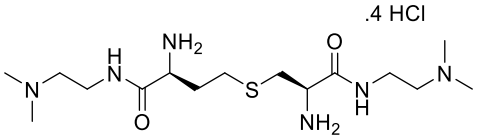
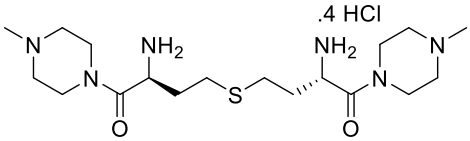
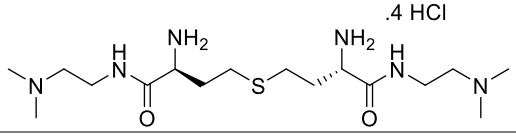
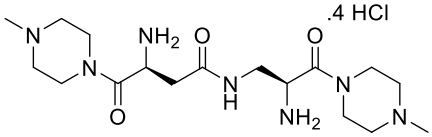
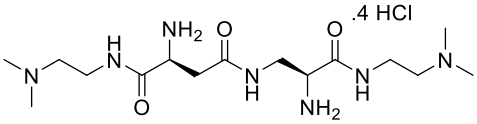
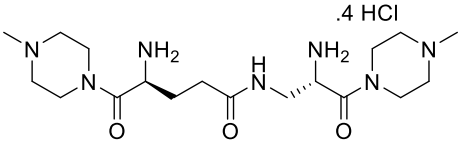
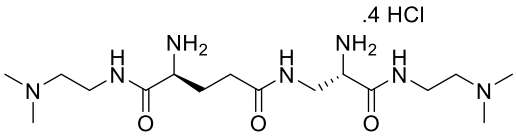
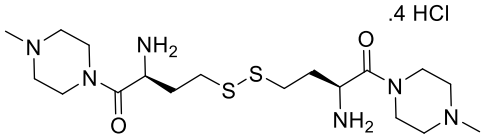
Unfortunately, all disulfide replaced analogues failed to inhibit the L-cystine crystallization even at 10 µM concentration as shown in Figure 15. With these results we

sought to synthesize the diamide of the L-homocystine to confirm if the reason behind the inactivity of the previous analogues is the absence of the disulfide or the altered distance between the two amino groups that account for most of the binding to the L-cystine crystal. Starting from the protected L-homocystine **60**, the diacid **93** was obtained in a good yield through a hydrolysis using lithium hydroxide. Then coupling with N-methyl piperazine using PyAOP in presence of DIPEA gave the diamide **94** in 70% yield. Finally, the corresponding HCl salt **95** was achieved via treatment with 4 M HCl in dioxane, as shown in Scheme 13. And as shown in Table 7 and Figure 15 the L-homocystine diamide failed to show inhibition even at 10 µM concentration which shows the importance of maintaining the distance between the two amino groups intact for optimal binding to the L-cystine crystal.



Scheme 13. Synthetic Route for Compound **95**.

Table 7. Aqueous solubility of L-cystine in presence of compounds **73-76**, **86**, **88**, **90**, **92** and **95**.

	Structure	Aqueous Solubility of L-Cystine		EC_{50} (nM)
		2 µM	10 µM	
9 (LH708)		2.89	2.84	42.8 ± 0.7
73 (LH1717)		0.93	1.14	>10,000
75 (LH1718)		1.11	1.18	>10,000
74 (LH1719)		1.09	1.13	>10,000
76 (LH1720)		1.14	1.20	>10,000
86 (LH1721)		1.10	1.04	>10,000
88 (LH1722)		1.07	1.20	>10,000
90 (LH1723)		1.15	1.14	>10,000
92 (LH1724)		1.04	1.20	>10,000
95 (LH1725)		0.75	0.80	>10,000

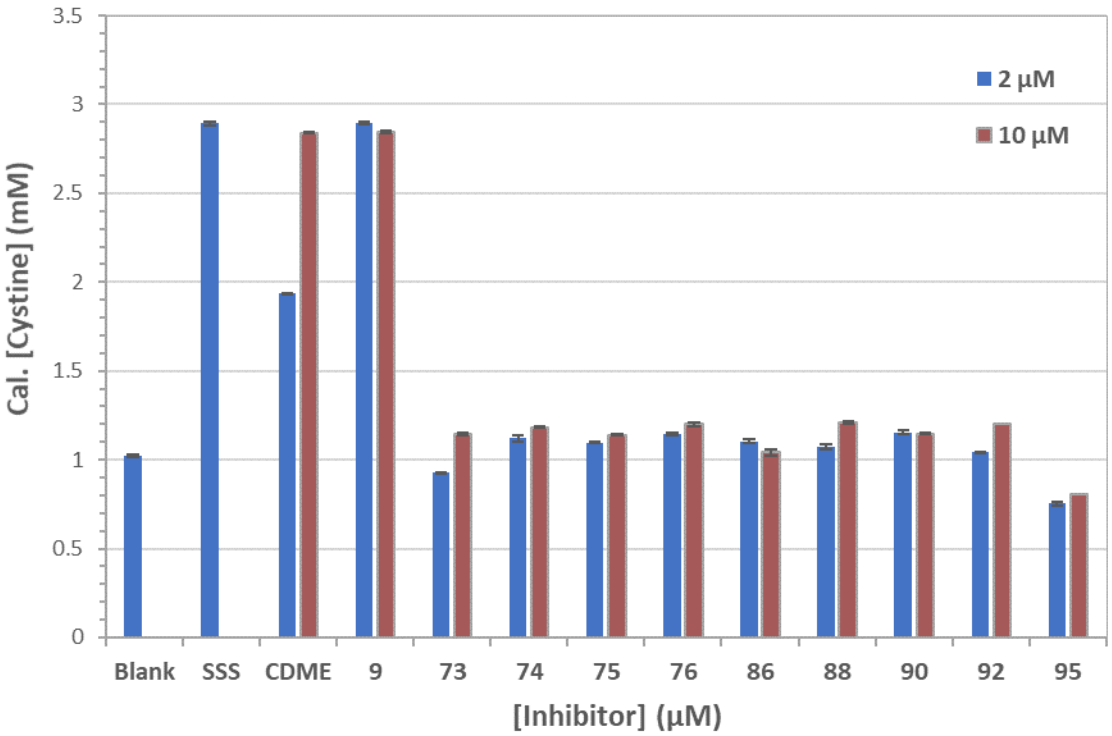
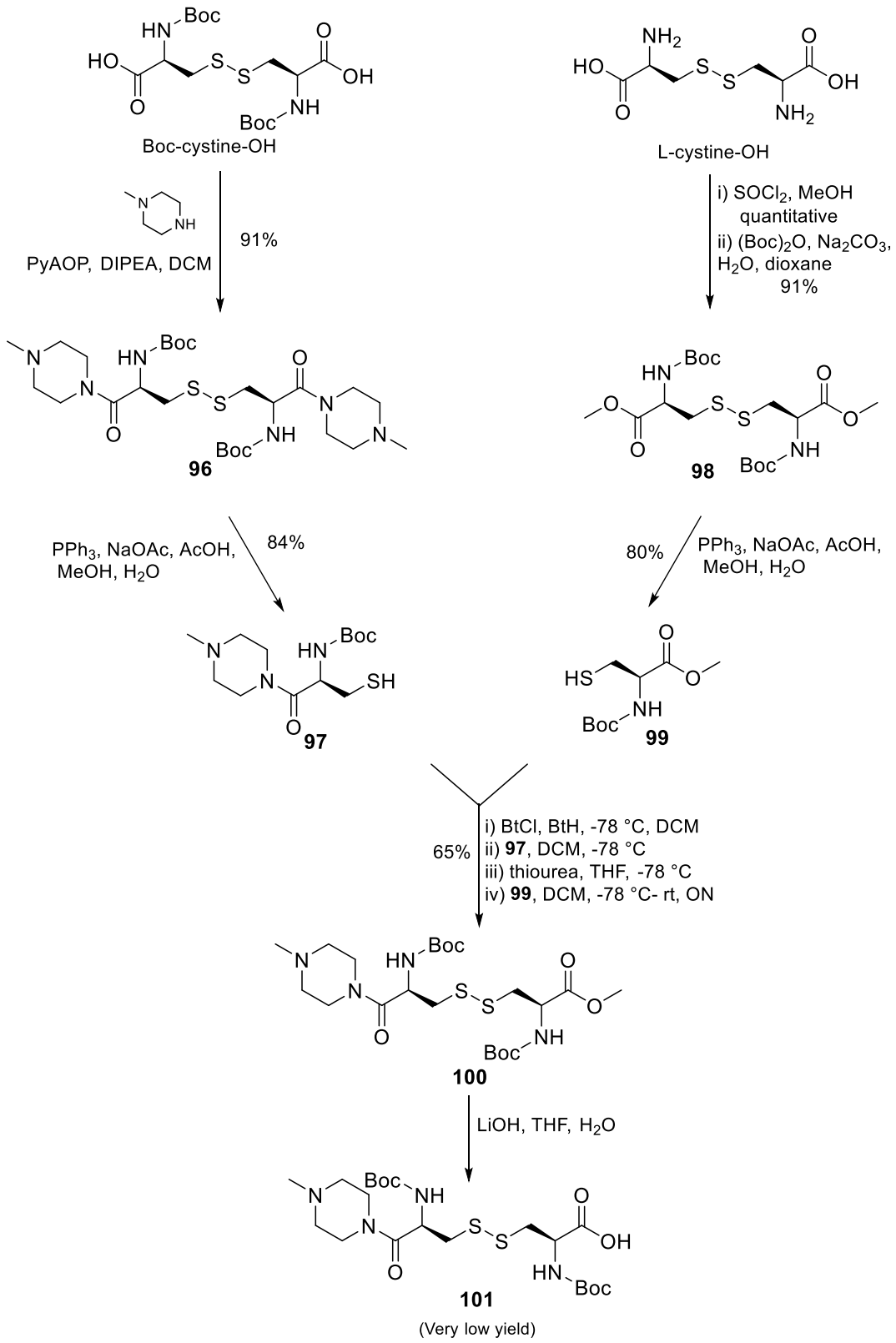


Figure 15. Aqueous Solubility of L-Cystine in Presence of **73-76**, **86**, **88**, **90**, **92** and **95** in Comparison to **9**, L-CDME. Also Water Control (Blank), and the Supersaturated Solution (SSS) Were Used.

2.7 Synthesis and Evaluation of L-Cystine Mixed Disulfide

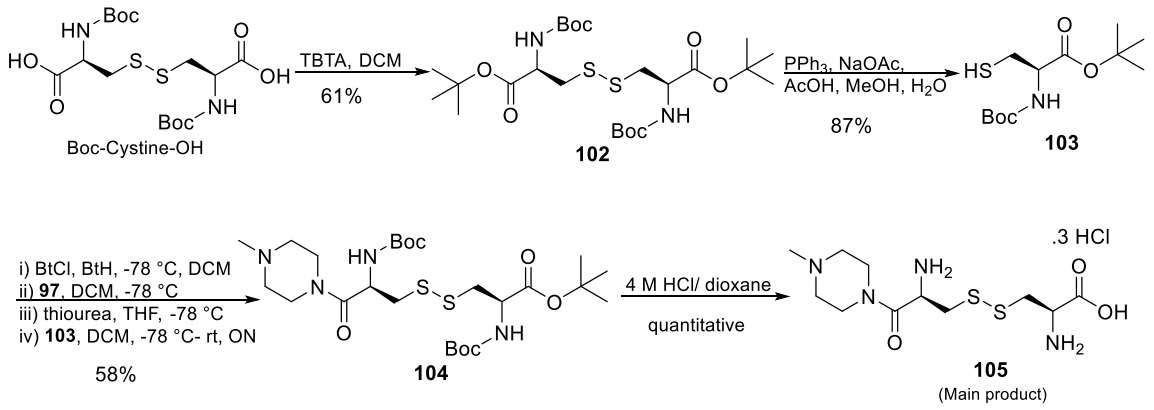
Since we have concerns about the stability of the disulfide bond in vivo, we decided to synthesize the possible asymmetrical disulfide metabolite **105** of **9**. For the synthesis we need both protected thiols of **9** and L-cysteine. Starting from Boc-L-cystine-OH, **96** was obtained in 91% yield through amide coupling with N-methyl piperazine using PyAOP and DIPEA. After that the disulfide was reduced using triphenyl phosphine to give the thiol **97** in a good yield. To prepare the other protected thiol we started from L-cystine-OH, the protected Boc-L-cystine-OMe **98** was obtained in two steps by treatment with thionyl chloride in MeOH followed by Boc protection using di-*tert*-butyl dicarbonate in

presence of sodium carbonate. The thiol **99** was obtained through reduction using triphenyl phosphine. The unsymmetrical disulfide was achieved in one-pot synthesis using 1-chloro benzotriazole through activation of the first thiol then addition of thiourea to quench any excess reagent, via formation of the isothiouronium salt, then addition of the second thiol. All steps were done at -78 °C then the reaction was warmed slowly to room temperature to give the desired mixed disulfide **100** as shown in Scheme 14.[69-71] The hydrolysis step to give the carboxylic acid **101** failed with many side product and very low yield of the desired product according to LC/MS which was hard to extract from the aqueous layer.



Scheme 14. Attempts at Obtaining Compound **105**.

With the failed attempt to achieve **105** we decided to repeat the synthesis with t-butyl ester protected acid instead which will be deprotected with Boc using acid. Boc-L-Cystine-OH was treated with *tert*-Butyl 2,2,2-trichloroacetimidate (TBTA) to give compound **102** in 61% yield.[72, 73] This was followed by disulfide reduction using triphenyl phosphine to give the thiol **103** in a good yield. The unsymmetrical disulfide **104** was achieved using 1-chloro benzotriazole in stepwise addition of thiols **97** and **103** using the same procedure mentioned previously. The deprotection of **104** using 4 M HCl in dioxane was done previously in MeOH which resulted in the methyl ester of **105**, so dioxane was used as solvent, as shown in Scheme 15. The desired product **105** was the main product with some symmetrical disulfide forming (~ 5:1), the mechanism of their formation is unknown. The deprotection step was tested with stronger acid (TFA/ DCM) at 0 °C, but the symmetrical disulfide still formed.



Scheme 15. Synthetic Route for Compound **105**.

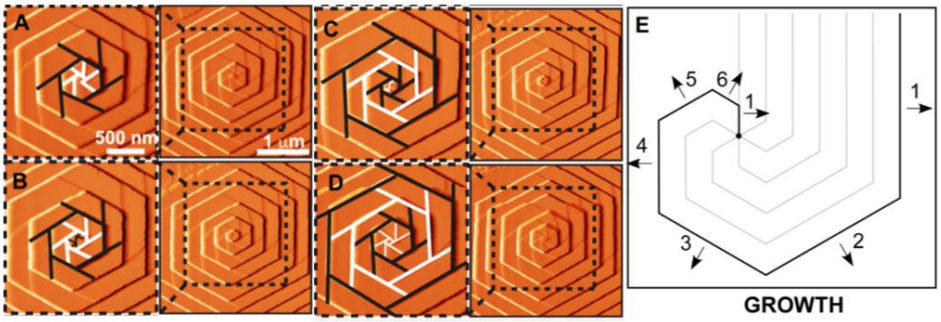
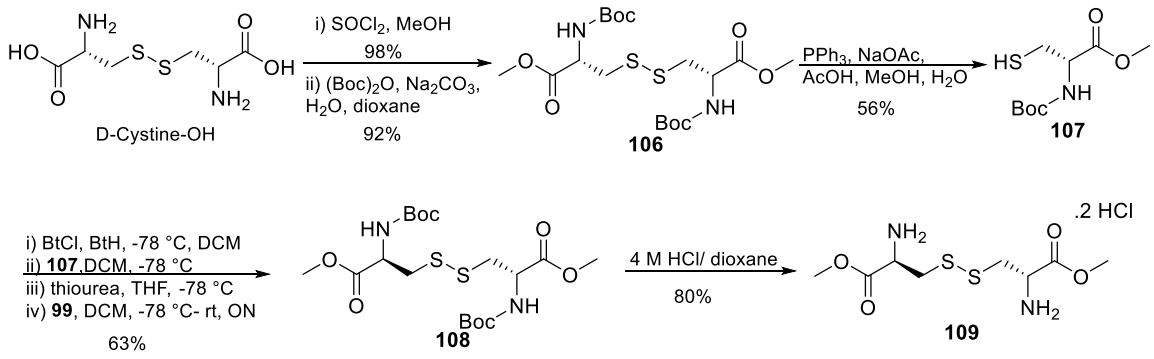


Figure 16. Series of AFM Images Depicting the Growth of the Hexagonal Spiral Morphology of L-Cystine.[74] (Reproduced with permission from American Chemical Society).

Studies of the L-cystine crystal growth using AFM images demonstrate that the evolution of the hexagonal spirals during growth represent a clockwise rotation (Figure 16) that spins out to form six interlacing spirals and a counterclockwise for D-cystine.[74] This encouraged us to synthesize the unsymmetrical L,D-cystine methyl ester as an analogue to L-CDME and the unsymmetrical L,D diacid where we thought could disrupt the spiral growth. D-Cystine-OH was treated with thionyl chloride and MeOH to give the methyl ester in 98% yield then amino protection using di-*tert*-butyl dicarbonate in presence of sodium carbonate to give **106** in a good yield. The disulfide was reduced using triphenyl phosphine to give the thiol **107** in 56% yield. The unsymmetrical disulfide **108** was achieved using 1-chlorobenzotriazole in stepwise addition of the thiol as described in Scheme 16. Compound **109** was obtained in 80% yield with amine deprotection using 4 M HCl in dioxane. Figure 17 illustrates the dose response curves of **105** and **109** in compare to **9** and L-CDME.



Scheme 16. Synthetic Route for Compound **109**.

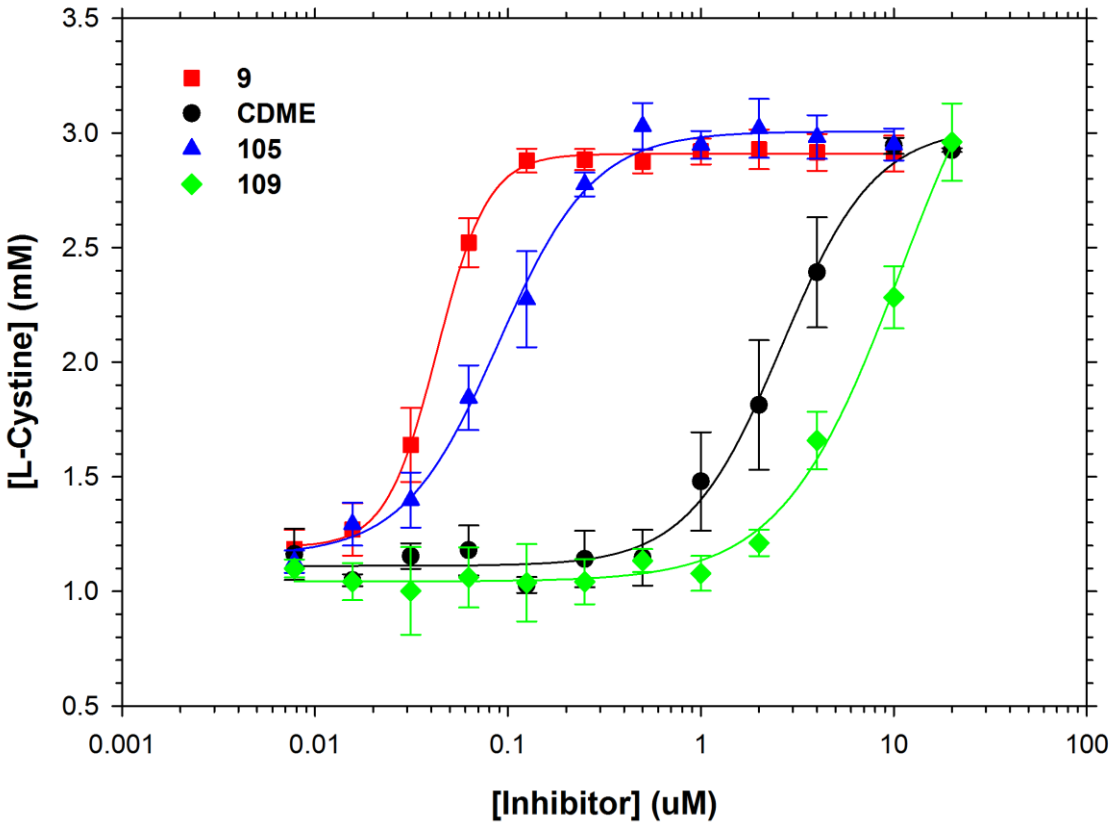
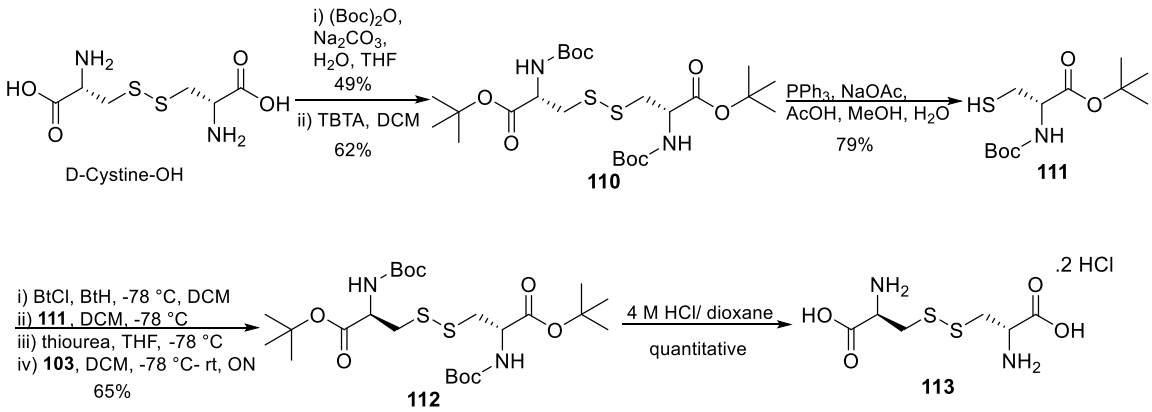


Figure 17. Dose Response Curves for Compounds **105** and **109** in Comparison to **9** and L-CDME.

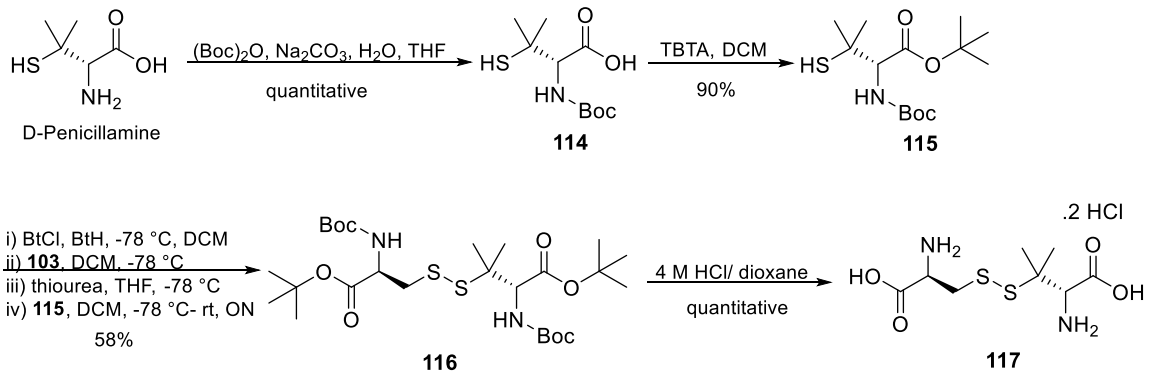
The unsymmetrical cystine **113** was synthesized starting with two steps protection of D-cystine-OH using di-*tert*-butyl dicarbonate in presence of sodium carbonate then treatment with *tert*-Butyl 2,2,2-trichloroacetimidate (TBTA) to give compound **110** in 62% yield. The thiol **111** was obtained in 79% yield through disulfide reduction with triphenyl phosphine. The protected unsymmetrical cystine was obtained using 1-chlorobenzotriazole. The deprotection was done using 4 M HCl in dioxane to give **113** in a quantitative yield as shown in Scheme 17.



Scheme 17. Synthetic Route for Compound **113**.

We also want to test the inhibition ability of the unsymmetrical cysteine disulfides products of the common thiol drugs used in clinics. Mixed disulfides of D-penicillamine and tiopronin were synthesized and tested for L-cystine crystallization inhibition. D-Penicillamine was treated with di-*tert*-butyl dicarbonate in presence of sodium carbonate to give compound **114** in a quantitative yield. That was followed by t-butyl ester formation using *tert*-butyl 2,2,2-trichloroacetimidate (TBTA) to give compound **115** in 90% yield. As shown in Scheme 18 the protected mixed disulfide **116** was achieved in

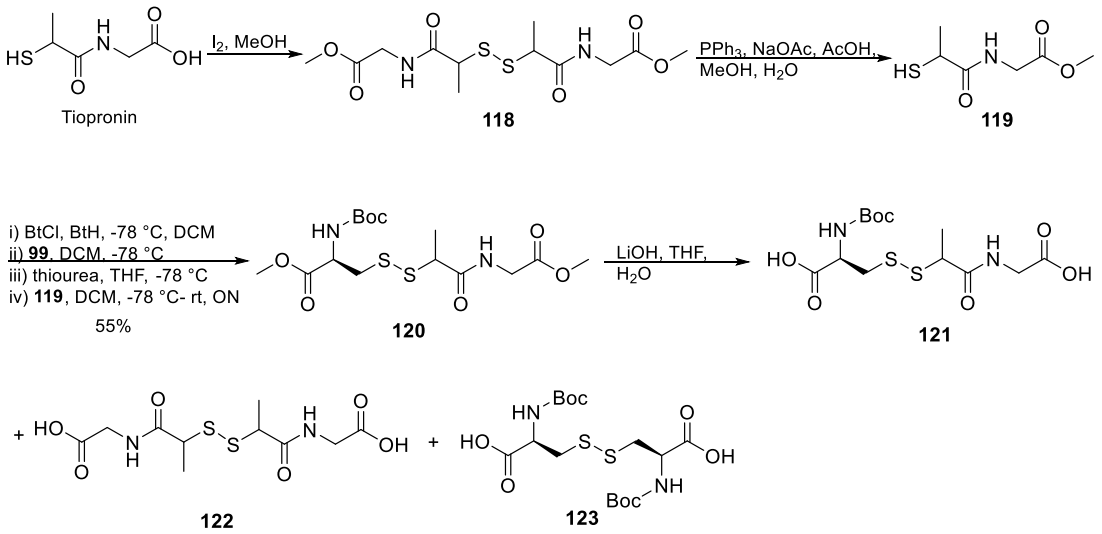
58% yield using 1-chlorobenzotriazole. The deprotection was done using 4 M HCl in dioxane to give **117** in a quantitative yield.



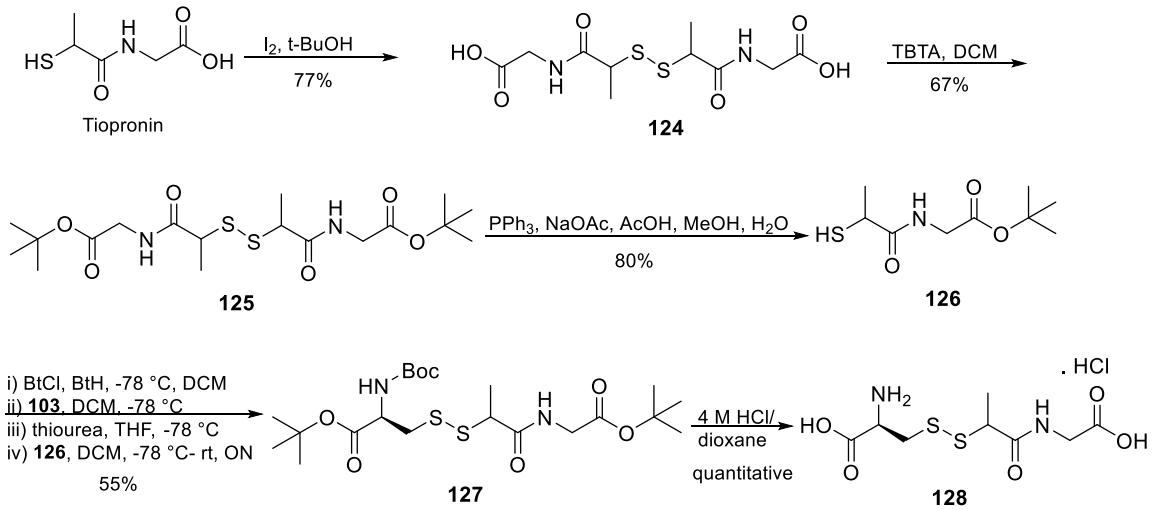
Scheme 18. Synthetic Route for Compound **117**.

For tiopronin the thiol group was needed to be protected first before the t-butyl ester formation. First the thiol was protected through S-acylation, but upon deprotection with base the disulfide was the only observed product. So we decided to proceed making the methyl ester disulfide **118** through oxidation using iodine in MeOH followed by reduction to give the thiol **119**.[75] The protected disulfide **120** was formed using 1-chlorobenzotriazole as shown in Scheme 19. The hydrolysis step which was done using two equivalents of lithium hydroxide at 0 °C then room temperature for one hour yielded the symmetrical disulfides **122** and **123** mainly. Other bases and solvent systems were used which led to the same result. The synthesis was proceeded then with the t-butyl ester as the carboxylic acid protecting group where first the disulfide of tiopronin **124** was obtained through oxidation using iodine in t-BuOH. Then treatment with *tert*-Butyl 2,2,2-trichloroacetimidate (TBTA) gave the protected disulfide **125** in 67% yield. The disulfide was reduced using triphenyl phosphine to offered the thiol **126** in a good yield. The protected mixed disulfide **127** was achieved in 55% yield using 1-chlorobenzotriazole as

shown in Scheme 20. The deprotection step was achieved using 4 M HCl in dioxane to give **128** in a quantitative yield.



Scheme 19. Attempts at Obtaining Compound **128**.

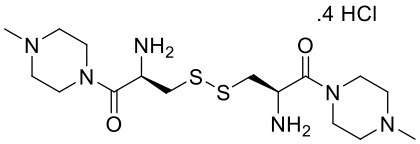
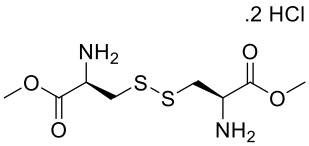
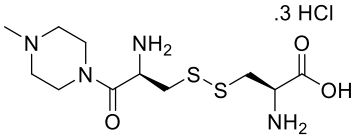
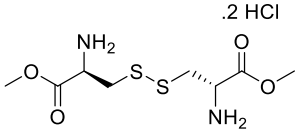


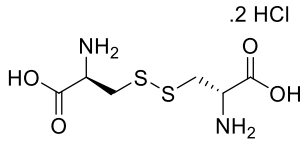
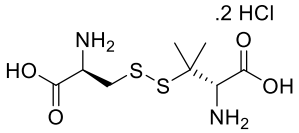
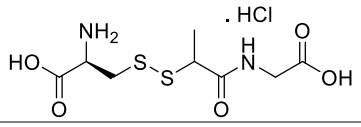
Scheme 20. Synthetic Route for Compound **128**.

The possible asymmetrical disulfide metabolite of **9** (**105**) has an EC_{50} of 88.9 nM which is two times lower than the inhibition achieved with the corresponding diamide **9**, but still

considered a very good inhibitor compared to first tested analogues as well as L-CDME. In addition to the good inhibitory profile of this possible metabolite its formation yields two equivalents of each molecule of the disulfide **9** which means it could be administered in low doses. Unfortunately, the unsymmetrical disulfide analogue of L-CDME **(109)** showed almost five fold reduced inhibition with an EC_{50} of 10.9 µM compared to 2.6 µM for L-CDME. The unsymmetrical cystine **113** as well did not show any inhibition even at 10 µM concentration. The two unsymmetrical disulfide of D-penecillamine **117** and tiopronin **128** has no inhibition even at 10 µM concentration as well. Table 8 and Figure 18 shows the aqueous solubility of L-cystine in presence of 2 and 10 µM of **105**, **109**, **113**, **117** and **128** compared to **9** and L-CDME.

Table 8. Aqueous Solubility of L-Cystine in Presence of Compounds **105**, **109**, **113**, **117** and **128** Compared to **9** and L-CDME.

	Structure	**Aqueous Solubility of L-Cystine**		**EC_{50} (nM)**
		2 µM	**10 µM**	
9 (LH708)		3.00	3.00	42.8 ± 0.7
1 (L-CDME)		1.37	2.90	2,621 ± 234
105 (LH1727)		3.03	2.93	88.9 ± 7.1
109 (LH1728)		1.25	2.37	10,900 ± 2800

113 (LH1765)		1.06	1.07	>10,000
117 (LH1755)		1.24	1.37	>10,000
128 (LH1756)		0.80	0.95	>10,000

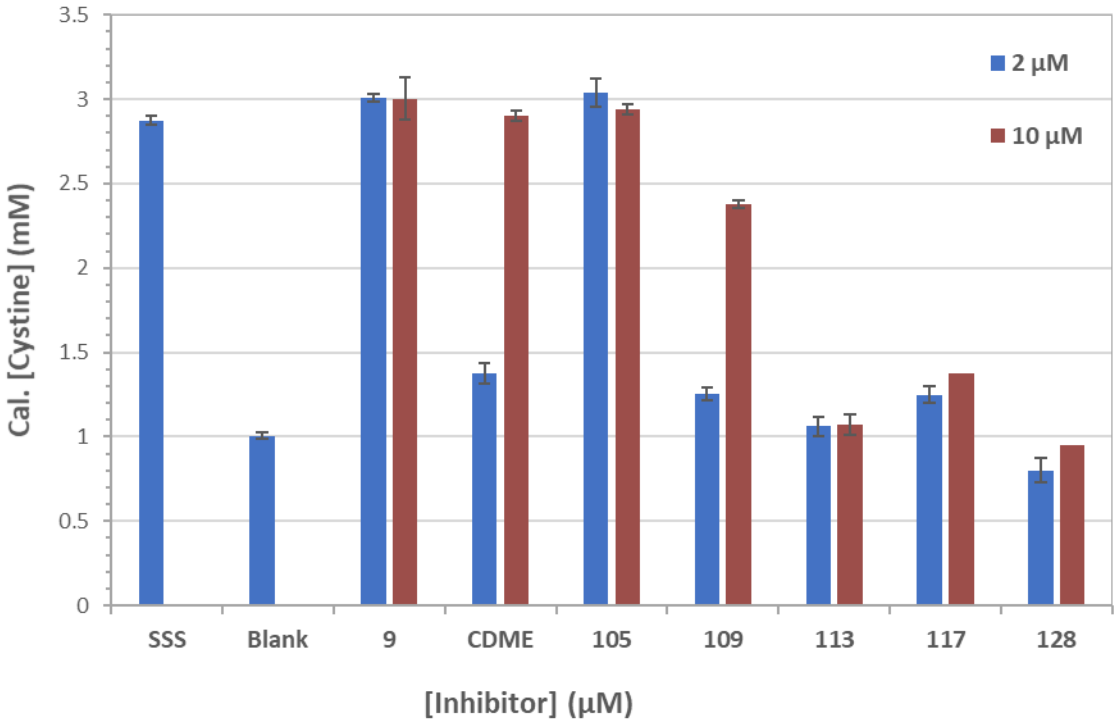
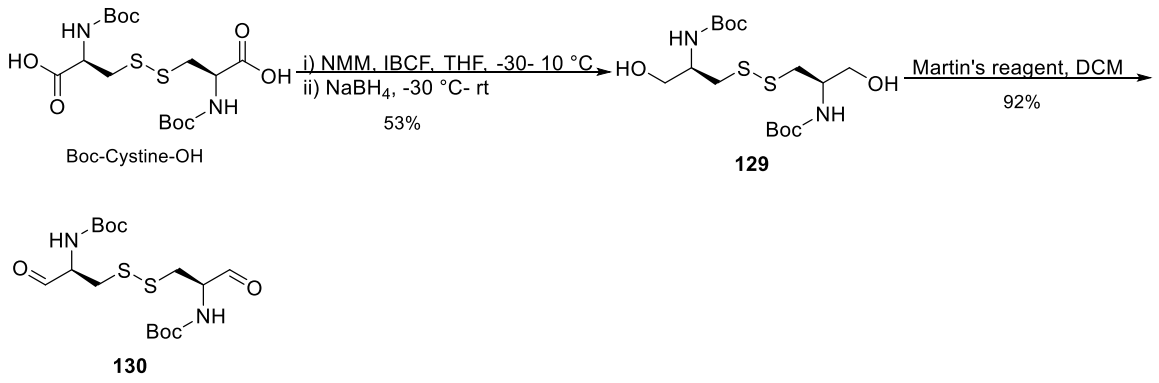


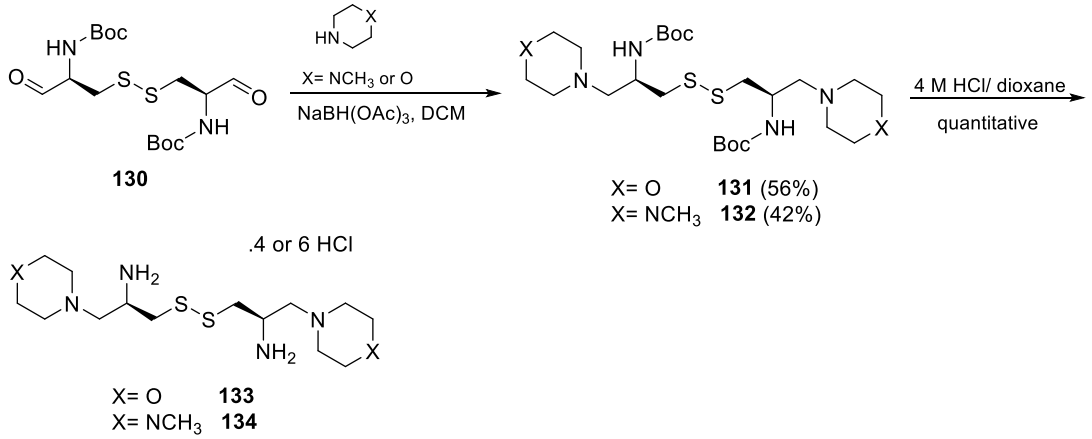
Figure 18. Aqueous Solubility of L-Cystine in Presence of **105**, **109**, **113**, **117** and **128** in Comparison to **9** and L-CDME. Also Water Control (Blank), and the Supersaturated Solution (SSS) Were Used.

2.8 Synthesis and Evaluation of Analogues Lacking Carbonyl or with Pseudo Reversed Amide as Analogues of Compounds 8 and 9

In addition, a new series of analogues were designed and synthesized having amine analogues instead of the amide or with different carbonyl position for the amide bond. The synthesis of the main two intermediates started by reducing the Boc protected L-cystine to the dialcohol analogue **129** using isobutyl chloroformate (IBCF) and N-methylmorpholine (NMM) followed by sodium borohydride.[76, 77] Then the dialcohol **129** was oxidized using Dess-Martin periodinane to give the corresponding dialdehyde **130** in 92% yield, as shown in Scheme 21.[78, 79]

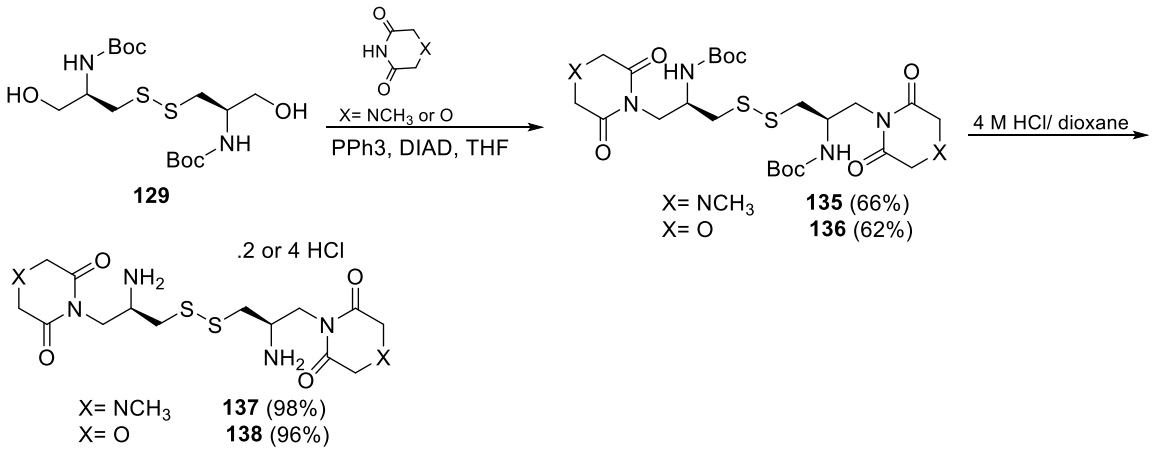


Scheme 21. Synthetic Route for Intermediates **129** and **130**.

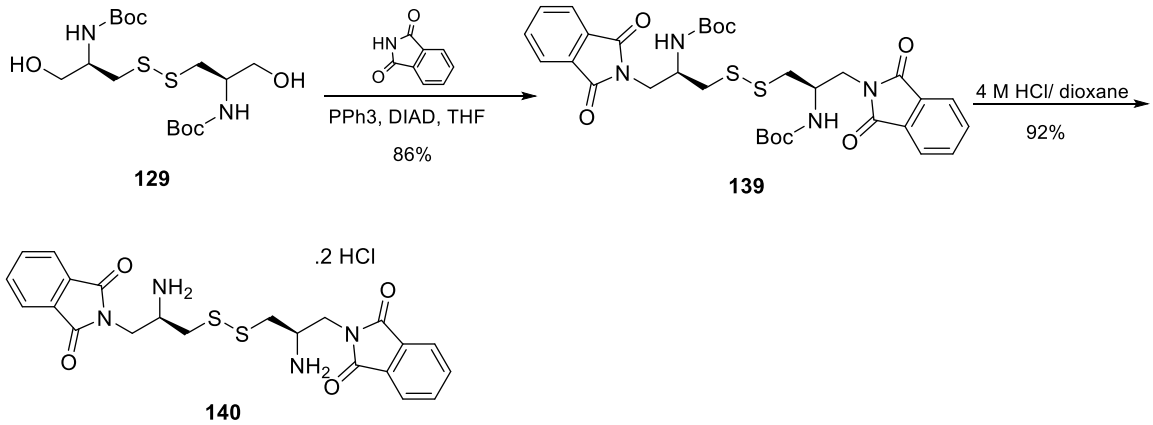


Scheme 22. Synthetic Route for Compounds **133** and **134**.

Two diamine analogues of **8** and **9** were synthesized starting from the dialdehyde **130** in a reductive amination with morpholine or N-methyl piperazine to give the protected diamines **131** and **132**, respectively.[80] As shown in Scheme 22, the HCl salts **133** and **134** of compounds **131** and **132**, respectively were obtained upon treatment with 4 M HCl in dioxane in a quantitative yields. These analogues had no inhibition ability apparently due to the lack of binding ability to the L-cystine crystals which shows the importance of the carbonyl oxygen in the binding to L-cystine crystals.

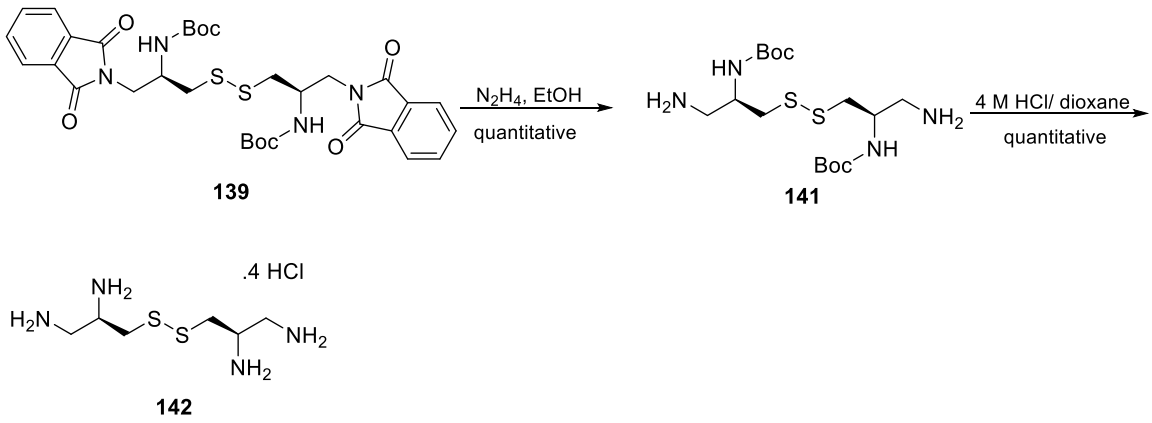


Scheme 23. Synthetic Route for Compounds **137** and **138**.



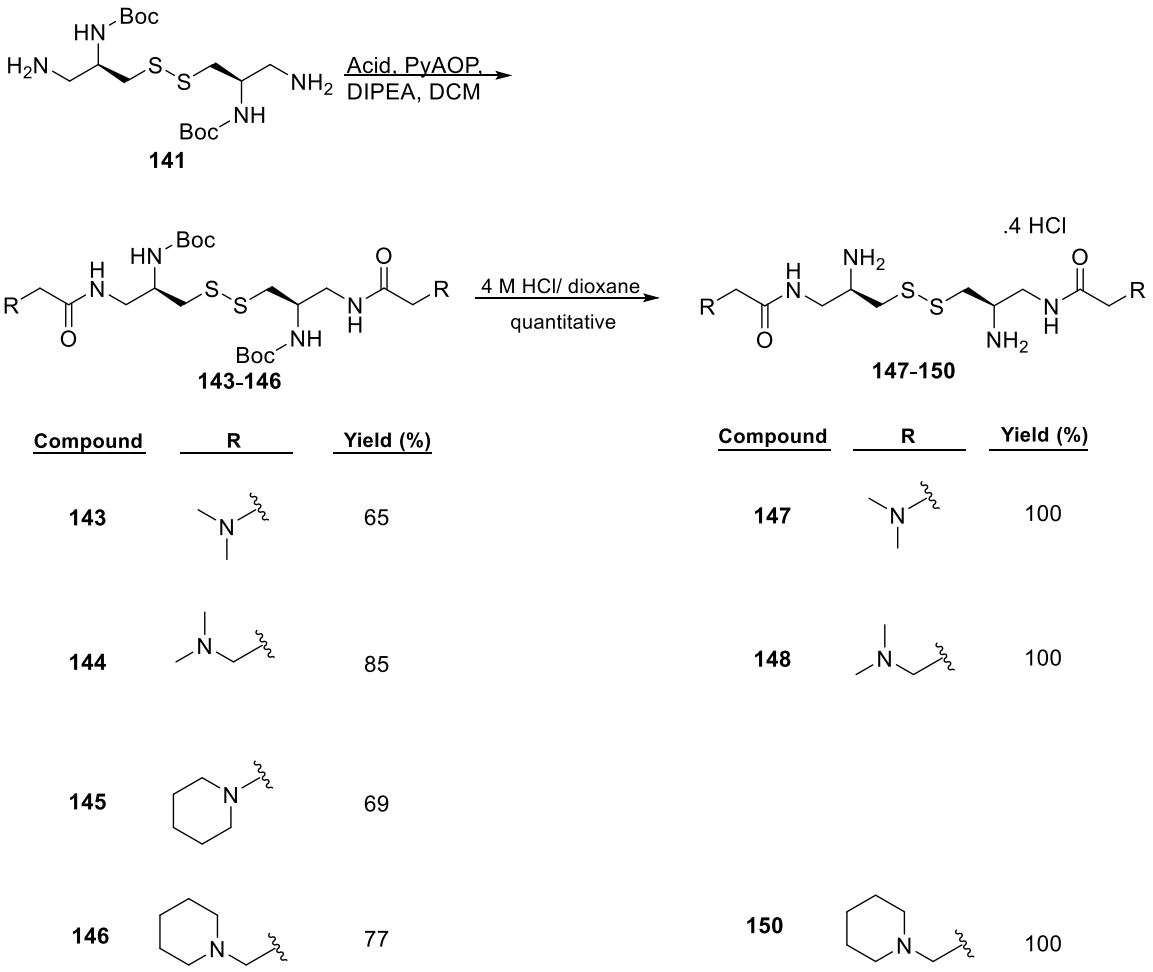
Scheme 24. Synthetic Route for Intermediate **139** and Compound **140**.

To test the effect of the position of the carbonyl on binding different analogues were synthesized and tested for their inhibition ability. Compounds **135** and **136** were obtained starting from the dialcohol **129** through a Mitsunobu reaction with 4-methyl-2,6-piperazinedione and 3,5-Morpholinedione, respectively in good yields.[81] These compounds were deprotected using 4 M HCl in dioxane to give the corresponding HCl salts **137** and **138** in good yields, as shown in Scheme 23.



Scheme 25. Synthetic Route for Intermediate **141** and Compound **142**.

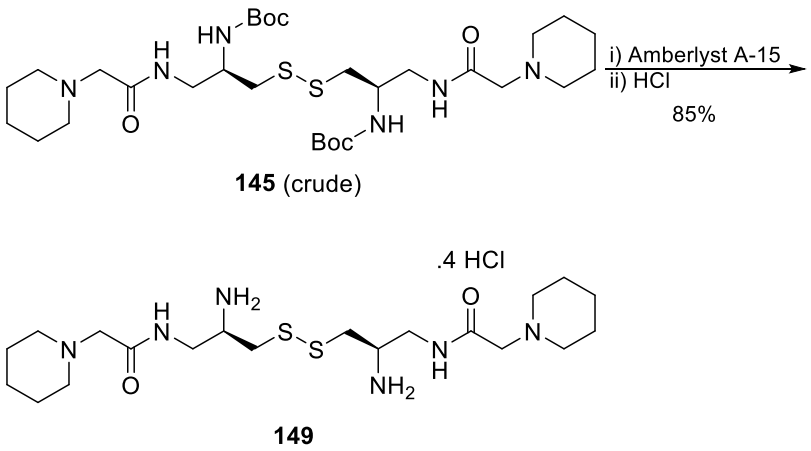
In addition, protected diphthalimido analogue **137** was achieved in 86% yield through a Mitsunobu reaction as described before starting from the dialcohol **129** and phthalimide. As shown in Scheme 24, the deprotection of compound **139** using 4 M HCl in dioxane offered **140** in 92% yield. The phthaloyl carbazates **139** was also cleaved with hydrazine to afford the corresponding diamine **141** in a quantitative yield.[81] The diamine was then deprotected using 4 M HCl in dioxane to give the HCl salt **142** in a quantitative yield, as shown in Scheme 25.



Scheme 26. Synthetic Route for Compounds **147-150**.

Also the diamine **141** was coupled with different acids to give the diamide analogues **147-150**. The amide coupling was achieved using PyAOP in presence of DIPEA for the diamine **141** and the different acids: dimethyl glycine, dimethyl alanine, 1-Piperidineacetic acid and 1-Piperidine-propanoic acid to give diamides **143-146**, respectively. Compounds **143**, **144** and **146** were then deprotected using 4 M HCl in dioxane to give the corresponding HCl salt **147**, **148** and **150** in a quantitative yield, as shown in Scheme 26. On the other hand compound **145** was very hard to purify from the reagent's side product tris(pyrrolidinophosphine) oxide, so the crude product was

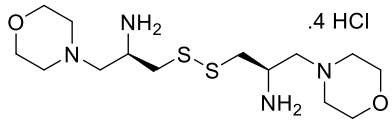
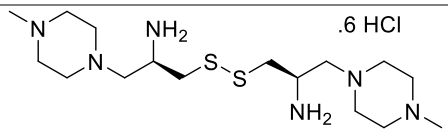
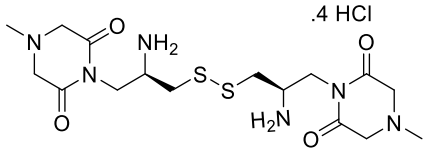
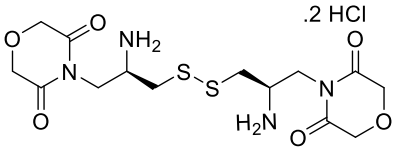
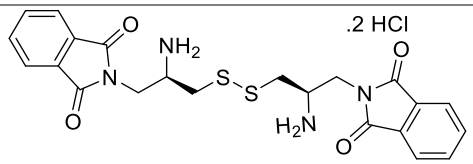
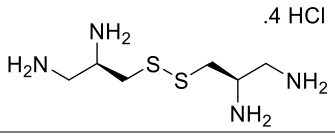
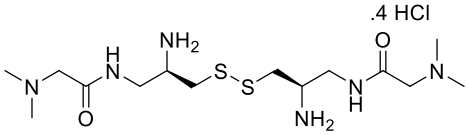
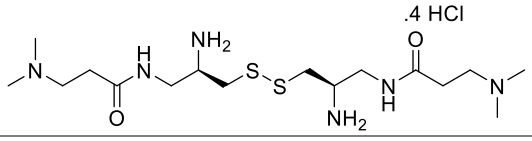
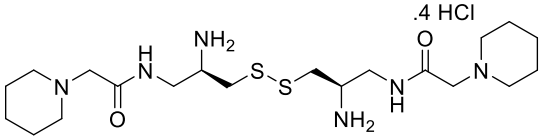
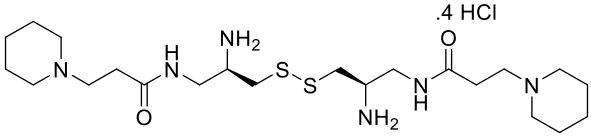
deprotected using Amberlyst A-15 in DCM where the deprotected desired compound was bound to the resin and the side product was washed before stirring of the resin in 4 M NH_3 in MeOH to give the desired product in solution which was dried and then treated with HCl in MeOH to give the corresponding HCl salt **149**, as shown in Scheme 27.[82]



Scheme 27. Synthetic Route for Compound **149**.

Unfortunately, all of these analogues did not show inhibitory activity even at 10 µM (Figure 19 and Table 9) which shows also that not only the presence of a carbonyl group is important as shown before but also the position is critical to the binding to L-cystine crystals. Indeed, the results obtained in this study show the importance of keeping the core structure of L-cystine intact in order to get a molecule with binding ability to the L-cystine crystal and inhibit crystal growth.

Table 9. Aqueous Solubility of L-Cystine in Presence of Compounds **133**, **134**, **137**, **138**, **140**, **142** and **147-150**.

	Structure	Aqueous Solubility of L-Cystine	
		2 μM	**10 μM**
133 (LH1750)		1.08	1.12
134 (LH1749)		1.25	1.37
137 (LH1742)		1.05	1.14
138 (LH1743)		1.02	1.04
140 (LH1744)		0.91	0.98
142 (LH1764)		1.12	1.28
147 (LH1745)		1.12	1.39
148 (LH1746)		1.18	1.41
149 (LH1748)		0.99	1.07
150 (LH1747)		1.07	1.25

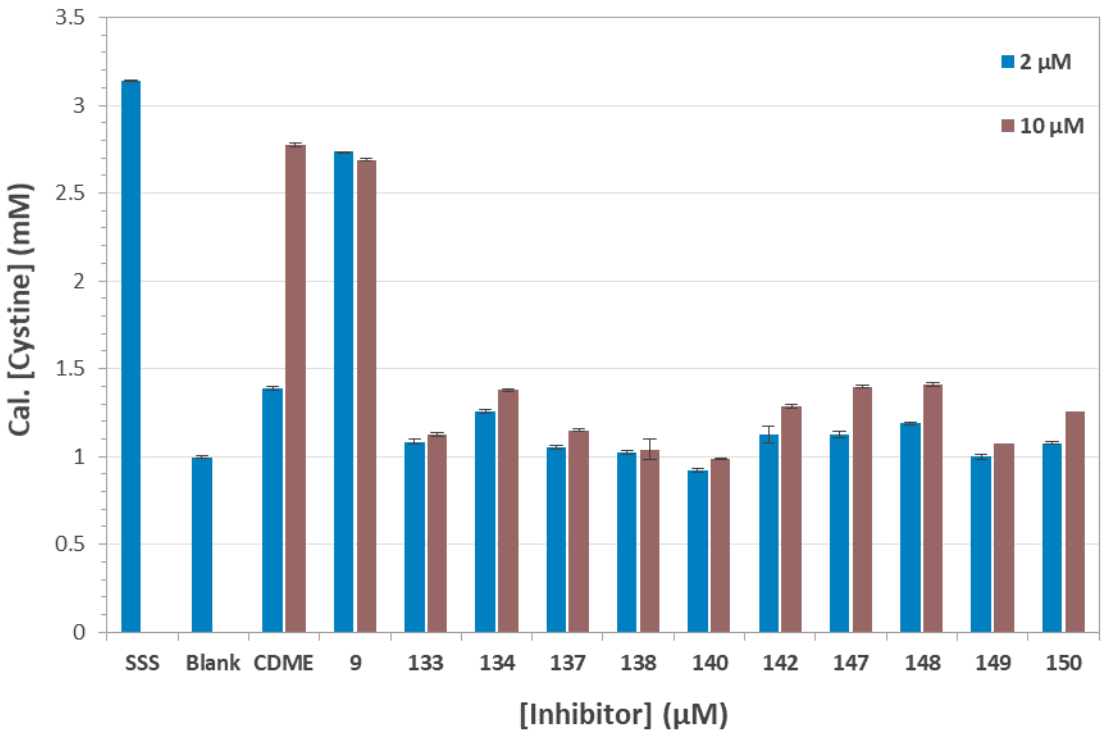


Figure 19. Aqueous Solubility of L-Cystine in Presence of **133**, **134**, **137**, **138**, **140**, **142** and **147-150** in Comparison to **9** and L-CDME. Also Water Control (Blank), and the Supersaturated Solution (SSS) Were Used.

CHAPTER THREE

Stability, Pharmacokinetic Studies and Prodrugs Synthesis and Evaluation

3.1 Chemical Stability of Compounds 8 and 9 Compared to L-CDME

We sought to determine the stability of diamide analogues **8** and **9** compared to L-CDME in term of their chemical stability which was determined in pH 7.4 phosphate buffered saline at 37 °C using LC-MS/MS by following the disappearance of the test compounds (Figure 20). The diamide analogues showed much greater chemical stability compared to L-CDME which has a half-life of about 2.7 h while **9** and **8** have half-lives of 19.8 and 46.2 days, respectively. Figure 20 depict the disappearance of each compound with time in days.

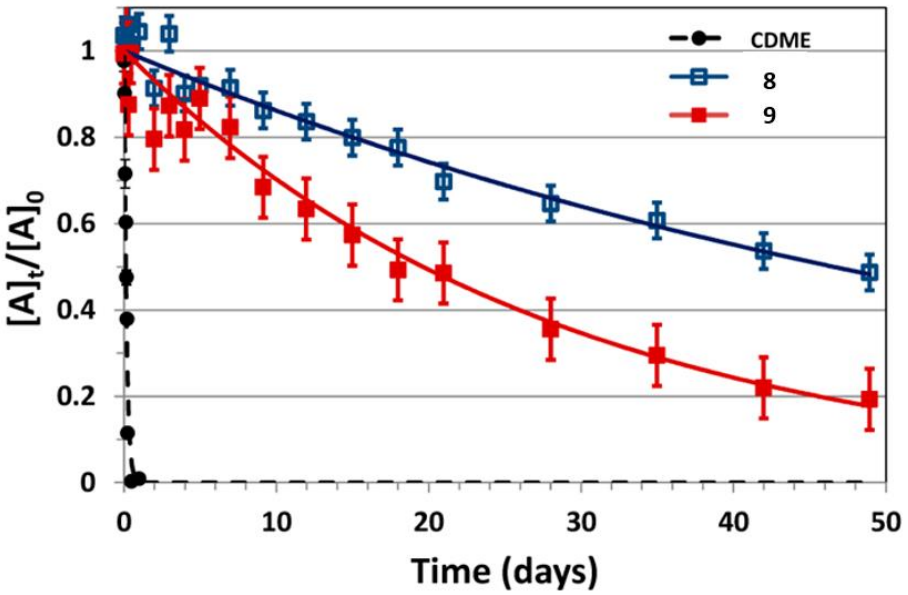


Figure 20. Chemical Stability of **8**, **9** and L-CDME in PBS at 37 °C.[44]

3.2 Bioavailability of L-Cystine Diamides in Knockout Mice Urine

We wanted to test the bioavailability of the L-cystine diamide in urine, the site of crystallization in the body. Three *SLC3A1* knockout male mice were treated with compound **9** at 8.26 mg/mL through daily gavage for 1 week and the urine of all mice were collected during the 4 h period immediately after the last dosing. The amounts of L-cystine diamide in the urine sample was determined using LC-MS/MS and shown in Figure 21 and compared to urine samples collected before feeding. While no test compound was found in urine samples collected prior to oral dosing (the small blue bar represent noise peaks), **9** was found in the urine of mice after dosing, suggesting that the compound is orally bioavailable.

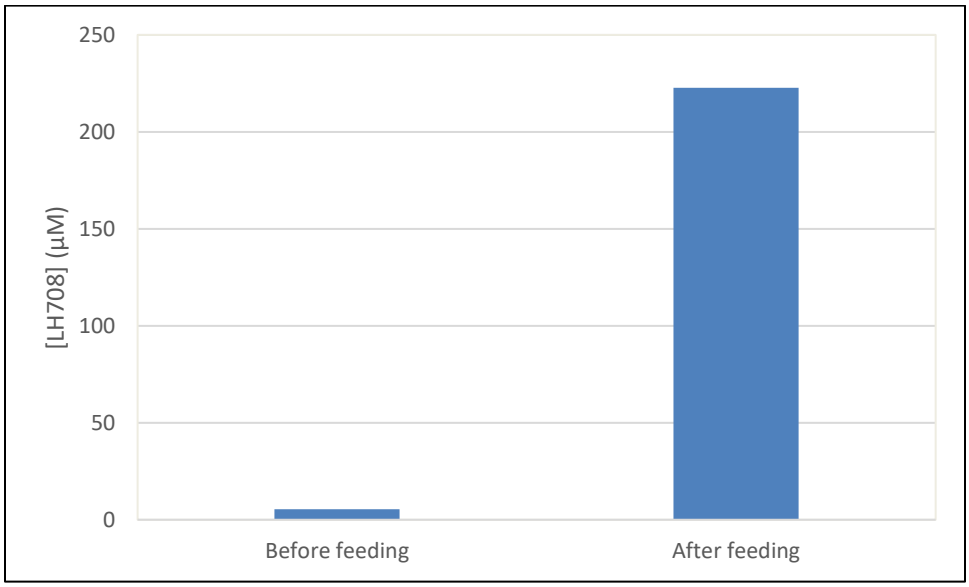


Figure 21. Drug Concentration of **9** in *SLC3A1* Knockout Mice Urine After Oral Dosing.

Indeed, a more detailed study analyzed by Dr. Herve Aloysius included two groups of 4−5 wild-type male mice and two groups of *SLC3A1* knockout male mice treated with either **8** or **9** at 29.3 µmol/kg through daily gavage for 1 week. The urine of each mouse

was collected individually during the 4 h period immediately after the last dosing and the amounts of L-cystine diamides in the urine samples were determined using LC-MS/MS as shown in Figure 22. There were no test compounds in urine samples collected before oral dosing (data not shown), while micromolar concentrations of **8** and **9** were found in the urine of each mouse after dosing. Which again show that the L-cystine diamides are orally bioavailable and even more significant higher concentrations of **9** were found in urine samples collected from the *SLC3A1* knockout cystinuria mouse group than those from the wild-type group (7.59 ± 1.34 μM vs 2.10 ± 0.57 μM for **9**). Also higher concentrations of **9** were found in *SLC3A1* knockout cystinuria mouse group as compared to **8** at equivalent oral doses (7.59 ± 1.34 μM for **9** vs 2.09 ± 0.62 μM for **8**). These unexpected results may indicate that the activities of other transporters are elevated after knocking out the *SLC3A1* gene which worked in our favor in the case of **9** but not in the case of **8**.[44]

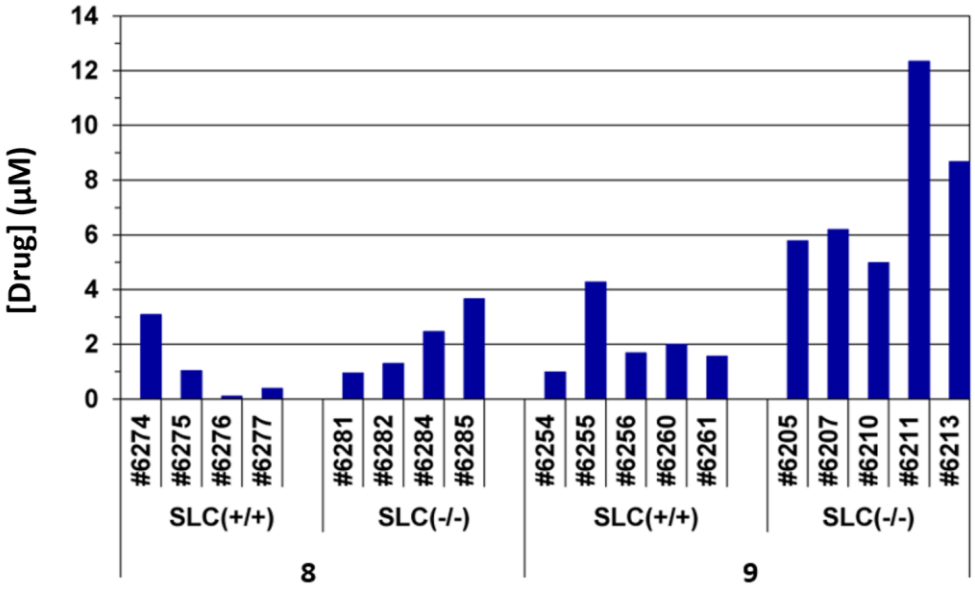


Figure 22. Drug Concentration in Mouse Urine After Oral Dosing of L-Cystine Diamides **8** and **9**.[44]

3.3 Bioavailability of L-Cystine Diamides in Knockout Mice Blood

In addition, we tested the bioavailability of the L-cystine diamide **9** in blood after oral or IV administration. A group of three *SLC3A1* knockout male mice were treated with compound **9** at 8.26 mg/mL (200 µL) through daily gavage for 1 week and another group of three *SLC3A1* knockout male mice were treated with compound **9** at 16.5 mg/mL (100 µL) through IV injection. The blood of each mouse was collected and the amounts of L-cystine diamide in the blood samples was determined using LC-MS/MS followed as the procedure shown in Figure 23. As illustrated in Figure 23 the half-life was calculated from the average of each group as 3.1 h and 2.5 h for IV bolus injection and oral feeding, respectively. The bioavailability of **9** was calculated to be 18% from the study.

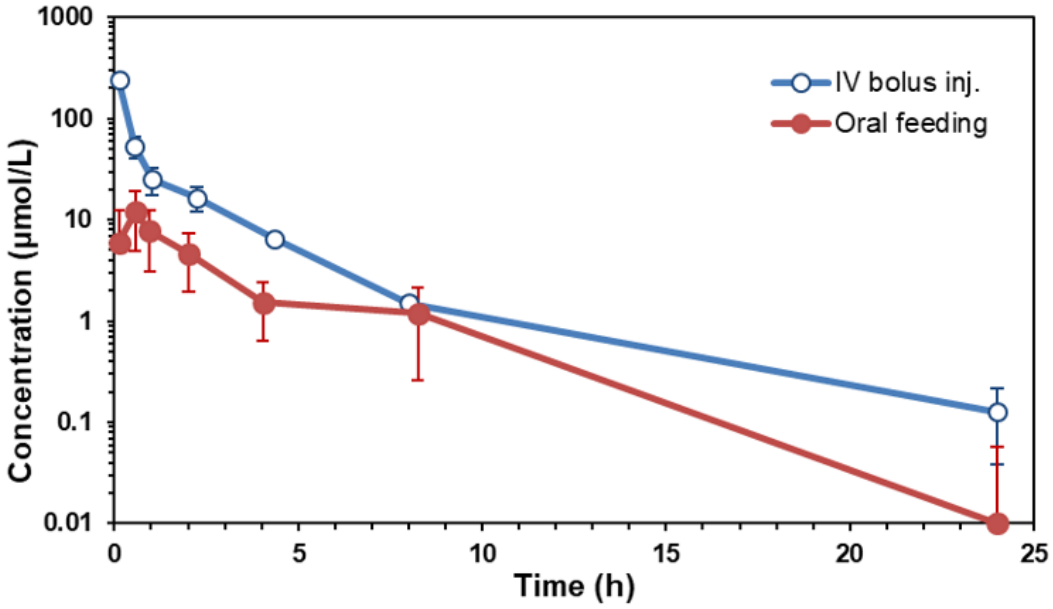


Figure 23. Drug Concentration of **9** in *SLC3A1* Knockout Mice Blood After IV and Oral Dosing.

3.4 Synthesis and Evaluation of L-Cystine Diamide Prodrugs

With the low bioavailability profile of compound **9** we sought to make prodrug analogues of the compound, which could be performed for the other analogues as well, to increase its absorption in vivo. Prodrugs are temporary modified forms of the active compound which requires in vivo transformation to give the actual drug. A promoiety could be linked chemically to a functional group on the parent compound to improve its drug-like profile. Amino groups like in our active compounds are known for their tendency to ionize under physiological conditions which cause physicochemical hurdles leading to the potential poor membrane penetration and hence a low bioavailability.[83, 84]

We decide to make different carbamate prodrugs since carbamate functionality displays a very good chemical and proteolytic stabilities in general. Carbamates are typically enzymatically more susceptible to hydrolysis than amides while the opposite holds true compared to esters. Their activation in vivo requires esterases to give the active compound after releasing the instable intermediate of carbamic acid.[83] Also the cascade-type prodrugs like the N-acyloxyalkoxycarbonyl derivatives or acyloxyalkyl carbamates which possess an esterase sensitive terminal group that upon hydrolysis triggers a spontaneous decomposition of the (hydroxyalkoxy)carbonyl intermediate to give the parent amine (Figure 24).[84] In addition, (Oxodioxolenyl)methyl carbamates system where its activation is achieved through two mechanisms: a base catalyzed cleavage or enzymatic hydrolysis (Figure 25).[84]

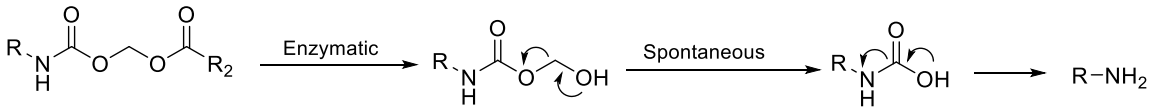


Figure 24. Mechanism of Acyloxyalkyl Carbamates In Vivo Activation.[84]

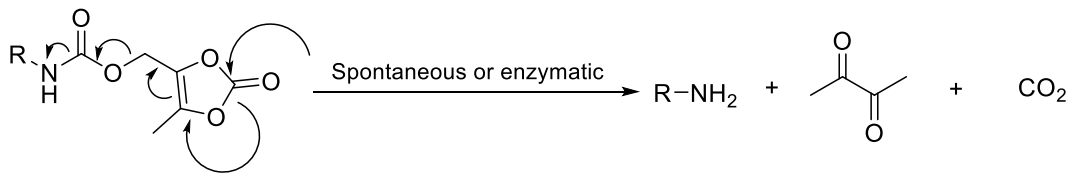
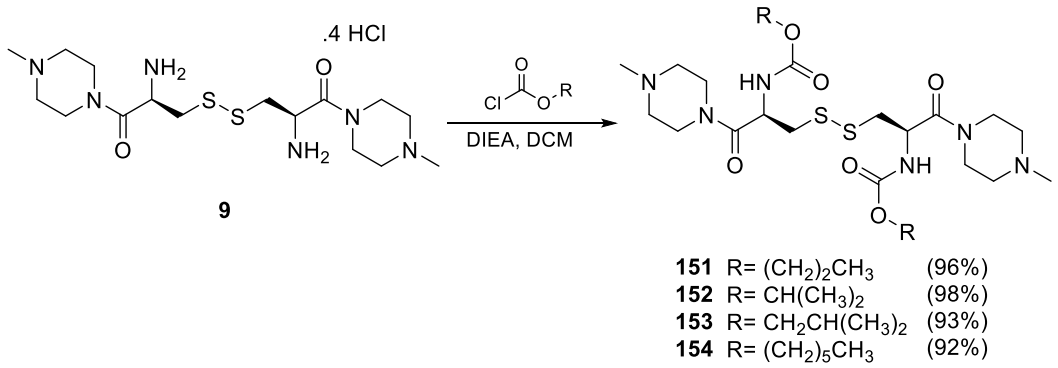


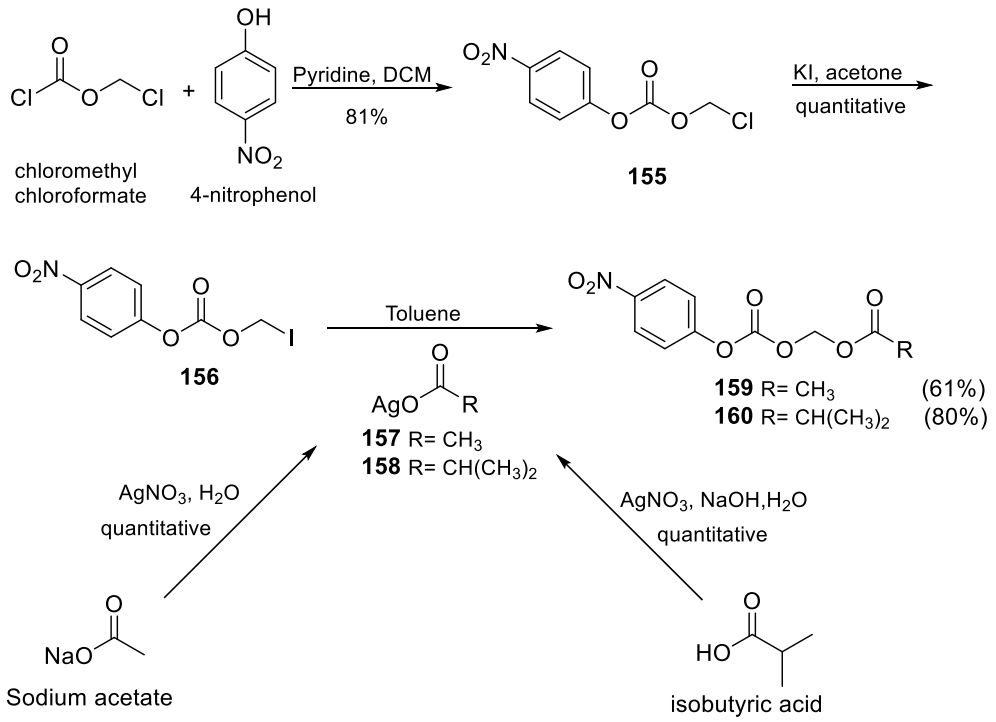
Figure 25. Mechanism of (Oxodioxolenyl)methyl Carbamates In Vivo Activation.[84]

Seven different analogues were synthesized and tested for their activation and stability in vitro. Alkyl carbamates including propyl, isopropyl, isobutyl and hexyl were synthesized by reacting **9** with the corresponding chloroformate in presence of DIEA to give prodrugs **151-154** in good yields, as shown in Scheme 28.[85]



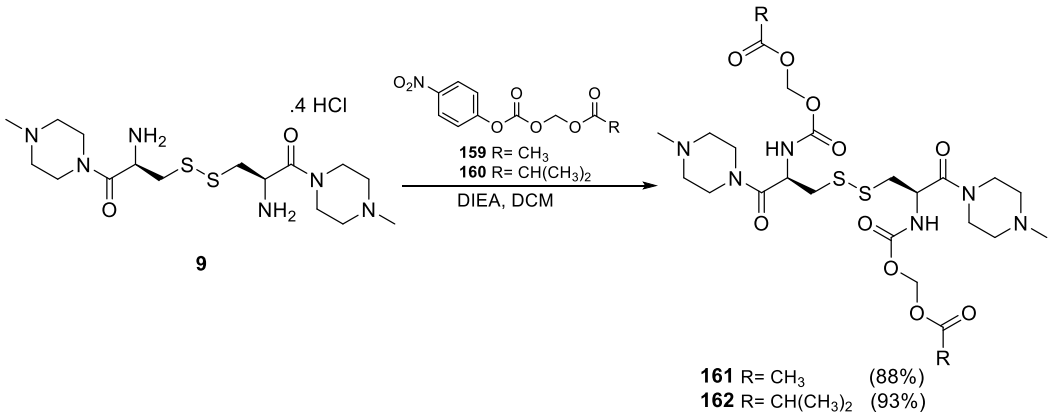
Scheme 28. Synthetic Route for Compounds **151-154**.

Prodrugs **161** and **162** with the acetoxy or isobutanoyloxy methyl carbamate analogues were obtained starting from commercially available chloromethyl chloroformate and 4-nitrophenol which gave nitrophenolate **155** in 81% yield then was followed by a Finkelstein reaction to give iodide **156** in a quantitative yield.[86] Starting from sodium acetate or isobutyric acid to get the silver analogues **157** and **158** in a quantitative yield through a reaction with silver nitrate.[87] The acetoxy or isobutanoyloxy methyl carbonate analogues **159** and **160** were obtained from reacting the corresponding 4-nitro phenol carbonate **156** with either **157** or **158**, respectively as shown in Scheme 29.[88, 89]



Scheme 29. Synthetic Route for Intermediates **159** and **160**.

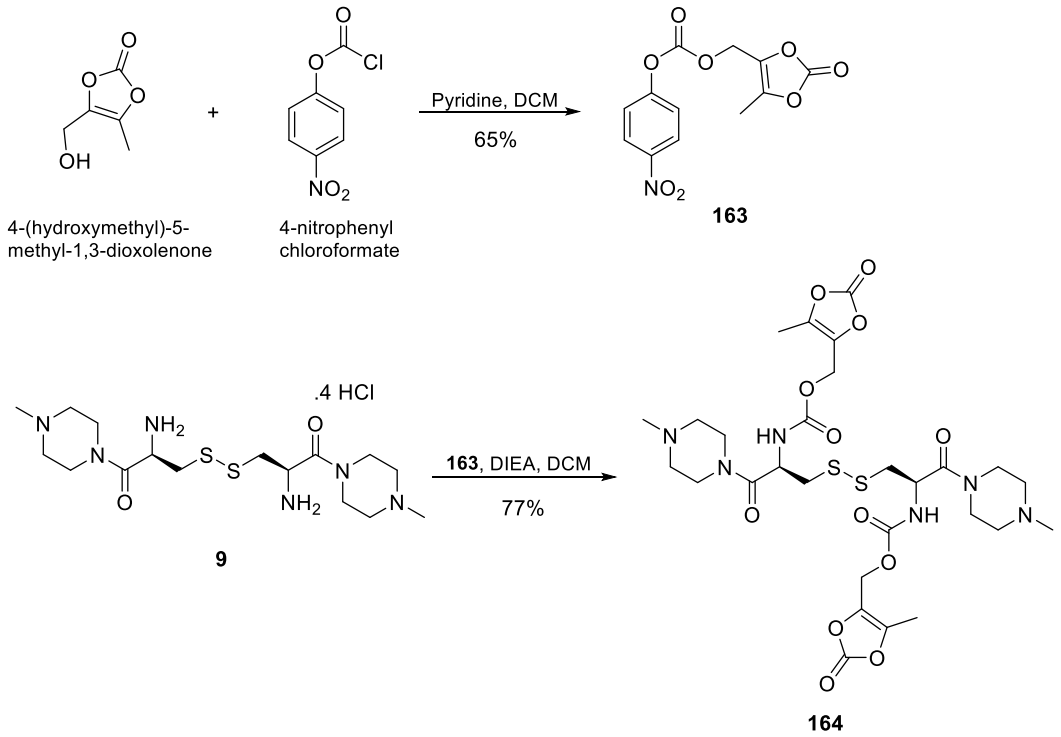
As shown in Scheme 30 prodrugs **161** and **162** were obtained in good yields by reacting **9** with the corresponding carbonate analogues **159** and **160** in presence of DIEA.[90, 91]



Scheme 30. Synthetic Route for Compounds **161** and **162**.

On the other hand 5-methyl-2-oxo-1,3-dioxol-4-yl)methoxycarbonyl analogue was synthesized starting from commercially available 4-(hydroxymethyl)-5-methyl-1,3-

dioxolenone and 4-nitrophenyl chloroformate in presence of pyridine which gave nitrophenolate **163** in 65% yield.[92] As shown in Scheme 31, compound **9** was then reacted with **163** in presence of DIEA to give prodrug **164** in 77% yield.[93, 94]



Scheme 31. Synthetic Route for Compound **164**.

3.4.1 Prodrugs Stability and Activation

For measuring the stability and activation of the prodrugs in buffer, serum or different liver microsomes we wanted to follow the formation of the final activated product (**9**) and monodeprotected amine. These molecules are very polar especially compound **9** which makes their analysis a real challenge. Different chromatographic systems were tried to retain the polar molecules and even HILIC separation failed to retain these molecules.[95-97] Size exclusion chromatography on the other hand was found to retain all components with good separation time.[98]

3.4.1.1 Prodrug Stability in 50 mM Phosphate Buffer

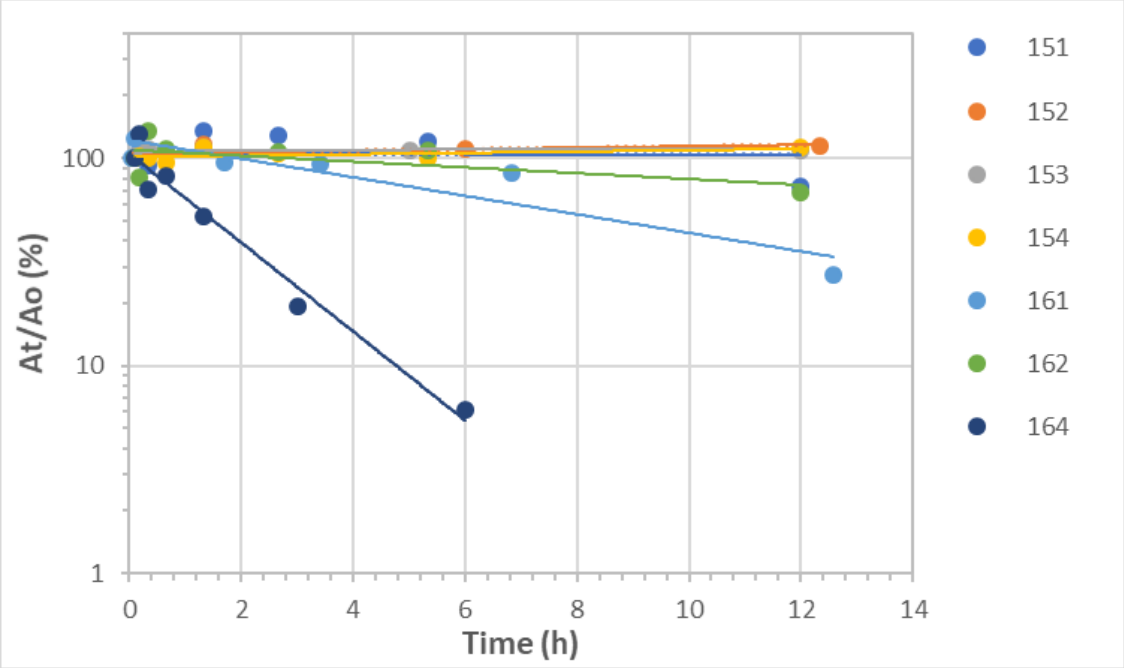


Figure 26. Prodrugs **151-154**, **161**, **162** and **164** Chemical Stability in 50 mM Phosphate Buffer Containing 1 mM EDTA at pH 7.4.

The stability of the different prodrugs was evaluated in 50 mM phosphate buffer containing 1 mM EDTA at pH 7.4, human liver microsomes and mouse liver microsomes. A 0.2 mM test compounds in 0.5 mg protein/mL of microsomes were used and the presence of the prodrugs, their mono activated intermediates and fully activated analogue (9) were determined using selective ion monitoring on LC/MS for higher sensitivity. The alkyl chloroformate prodrugs **151-154** were totally stable for more than two days in the buffer system. The acetoxy methyl carbamate analogue **161** afforded 14.1 h half-life while the isobutanoyloxy methyl analogue **162** had a half-life of 22.4 h in buffer. Prodrug **164** decomposed most rapidly with a half-life value of 1.4 h. Figure 26 illustrates the plotted ionization intensity of prodrugs **151-154**, **161**, **162** and **164** versus time in hour.

3.4.1.2 Prodrug Activation in 0.5 mg protein/mL of Mouse Liver Microsomes

Activation of 0.2 mM of prodrugs were evaluated in 0.5 mg protein/mL of mouse liver microsomes in 50 mM phosphate buffer containing 1 mM EDTA at pH 7.4. The alkyl chloroformate prodrugs **151-154** were also stable for more than two days with mouse liver microsomes. The acetoxy methyl carbamate analogue **161** afforded 0.35 h half-life while the isobutanoyloxy methyl carbamate analogue **162** had a half-life of less than 5 m in presence of mouse liver microsomes. Prodrug **164** on the other hand obtained a half-life value of 1.1 h with close rate of decomposition compared to the buffer. Figure 27 illustrates the plotted ionization intensity of prodrugs **151-154**, **161**, **162** and **164** versus time in hour.

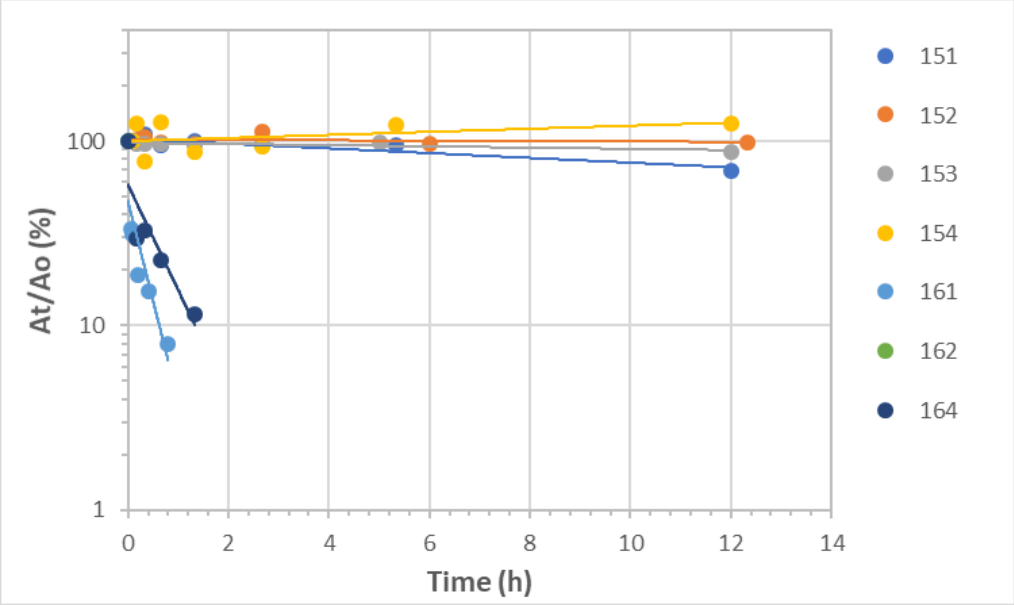


Figure 27. Prodrugs **151-154**, **161**, **162** and **164** Activation in 50 mM Phosphate Buffer Containing 1 mM EDTA at pH 7.4 in Presence of Mouse Liver Microsomes.

3.4.1.3 Prodrug Activation in 0.5 mg protein/mL of Human Liver Microsomes

Activation of 0.2 mM of prodrugs was also evaluated in 0.5 mg protein/mL of human liver microsomes in 50 mM phosphate buffer containing 1 mM EDTA at pH 7.4. The alkyl chloroformate prodrugs **151-154** again were totally stable for more than two days in presence of human liver microsomes. The acetoxy methyl carbamate analogue **161** afforded 0.23 h half-life while the isobutanoyloxy methyl carbamate analogue **162** had a half-life of less than 5 m in presence of human liver microsomes. The (Oxodioxolenyl)methyl carbamate analogue **164** on the other hand obtained a half-life value of 0.63 h. Figure 28 illustrates the plotted ionization intensity of prodrugs **151-154**, **161**, **162** and **164** versus time in hour.

3.4.1.4 Prodrug Stability in 50% Phosphate Buffer

We wanted to test the activation of the synthesized prodrugs in plasma as well. 0.2 mM of each prodrug in 50% plasma or water, for comparison, using 50 mM phosphate buffer containing 1 mM EDTA at pH 7.4 was used. In the diluted buffer the alkyl chloroformate prodrugs **151-154** were totally stable for more than two days as seen before. The acetoxy methyl carbamate analogue **161** afforded 11 h half-life while the isobutanoyloxy methyl carbamate analogue **162** had a half-life of 16.5 h. The (Oxodioxolenyl)methyl carbamate analogue **164** on the other hand obtained a half-life value of 1.3 h. Figure 29 illustrates the plotted ionization intensity of prodrugs **151-154**, **161**, **162** and **164** versus time in hour.

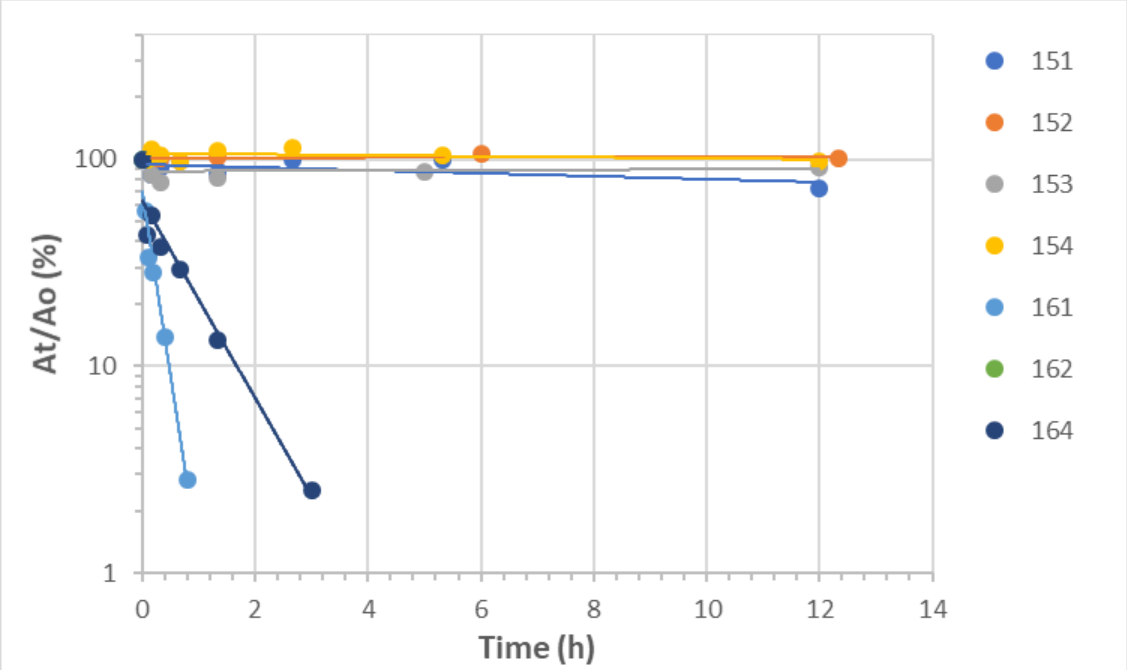


Figure 28. Prodrugs **151-154**, **161**, **162** and **164** Activation in 50 mM Phosphate Buffer Containing 1 mM EDTA at pH 7.4 in Presence of Human Liver Microsomes.

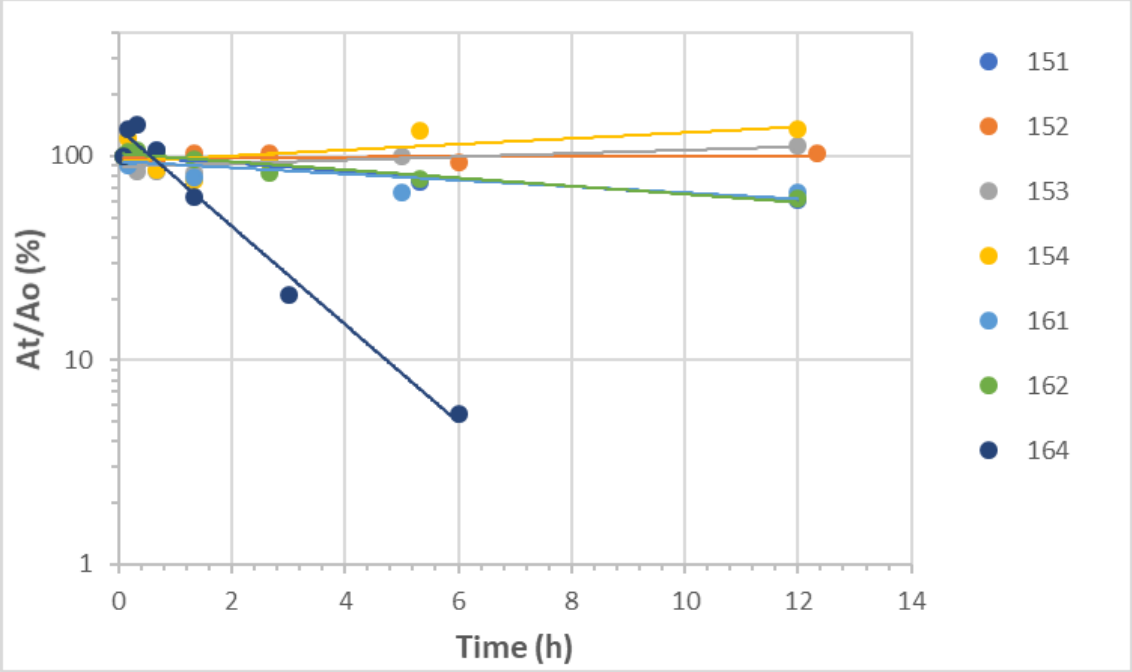


Figure 29. Prodrugs **151-154**, **161**, **162** and **164** Chemical Stability in 50% Phosphate Buffer.

3.4.1.5 Prodrug Activation in 50% Plasma

The activation of the synthesized prodrugs in 50% plasma using 0.2 mM concentration of each prodrug and 50 mM phosphate buffer containing 1 mM EDTA at pH 7.4 was done. The alkyl chloroformate prodrugs **151-154** were totally stable for more than two days in plasma as well. The acetoxy methyl carbamate analogue **161** afforded 1.1 h half-life while the isobutanoyloxy methyl carbamate analogue **162** had a half-life of about half an hour. Prodrug **164** on the other hand obtained a half-life value of less than 5 m in plasma. Figure 30 illustrates the plotted ionization intensity of prodrugs **151-154**, **161**, **162** and **164** versus time in hour.

Table 10 lists the stability and activation half-lives in hour of prodrugs **151-154**, **161**, **162** and **164.** The simple alkyl carbamate prodrugs **151-154** were stable for more than two days in buffer, serum as well as the different liver microsomes. Prodrugs **161** and **162** have a half-lives of 14.1 and 22.4 h in buffer, respectively, while prodrug **164** has a very low stability in buffer with a half-life of 1.41 h. In mouse and human liver microsomes prodrug **161** obtained a half-lives of 0.35 and 0.23 h, respectively. Prodrug **162** has a half-life of less than 5 minutes in both mouse and human liver microsomes. prodrug **164** obtained a half-lives of 1.1 and 0.63 h in mouse and human liver microsomes, respectively. In 50% serum prodrugs **161** and **162** have half-lives of 1.1 and 0.53 h, respectively, while prodrug **164** has a half-life of less than 5 minutes.

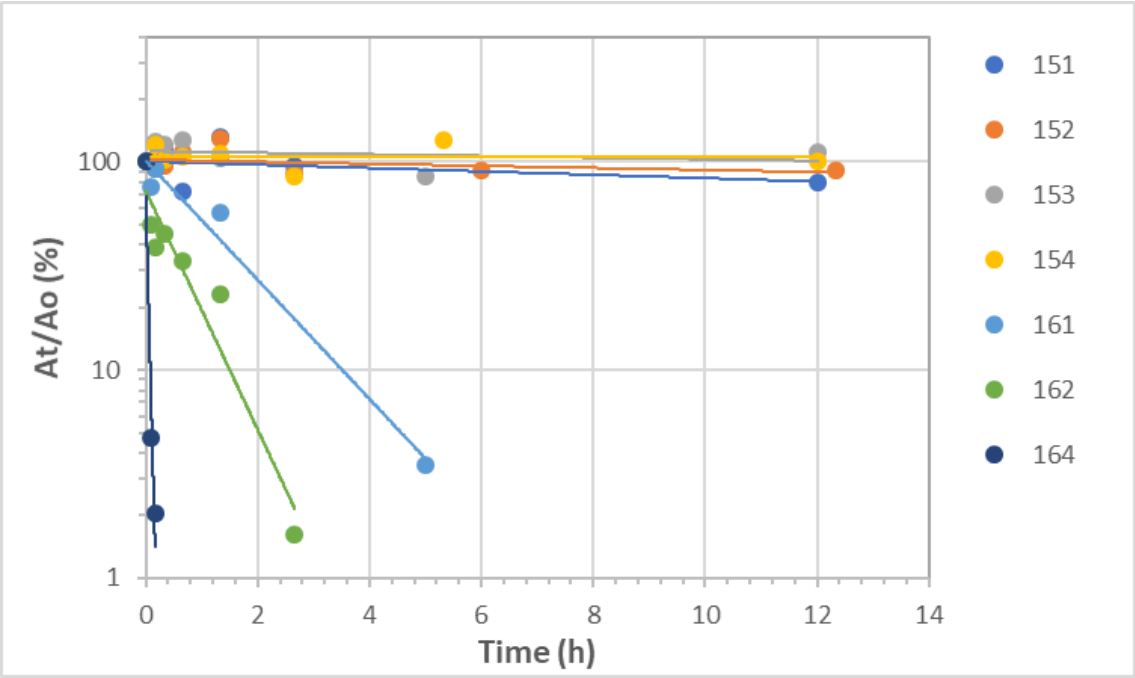
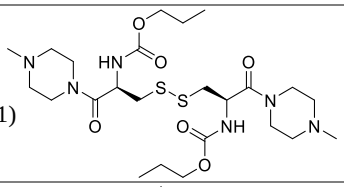
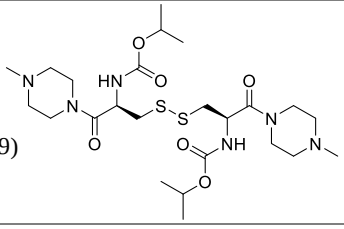
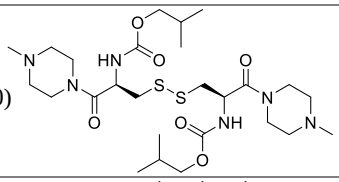
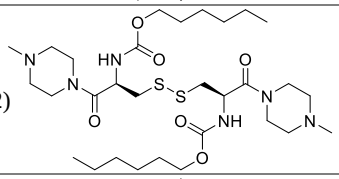
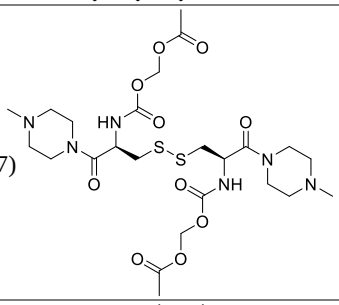
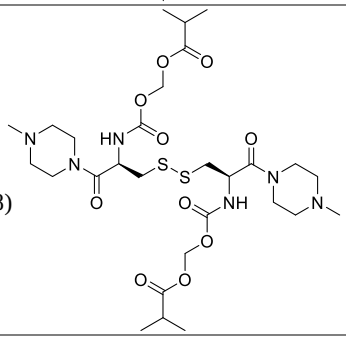
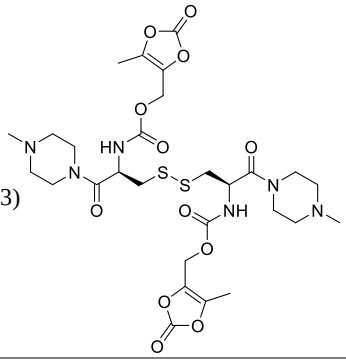


Figure 30. Prodrugs **151-154**, **161**, **162** and **164** Activation in 50% Plasma in Phosphate Buffer.

Table 10. Prodrugs **151-154**, **161**, **162** and **164** Stability and Activation Half-lives in Hour.

Structure	Buffer	Mouse Liver Microsomes	Human Liver Microsomes	50% Buffer	50% Plasma
151 (LH1761) 	>48	>48	>48	>48	>48
152 (LH1759) 	>48	>48	>48	>48	>48

153 (LH1760) 	>48	>48	>48	>48	>48
154 (LH1762) 	>48	>48	>48	>48	>48
161 (LH1757) 	14.1	0.35	0.23	11.6	1.1
162 (LH1758) 	22.4	<0.08	<0.08	16.5	0.53
164 (LH1763) 	1.41	1.1	0.63	1.3	<0.08

3.5 Conclusion

The L-cystine diamides were found to obtain higher crystallization inhibition ability with enhanced chemical as well as in vivo stability. The presence of a basic amine in the perturbing moieties, at both end of the molecules, was further confirmed to be very

important for the inhibition ability. Our research showed that in order to achieve binding to the L-cystine crystal surfaces, the L-cystine core should be maintained in the inhibitor's structure. Our research led to compound 8-L-cystinyl bis(1,8-diazaspiro[4.5]decane) (**56**) with an EC_{50} of 25.1 nM which is 1.7 fold more potent than LH708 (**9**) in our crystallization inhibition assay. The bioavailability of LH708 (**9**) was confirmed in urine and blood and prodrugs were synthesized and evaluated to solve the low bioavailability of the compound (18%). Out of the seven tested prodrugs, the *N*-isobutanoyloxy methyl carbamate analogue (**162**) was found to exhibit a good chemical stability while readily activated in liver microsomes.

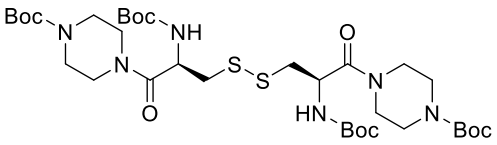
CHAPTER FOUR

EXPERIMENTAL SECTION

The reagents used in synthesis and assays were of ACS grade and were used without further purification. Solvents were either HPLC grade or ACS grade and were used without further purification. All moisture and air-sensitive reactions were carried out under argon or nitrogen atmosphere and reaction monitoring and follow-up were done using aluminum backed Silica G TLC plates with ultraviolet light visualization and Shimadzu 2010 LC-MS system or Agilent 1200 LC-MS system. Purification was done using Combi Flash Rf Teledyne ISCO flash chromatography system using ethyl acetate, dichloromethane, hexane, and methanol as a mobile phase, as indicated in each procedure, with prepacked silica gel columns as a stationary phase. VirTis freezemobile freeze dryer was used to lyophilized the final compounds. Proton ^{1}H (400 MHz) and carbon ^{13}C (100 MHz) were recorded using Bruker Avance III (400 MHz) Multinuclear NMR Spectrometer using deuterated solvents like: D_2O, $CDCl_3$, acetone-d_6, methanol-d_4, and DMSO-d_6. Compound **9** was recorded using NMR spectra at 100 °C, ^{1}H NMR spectra (500 MHz) and ^{13}C NMR spectra (125 MHz) were recorded on Bruker 500 MHz Multinuclear NMR spectrometers using DMSO-d6 as a solvent. Chemical shift data was expressed in parts per million (δ) relative to residual nondeuterated solvent signal and spin multiplicities were abbreviated as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet), b (broad). Coupling constants were reported in Hertz (Hz). High-resolution mass spectra (HRMS) data were conducted by the Center for Integrative Proteomics Research (CIPR) at Rutgers University.

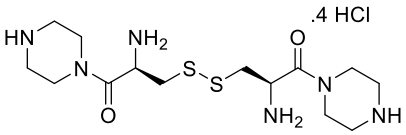
4.1 Synthesis

N,N'-Bis(*tert*-butoxycarbonyl)-*L*-cystine bispiperazide (23)



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then *tert*-butyl piperazine-1-carboxylate (90 mg, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (5% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 42%. The solvent was evaporated under reduced pressure to give a light yellow solid (74 mg, 60% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 18H), 1.47 (s, 18H), 3.01 (b, 4H), 3.40-3.67 (m, 16H), 4.93 (t, 2H, *J*= 8 Hz), 5.44 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.16, 154.99, 154.44, 80.39, 49.13, 45.75, 42.23, 41.52, 28.33. LC-MS (ESI$^+$) m/z 777.1 [M + H]$^+$.

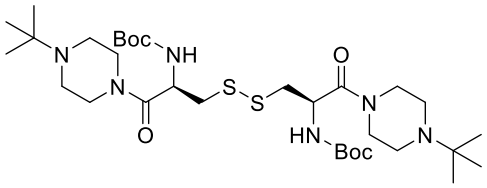
L-Cystine bispiperazide tetrahydrochloride (29)



Compound **26A** (12 mg, 0.015 mmol) was dissolved in 60 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (70 uL, 0.28 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room

temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (70 uL, 0.28 mmol) was added in two portions to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a yellowish white solid (6 mg, 85% yield). ^{1}H NMR (400 MHz, D_2O) δ 3.34−3.44 (m, 12H), 3.71-3.77 (m, 2H), 3.94-4.10 (m, 8H). ^{13}C NMR (100 MHz, D_2O) δ 166.60, 49.70, 42.71, 39.36, 37.88. LC-MS (ESI$^+$) m/z 377.2 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{14}H_{29}N_6O_2S_2^+$ [M + H]$^+$ 377.1788, found 377.1799.

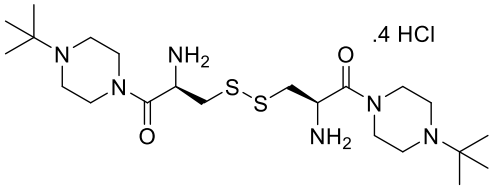
***N,N′*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(*N′*-tert-butylpiperazide) (18)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then 1-*tert*-butyl piperazine (77 uL, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 25%. The solvent was evaporated under reduced pressure to give a light

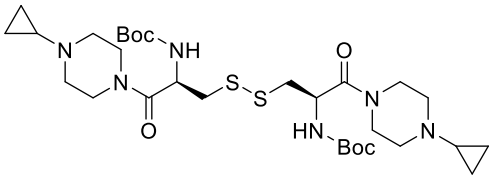
yellow solid (80 mg, 73% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.06 (s, 18H), 1.42 (s, 18H), 2.56 (b, 8H), 2.94-3.02 (m, 4H), 3.64 (b, 8H), 4.93 (b, 2H), 5.46 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.62, 155.04, 80.03, 49.05, 46.35, 45.51, 42.89, 41.84, 28.35, 25.84. LC-MS (ESI$^+$) m/z 689.22 [M + H]$^+$.

***L*-Cystine bis(*N'*-*tert*-butylpiperazide) tetrahydrochloride (24)**



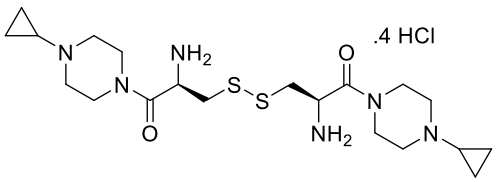
Compound **21A** (27 mg, 0.04 mmol) was dissolved in 120 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (116 uL, 0.46 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (58 uL, 0.23 mmol) was added to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a yellowish white solid (21 mg, 84% yield). ^{1}H NMR (400 MHz, D_2O) δ 1.46 (s, 18H), 3.21−3.44 (m, 12H), 3.76 (b, 8H), 4.29 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 166.33, 64.82, 49.74, 45.66, 42.94, 39.88, 37.55, 23.62. LC-MS (ESI$^+$) m/z 489.23 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{22}H_{45}N_6O_2S_2^+$ [M + H]$^+$ 489.3040, found 489.3075.

***N,N′*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(*N′*-cyclopropylpiperazide) (19)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then 1-cyclopropyl piperazine (58 uL, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the dark yellow residue was purified via ISCO (5% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 25%. The solvent was evaporated under reduced pressure to give a yellow solid (70 mg, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.40-0.46 (m, 8H), 1.41 (s, 18H), 1.60 (b, 2H), 2.57-2.62 (m, 8H), 2.96-3.03 (m, 4H), 3.56 (b, 8H), 4.93 (t, 2H, *J*= 8 Hz), 5.47 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.76, 155.02, 80.06, 53.10, 49.11, 45.84, 41.81, 38.20, 28.35, 5.93. LC-MS (ESI^+) m/z 657.09 $[M + H]^+$.

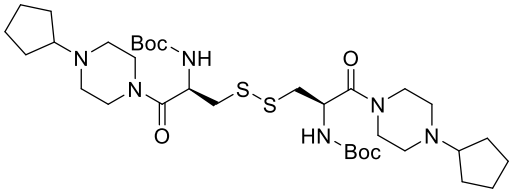
***L*-Cystine bis(*N′*-cyclopropylpiperazide) tetrahydrochloride (25)**



Compound **22A** (28 mg, 0.04 mmol) was dissolved in 120 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (192 uL, 0.77 mmol) in four portions was added to the chilled solution every half an hour and the solution was

warmed to room temperature after each addition. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a yellowish white solid (19 mg, 80%). ^{1}H NMR (400 MHz, D_2O) δ 1.03 (s, 8H), 2.91 (b, 2H), 3.30-3.86 (m, 20H), 4.69 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 166.51, 51.88, 49.81, 42.57, 39.47, 37.86, 3.64. LC-MS (ESI$^+$) m/z 457.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{20}H_{37}N_6O_2S_2^+$ [M + H]$^+$ 457.2414, found 457.2444.

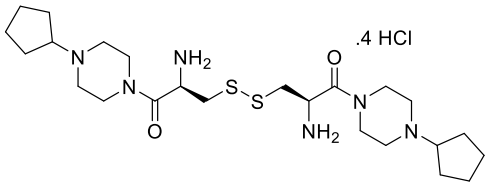
***N,N′*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(*N′*-cyclopentylpiperazide) (20)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then 1-cyclopentyl piperazine (75 uL, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 25%. The solvent was evaporated under reduced pressure to give a light yellow solid (85 mg, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 18H), 1.48-1.58 (m, 8H), 1.74 (b, 4H), 1.89 (b, 4H), 2.68 (b, 8H), 2.98 (b, 4H), 3.01-3.15 (m, 2H), 3.75 (b, 8H), 4.91 (b, 2H), 5.44 (d, 2H, *J*=

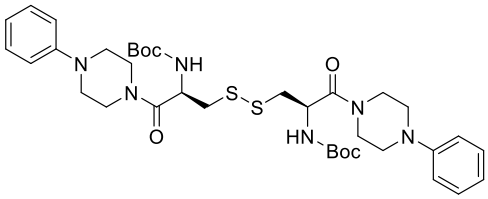
8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.80, 155.05, 80.26, 67.55, 52.12, 51.58, 48.99, 45.82, 41.49, 29.74, 28.34, 23.92. LC-MS (ESI$^+$) m/z 713.19 [M + H]$^+$.

_L_-Cystine bis(_N′_-cyclopentylpiperazide) tetrahydrochloride (26)



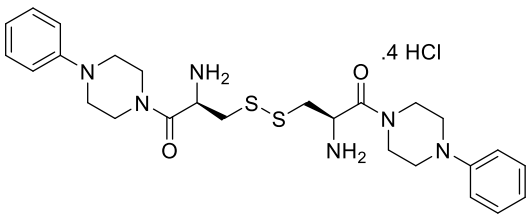
Compound **23A** (36 mg, 0.05 mmol) was dissolved in 150 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (150 uL, 0.6 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (75 uL, 0.3 mmol) was added to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a yellowish white solid (25 mg, 76% yield). ^{1}H NMR (400 MHz, D_2O) δ 1.64-1.83 (m, 14H), 2.18 (b, 4H), 3.16-3.43 (m, 10H), 3.60-3.82 (m, 10H), 4.24 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 166.23, 68.22, 50.17, 49.74, 42.77, 39.72, 27.84, 23.34. LC-MS (ESI$^+$) m/z 513.32 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{24}H_{45}N_6O_2S_2^+$ [M + H]$^+$ 513.3040, found 513.3069.

***N,N′*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(*N′*-phenylpiperazide) (21)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then 1-phenyl piperazine (74 uL, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the dark yellow residue was purified via ISCO (5% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 33%. The solvent was evaporated under reduced pressure to give a dark yellow solid (84 mg, 73% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 18H), 3.02 (b, 4H), 3.17 (b, 8H), 3.77 (b, 8H), 5.00 (b, 2H), 5.48 (d, 2H, *J*= 8 Hz), 6.89 (d, 6H, *J*= 8 Hz), 7.23-7.27 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.97, 155.04, 150.77, 129.26, 120.59, 116.64, 80.24, 55.87, 49.82, 45.83, 42.28, 41.69, 28.35. LC-MS (ESI$^+$) m/z 729.12 $[M + H]^+$.

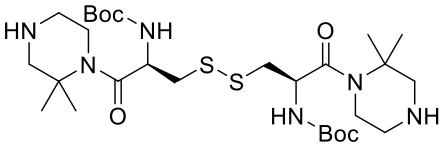
***L*-Cystine bis(*N′*-phenylpiperazide) tetrahydrochloride (27)**



Compound **24A** (28 mg, 0.04 mmol) was dissolved in 120 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (144 uL, 0.58 mmol) in four portions was added to the

chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (144 uL, 0.58 mmol) was added in two portions to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a yellowish white solid (23 mg, 88% yield). ^{1}H NMR (400 MHz, D_2O) δ 3.33-3.66 (m, 12H), 3.86-4.05 (m, 8H), 4.94 (t, 2H, *J*= 4 Hz), 7.34-7.52 (m, 10H). ^{13}C NMR (100 MHz, D_2O) δ 166.35, 144.72, 130.29, 127.08, 119.52, 52.25, 49.76, 44.17, 41.09, 37.93. LC-MS (ESI$^+$) m/z 529.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{26}H_{37}N_6O_2S_2^+$ [M + H]$^+$ 529.2414, found 529.2446.

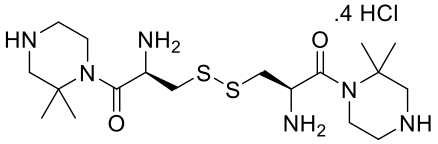
***N,N'*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(2',2'-dimethylpiperazide) (22)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then *tert*-butyl 2,2-dimethyl piperazine-1-carboxylate (103 mg, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (5% MeOH: DCM + 0.1% TEA/ DCM)

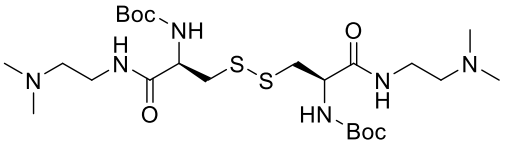
and the product was eluted at 45%. The solvent was evaporated under reduced pressure to give a colorless oil (80 mg, 60% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.36 (s, 12H), 1.41 (s, 18H), 1.46 (s, 18H), 2.99 (b, 4H), 3.48-3.88 (m, 12H), 4.88 (b, 2H), 5.32 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.93, 155.11, 154.80, 80.31, 56.11, 54.05, 50.56, 49.34, 45.32, 43.96, 39.81, 28.35, 25.03. LC-MS (ESI$^+$) m/z 832.98 [M + H]$^+$.

***L*-Cystine bis(2',2'-dimethylpiperazide) tetrahydrochloride (28)**



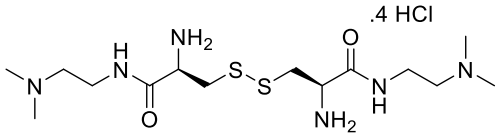
Compound **25A** (7 mg, 0.009 mmol) was dissolved in 50 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (50 uL, 0.2 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (25 uL, 0.1 mmol) was added to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a white solid (4 mg, 80% yield). ^{1}H NMR (400 MHz, D_2O) δ 1.41-1.50 (m, 12H), 3.34-3.50 (m, 10H), 3.65-4.02 (m, 6H), 4.28 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 167.11, 55.09, 52.51, 49.72, 42.30, 39.06, 38.03, 22.45, 20.65. LC-MS (ESI$^+$) m/z 433.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{18}H_{37}N_6O_2S_2^+$ [M + H]$^+$ 433.2414, found 433.2444.

***N,N'*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(*N',N'*-dimethylethyleneamide) (30)**



Boc-L-cystine-OH (200 mg, 0.45 mmol) was suspended in 6 mL of dry DCM. DIEA (488 uL, 2.8 mmol) was added and gave a clear solution. Then N,N-dimethylethylenediamine (151 uL, 1.38 mmol) and PyAOP (522 mg, 1 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (40 mL) of water and extracted with (3 x 80 mL) of DCM. The combined organic layers were washed with (30 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 70%. The solvent was evaporated under reduced pressure to give a yellowish white solid (250 mg, 95% yield). ^{1}H NMR (400 MHz, DMSO) δ 1.36 (s, 18H), 2.40 (s, 12H), 2.64 (t, 4H, *J*= 4 Hz), 2.83 (dd, 2H, *J*= 8, 12 Hz), 3.08 (dd, 2H, *J*= 4, 12 Hz), 3.24 (b, 4H), 4.14 (b, 2H), 7.05 (d, 2H, *J*= 8 Hz), 8.01 (b, 2H). ^{13}C NMR (100 MHz, DMSO) δ 170.44, 155.24, 78.36, 56.77, 53.64, 43.83, 40.54, 35.63, 28.12. LC-MS (ESI$^+$) m/z 581.2 [M + H]$^+$.

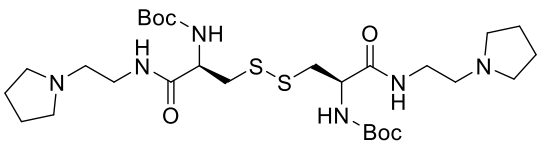
***L*-Cystine bis(*N',N'*-dimethylethyleneamide) tetrahydrochloride (38)**



Compound **27A** (241 mg, 0.42 mmol) was dissolved in 1 mL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (1.3 mL, 5.1 mmol) in

two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (1.3 mL, 5.1 mmol) was added to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then was filtrated with additional washes of diethyl ether. The residue was collected and dried to give a yellowish white solid (160 mg, 73% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.99 (s, 12H), 3.23-3.29 (m, 2H), 3.39 (t, 4H, *J*= 4 Hz), 3.45-3.50 (m, 2H), 3.58-3.68 (m, 2H), 3.77-3.83 (m, 2H), 4.44 (dd, 2H, *J*= 8, 12 Hz). ^{13}C NMR (100 MHz, D_2O) δ 168.79, 56.14, 52.03, 43.25, 37.14, 35.00. LC-MS (ESI$^+$) m/z 381.21 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{14}H_{33}N_6O_2S_2^+$ [M + H]$^+$ 381.2101, found 381.2125.

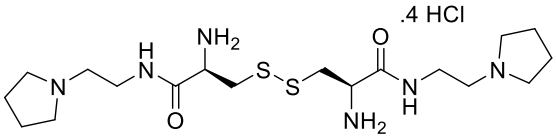
***N,N′*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(*N′*-pyrrolidinoethyleneamide) (31)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then N-(2-aminoethyl)pyrrolidine (61 uL, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (2 x 50 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (10% MeOH: DCM + 0.1% TEA/ DCM)

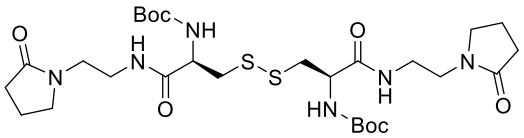
and the product was eluted at 60%. The solvent was evaporated under reduced pressure to give a white solid (82 mg, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26 (s, 18H), 1.68 (b, 8H), 2.60 (b, 12H), 2.86-3.10 (m, 4H), 3.30 (b, 4H), 4.47 (b, 2H), 5.65 (b, 2H), 7.64 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.50, 155.54, 79.86, 54.70, 54.24, 53.96, 44.24, 37.89, 28.28, 23.31. LC-MS (ESI$^+$) m/z 633.26 [M + H]$^+$.

_L_-Cystine bis(_N′_-pyrrolidinoethyleneamide) tetrahydrochloride (39)



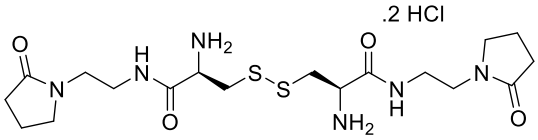
Compound **28A** (31 mg, 0.05 mmol) was dissolved in 150 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (330 uL, 1.3 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (330 uL, 1.3 mmol) was added to the solution in two portions at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The yellow residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated six times. The residue was dried to give a yellow solid (25 mg, 86%). ^{1}H NMR (400 MHz, D_2O) δ 2.01 (b, 4H), 2.16 (b, 4H), 3.15-3.25 (m, 6H), 3.45 (b, 6H), 3.57 (b, 2H), 3.73 (b, 6H), 4.41 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 168.70, 54.66, 53.34, 51.99, 37.07, 36.08, 22.68. LC-MS (ESI$^+$) m/z 433.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{18}H_{37}N_6O_2S_2^+$ [M + H]$^+$ 433.2414, found 433.2439.

***N*,*N′*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(*N′*-pyrrolidin-2-oneethyleneamide) (32)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then N-(2-aminoethyl)pyrrolidine (61 uL, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (2 x 50 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (10% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 60%. The solvent was evaporated under reduced pressure to give a white solid (82 mg, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 18H), 1.95-2.01 (m, 4H), 2.34 (t, 4H, *J*= 8 Hz), 2.98 (b, 4H), 3.34-3.52 (m, 12H), 4.60 (b, 2H), 5.58 (b, 2H), 7.94 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.86, 170.69, 155.61, 80.24, 54.52, 47.34, 44.77, 42.13, 37.08, 31.02, 28.38, 18.03. LC-MS (ESI$^+$) m/z 661.41 [M + H]$^+$.

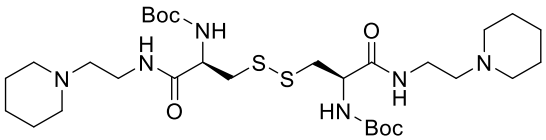
***L*-Cystine bis(*N′*-pyrrolidin-2-oneethyleneamide) dihydrochloride (40)**



Compound **29A** (90 mg, 0.13 mmol) was dissolved in 350 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (920 uL, 3.68 mmol) in four portions was added to the chilled solution every half an hour and the

solution was warmed to room temperature after each addition. The solvent was evaporated under reduced pressure and the residue was dried on pump. The yellow residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a yellowish white solid (67 mg, 99%). ^{1}H NMR (400 MHz, D_2O) δ 2.04 (b, 4H), 2.44 (b, 4H), 3.12-3.19 (m, 2H), 3.28-3.61 (m, 14H), 4.32 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 178.87, 167.90, 51.93, 47.91, 41.84, 37.22, 36.93, 31.13, 17.39. LC-MS (ESI$^+$) m/z 461.43 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{18}H_{33}N_6O_2S_2^+$ [M + H]$^+$ 461.2000, found 461.2028.

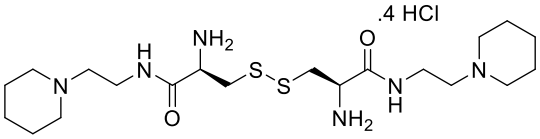
***N,N′*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(*N′*-piperidinoethyleneamide) (33)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then 1-(2-aminoethyl)piperidine (69 uL, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (2 x 50 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (5% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 80%. The solvent was evaporated under reduced pressure to give a yellowish white solid (95 mg, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.37 (s, 22H),

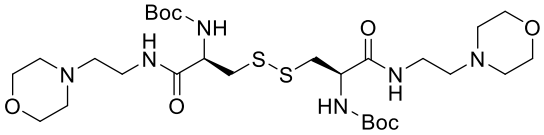
1.59 (b, 8H), 2.54 (b, 12H), 2.96-3.10 (m, 4H), 3.38 (b, 4H), 4.53 (b, 2H), 5.65 (d, 2H, *J*= 8 Hz), 7.65 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.42, 155.53, 79.99, 57.22, 55.24, 54.22, 43.89, 36.11, 28.34, 25.06, 23.62. LC-MS (ESI$^+$) m/z 661.33 [M + H]$^+$.

_L_-Cystine bis(_N′_-piperidinoethyleneamide) tetrahydrochloride (41)



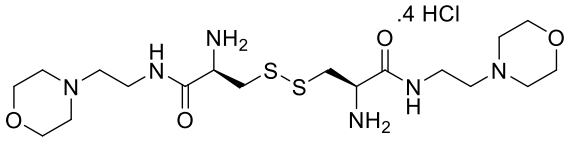
Compound **30A** (38 mg, 0.06 mmol) was dissolved in 150 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (404 uL, 1.62 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (404 uL, 1.62 mmol) was added to the solution in two portions at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated six times. The residue was dried to give a yellow solid (31 mg, 86%). ^{1}H NMR (400 MHz, D_2O) δ 1.15-1.19 (m, 2H), 1.46-1.53 (m, 6H), 1.96 (b, 4H), 3.00 (b, 4H), 3.19-3.25 (m, 2H), 3.32 (b, 4H), 3.41-3.47 (m, 2H), 3.55-3.64 (m, 6H), 3.71-377 (m, 2H), 4.41 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 168.62, 55.06, 53.64, 51.98, 37.08, 34.47, 22.76, 21.00. LC-MS (ESI$^+$) m/z 461.4 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{20}H_{41}N_6O_2S_2^+$ [M + H]$^+$ 461.2727, found 461.2755.

***N*,*N'*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(*N'*-morpholinoethyleneamide) (34)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then 4-(2-aminoethyl)morpholine (63 uL, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (2 x 50 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 35%. The solvent was evaporated under reduced pressure to give a white solid (85 mg, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 18H), 2.44-2.50 (m, 12H), 2.97-3.01 (m, 4H), 3.29-3.45 (m, 4H), 3.67 (t, 8H, *J*= 4 Hz), 4.66 (b, 2H), 5.63 (d, 2H, *J*= 12 Hz), 7.51 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.18, 155.73, 80.16, 66.91, 57.45, 54.49, 53.53, 45.29, 36.43, 28.42. LC-MS (ESI$^+$) m/z 665.3 $[M + H]^+$.

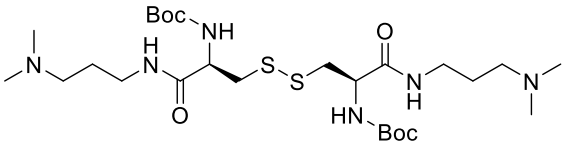
***L*-Cystine bis(*N'*-morpholinoethyleneamide) tetrahydrochloride (42)**



Compound **31A** (55 mg, 0.08 mmol) was dissolved in 300 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (376 uL, 1.5 mmol) in four portions was added to the chilled solution every half an hour and the

solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (188 uL, 0.74 mmol) was added to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 30 m. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated four times. The residue was dried to give a yellow solid (49 mg, 100%). ^{1}H NMR (400 MHz, D_2O) δ 3.20-3.28 (m, 6H), 3.43-3.50 (m, 6H), 3.60-3.68 (m, 6H), 3.77-3.89 (m, 6H), 4.13 (b, 4H), 4.42-4.45 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 168.70, 63.71, 55.57, 52.06, 51.95, 37.01, 34.11. LC-MS (ESI$^+$) m/z 465.37 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{18}H_{37}N_6O_4S_2^+$ [M + H]$^+$ 465.2313, found 465.2340.

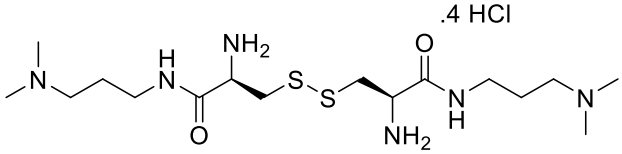
***N,N'*-Bis(*tert*-butoxycarbonyl)-*L*-cystine-bis(*N',N'*-dimethyl-1',3'-diaminopropane-amide) (35)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then 3-(dimethylamino)-1-propylamine (61 uL, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under

reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 75%. The solvent was evaporated under reduced pressure to give a light yellow solid (55 mg, 58% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 18H), 1.64-1.72 (m, 4H), 2.20 (s, 12H), 2.31 (t, 4H, *J*= 8 Hz), 2.94-3.02 (m, 4H), 3.25-3.33 (m, 4H), 4.69 (b, 2H), 5.61 (d, 2H, *J*= 8 Hz), 7.90 (b, 2H). ^{13}C NMR (100 MHz, DMSO) δ 170.07, 155.76, 80.08, 57.68, 54.51, 45.88, 45.41, 38.53, 28.41, 27.23. LC-MS (ESI^{+}) m/z 609.23 [M + H]$^{+}$.

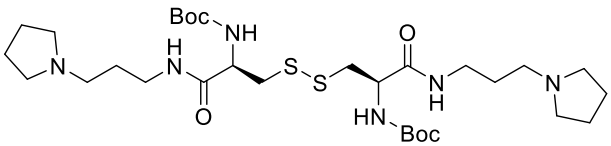
***L*-Cystine bis(*N'*,*N'*-dimethyl-1',3'-diaminopropaneamide) tetrahydrochloride (43)**



Compound **32A** (18 mg, 0.03 mmol) was dissolved in 100 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (90 uL, 0.35 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (90 uL, 0.35 mmol) was added in two portions to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a yellowish white solid (11 mg, 68% yield). ^{1}H NMR (400 MHz, D_2O) δ 1.99-2.03 (m, 4H), 2.91 (s, 12H), 3.19-3.28 (m, 6H), 3.35-3.43 (m, 6H), 4.36 (t, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, D_2O) δ 168.11, 55.34, 52.07, 42.87, 37.39, 36.68, 23.91. LC-MS (ESI^{+})

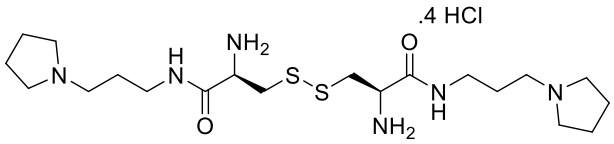
m/z 409.26 $[M + H]^+$. HRMS (ESI^+) m/z calculated for $C_{16}H_{37}N_6O_2S_2^+$ $[M + H]^+$ 409.2414, found 409.2438.

***N,N'*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(1'-pyrrolidine-3'-aminopropaneamide) (36)**



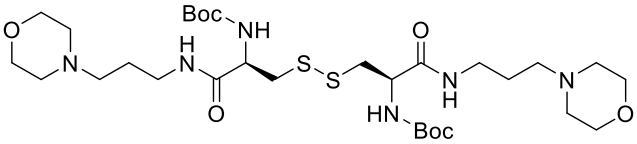
Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then 3-pyrrolidinopropylamine (66 uL, 0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (2 x 50 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 80%. The solvent was evaporated under reduced pressure to give a yellow solid (85 mg, 81%). ^1H NMR (400 MHz, $CDCl_3$) δ 1.31 (s, 18H), 2.02 (b, 12H), 2.93 (b, 4H), 3.18 (b, 4H), 3.30 (b, 4H), 3.60 (b, 4H), 4.40 (b, 2H), 5.78 (b, 2H), 8.35 (b, 2H). ^{13}C NMR (100 MHz, DMSO) δ 171.23, 155.33, 79.70, 54.14, 53.58, 52.94, 42.70, 36.55, 28.29, 25.59, 23.23. LC-MS (ESI^+) m/z 661.33 $[M + H]^+$.

***L*-Cystine bis(1'-pyrrolidine-3'-aminopropaneamide) tetrahydrochloride (44)**



Compound **33A** (39 mg, 0.06 mmol) was dissolved in 150 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (400 uL, 1.6 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated six times. The residue was dried to give a yellow solid (16 mg, 45%). ^{1}H NMR (400 MHz, D_2O) δ 2.02-2.09 (m, 8H), 2.16-2.24 (m, 4H), 3.11-3.17 (m, 4H), 3.27-3.33 (m, 6H), 3.36-3.46 (m, 6H), 3.70-3.76 (m, 4H), 4.40 (t, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, D_2O) δ 168.10, 54.34, 52.59, 52.16, 37.52, 36.89, 25.27, 22.73. LC-MS (ESI$^+$) m/z 461.46 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{20}H_{41}N_6O_2S_2^+$ [M + H]$^+$ 461.2727, found 461.2757.

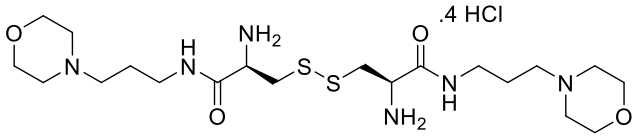
***N,N'*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(1'-morpholine-3'-aminopropaneamide) (37)**



Boc-L-cystine-OH (70 mg, 0.16 mmol) was suspended in 2 mL of dry DCM. DIEA (168 uL, 0.96 mmol) was added and gave a clear solution. Then 4-(2-aminoethyl)morpholine (63 uL,

0.48 mmol) and PyAOP (174 mg, 0.33 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (2 x 50 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 35%. The solvent was evaporated under reduced pressure to give a white solid (85 mg, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.30 (s, 18H), 1.60-1.68 (m, 4H), 2.40 (b, 12H), 2.88 (b, 4H), 3.15-3.20 (m, 4H), 3.61 (b, 8H), 4.49 (b, 2H), 5.60 (b, 2H), 7.76 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.27, 155.61, 79.99, 66.30, 56.48, 54.45, 53.46, 45.02, 38.09, 28.30, 25.56. LC-MS (ESI$^+$) m/z 693.32 $[M + H]^+$.

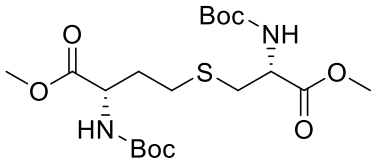
***L*-Cystine bis(1'-morpholine-3'-aminopropaneamide) tetrahydrochloride (45)**



Compound **34A** (60 mg, 0.09 mmol) was dissolved in 250 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (584 uL, 1.5 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. The solvent was evaporated under reduced pressure and the residue was dried on pump. The yellow residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated six times. The residue was dried to give a yellow solid (40 mg, 85%). ^{1}H NMR (400 MHz, D_2O) δ 2.06-2.12 (m, 4H), 3.27-3.33 (m,

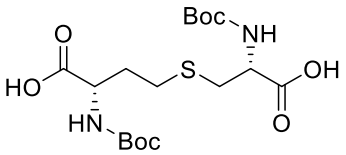
10H), 3.41-3.48 (m, 6H), 3.62 (b, 4H), 3.89 (t, 4H, *J*= 12 Hz), 4.20 (b, 4H), 4.42 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 168.16, 63.88, 54.89, 52.14, 51.91, 37.50, 36.82, 23.09. LC-MS (ESI$^+$) m/z 493.47 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{20}H_{41}N_6O_4S_2^+$ [M + H]$^+$ 493.2625, found 493.2662.

Methyl-*N*-(*tert*-butoxycarbonyl)-*S*-((*R*)-2-((*tert*-butoxycarbonyl)amino)-3-methoxy-3-oxo-propyl)-*L*-homocysteinate (65)



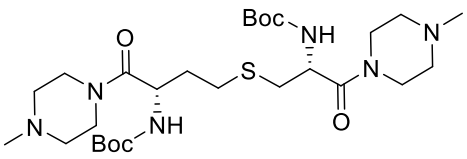
Compound **49** (255 mg, 0.7 mmol) and Compound **47** (209.4 mg, 0.84 mmol) were dissolved in 9 mL of degassed anhydrous DMF and cooled to 0 °C. NaH (28.6 mg, 1.19 mmol) and the reaction mixture was left to stir at 0 °C for 30 m then was brought to room temperature and left to stir under Argon for 4 h. The mixture was diluted with 10 mL of water. The organic layer was washed with (3 x 40 mL) of DCM. The combined organic layers were washed with (20 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the brown residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 25%. The solvent was evaporated under reduced pressure to give a yellow solid (150 mg, 48% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 18H), 1.88-2.12 (m, 2H), 2.54-2.58 (m, 2H), 2.94 (b, 2H), 3.75 (s, 6H), 4.38 (b, 1H), 4.51 (b, 1H), 5.13 (b, 1H), 5.36 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.62, 171.47, 155.12, 80.23, 53.23, 52.56, 52.44, 34.60, 32.60, 28.30. LC-MS (ESI$^+$) m/z 451.33 [M + H]$^+$.

***N*-(*tert*-Butoxycarbonyl)-*S*-((*R*)-2-((*tert*-butoxycarbonyl)amino)-2-carboxyethyl)-*L*-homo-cysteine (67)**



Compound **51** (60 mg, 0.13 mmol) was dissolved in 900 uL of THF and cooled to 0 °C. LiOH (7 mg, 0.28 mmol) was dissolved in 900 uL of water then was added to the chilled solution and the reaction mixture was left to stir at room temperature for 1 h. The reaction mixture was diluted with (5 mL) of water and was acidified by 0.5 N $KHSO_4$ to pH=2 then was extracted with (3 x 10 mL) of EtOAc. The combined organic layers were washed with (5 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to give a yellow solid (56 mg, 100% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 18H), 1.86-2.10 (m, 2H), 2.63 (b, 2H), 3.03 (b, 2H), 4.42 (b, 1H), 4.57 (b, 1H), 5.45 (b, 1H), 5.56 (b, 1H), 9.20 (b, 2H). ^{13}C NMR (100 MHz, DMSO) δ 173.74, 172.48, 155.30, 78.16, 53.83, 52.53, 32.67, 30.90, 28.15. LC-MS (ESI$^+$) m/z 423.17 $[M + H]^+$.

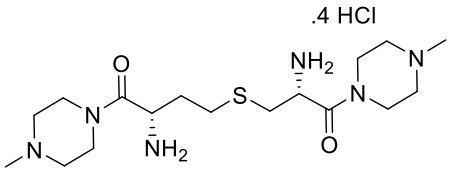
***tert*-Butyl ((*R*)-3-(((*S*)-3-((*tert*-butoxycarbonyl)amino)-4-(4-methylpiperazin-1-yl)-4-oxobutyl)-thio)-1-(4-methylpiperazin-1-yl)-1-oxopropan-2-yl)carbamate (69)**



To a solution of compound **53** (55 mg, 0.13 mmol) in 300 uL of anhydrous DMF, HATU (104 mg, 0.273 mmol) and DIEA (140 uL, 0.8 mmol) were added. Then 1-methyl piperazine (36 uL, 0.32 mmol) was added to the mixture and the reaction mixture was left to stir at room temperature for 3 h. The mixture was diluted with (20 mL) of EtOAc and washed with (3 x 10 mL) of water, (5 mL) of

brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 55%. The solvent was evaporated under reduced pressure to give a yellow oil (28 mg, 37% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 18H), 1.74-1.81 (m, 2H), 2.38 (s, 6H), 2.57 (b, 10H), 2.73-2.88 (m, 2H), 3.48 (b, 8H), 4.72 (b, 2H), 5.40 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.05, 155.55, 79.86, 54.88, 54.39, 49.50, 48.94, 45.61, 41.70, 35.28, 33.61, 28.34. LC-MS (ESI^+) m/z 587.18 $[M + H]^+$.

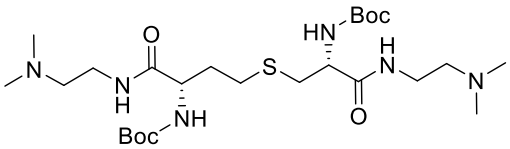
(*S*)-2-Amino-4-(((*R*)-2-amino-3-(4-methylpiperazin-1-yl)-3-oxopropyl)thio)-1-(4-methyl-piperazin-1-yl)butan-1-one tetrahydrochloride (73)



Compound **55** (23 mg, 0.04 mmol) was dissolved in 200 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (90 uL, 0.36 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (45 uL, 0.18 mmol) was added in two portions to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 5 h then in fridge for overnight. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a white solid (18 mg, 85% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.19 (b, 2H), 2.78 (b, 2H), 2.97 (s, 6H), 3.10-3.25 (m, 10H), 3.66 (b, 8H), 4.19 (b, 2H). ^{13}C NMR

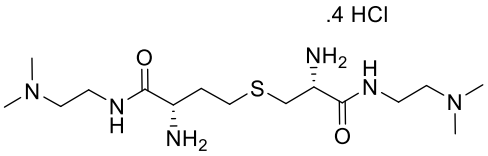
(100 MHz, D_2O) δ 167.77, 166.94, 52.69, 49.67, 43.11, 42.84, 39.65, 31.87, 29.80, 27.06. LC-MS (ESI^+) m/z 387.19 $[M + H]^+$. HRMS (ESI^+) m/z calculated for $C_{17}H_{35}N_6O_2S^+$ $[M + H]^+$ 387.2537, found 387.2562.

Di-*tert*-butyl ((7*R*,12*S*)-2,17-dimethyl-6,13-dioxo-9-thia-2,5,14,17-tetraazaoctadecane-7,12-di-yl)dicarbamate (71)



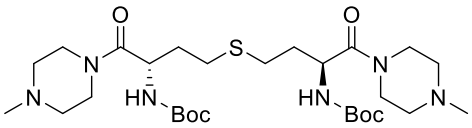
To a solution of compound **53** (250 mg, 0.59 mmol) in 2.5 mL of anhydrous DMF, HATU (718 mg, 1.89 mmol) and DIEA (924 uL, 5.31 mmol) were added. Then N,N-dimethylethylenediamine (216 uL, 2.1 mmol) was added to the mixture and the reaction mixture was left to stir at room temperature for 3 h. The mixture was diluted with (20 mL) of EtOAc and washed with (3 x 10 mL) of water, (5 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the light brown oil was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 80%. The solvent was evaporated under reduced pressure to give white crystals (189 mg, 57% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 18H), 1.93-2.03 (m, 2H), 2.34 (s, 12H), 2.56 (b, 4H), 2.67-2.95 (m, 2H), 3.02-3.08 (m, 2H), 3.40 (b, 4H), 4.26 (b, 2H), 5.61 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.84, 170.78, 155.64, 79.85, 57.67, 53.40, 50.34, 44.80, 36.71, 34.88, 34.20, 33.08, 28.45. LC-MS (ESI^+) m/z 563.19 $[M + H]^+$.

(*S*)-2-Amino-4-(((*R*)-2-amino-3-((2-(dimethylamino)ethyl)amino)-3-oxopropyl)thio)-*N*-(2-(di-methylamino)ethyl)butanamide tetrahydrochloride (75)



Compound **57** (165 mg, 0.3 mmol) was dissolved in 1.5 mL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (900 uL, 3.6 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated five times. The residue was dried to give a white solid (133 mg, 87% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.18-2.26 (m, 2H), 2.75 (t, 2H, *J*= 8 Hz), 2.97 (s, 12H), 3.05-3.22 (m, 2H), 3.38 (t, 4H, *J*= 8 Hz), 3.55-3.85 (m, 4H), 4.15 (t, 1H, *J*= 8 Hz), 4.24 (t, 1H, *J*= 8 Hz). ^{13}C NMR (100 MHz, D_2O) δ 170.78, 56.32, 51.88, 43.66, 34.54, 32.76, 29.89, 26.56. LC-MS (ESI$^+$) m/z 363.32 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{15}H_{35}N_6O_2S^+$ [M + H]$^+$ 363.2537, found 363.2567.

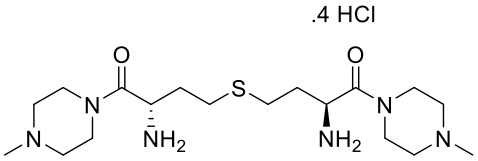
Di-*tert*-butyl ((2*S*,2′*S*)-thiobis(1-(4-methylpiperazin-1-yl)-1-oxobutane-4,2-diyl))di-carbamate (70)



To a solution of compound **54** (150 mg, 0.344 mmol) in 1 mL of anhydrous DMF, HATU (274 mg, 0.72 mmol) and DIEA (360 uL, 2.1 mmol) were added. Then 1-methyl piperazine

(96 uL, 0.86 mmol) was added to the mixture and the reaction mixture was left to stir at room temperature for 3 h. The mixture was diluted with (25 mL) of EtOAc and washed with (3 x 10 mL) of water, (5 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the light brown oil was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 65%. The solvent was evaporated under reduced pressure to give white crystals (155 mg, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 18H), 1.75-1.93 (m, 4H), 2.55 (b, 4H), 2.63 (s, 6H), 2.90 (b, 8H), 3.86 (b, 8H), 4.70 (b, 2H), 5.36 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.45, 155.56, 80.10, 54.12, 48.86, 45.93, 44.49, 43.78, 40.32, 33.28, 28.34. LC-MS (ESI$^+$) m/z 601.16 $[M + H]^+$.

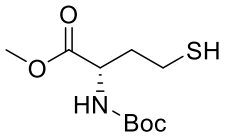
(2*S*,2′*S*)-4,4'-Thiobis(2-amino-1-(4-methylpiperazin-1-yl)butan-1-one) tetrahydro-chloride (74)



Compound **56** (23 mg, 0.04 mmol) was dissolved in 250 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (42 uL, 0.17 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still starting material and mono-deprotected product so additional 4 M HCl/ dioxane (42 uL, 0.17 mmol) was added in four portions to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 5 h then in fridge for overnight. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of

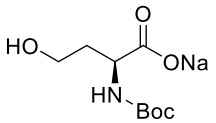
diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a white solid (6 mg, 29% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.18 (b, 4H), 2.72 (b, 4H), 2.96 (s, 6H), 3.14-3.26 (m, 8H), 3.64 (b, 8H), 4.19 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 167.90, 52.60, 49.70, 43.08, 42.56, 39.60, 29.74, 26.06. LC-MS (ESI$^+$) m/z 401.33 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{18}H_{37}N_6O_2S^+$ [M + H]$^+$ 401.2693, found 401.2722.

Methyl (*tert*-butoxycarbonyl)-*L*-homocysteinate (61)



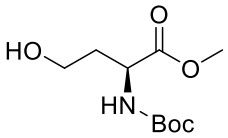
Compound **46** (838 mg, 1.69 mmol), triphenyl phosphine (499 mg, 1.9 mmol), sodium acetate (58 mg, 0.7 mmol) were suspended in a mixture of 8 mL of MeOH, 3.5 mL of water, and 58 uL of glacial acetic acid and heated to 60 °C for 75 m. The mixture was diluted with (80 mL) of EtOAc and washed with (2 x 40 mL) of water, (10 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the colorless oil was purified via ISCO (EtOAc/ hexane) and the product was eluted at 15%. The solvent was evaporated under reduced pressure to give a colorless oil (778 mg, 92% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 9H), 1.55 (t, 1H, *J*= 8 Hz), 1.84-2.08 (m, 2H), 2.55 (t, 2H, *J*= 8 Hz), 3.72 (s, 3H), 4.42 (b, 1H), 5.12 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.80, 155.39, 80.08, 52.42, 52.24, 37.15, 28.27, 20.70. LC-MS (ESI$^+$) m/z 250.21 [M + H]$^+$.

Sodium (*tert*-butoxycarbonyl)-*L*-homoserinate (62i)



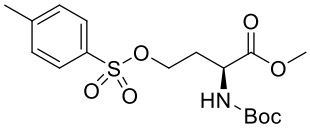
A flask was charged with L-homoserine (600 mg, 5.04 mmol) and 5 mL of water and 5 mL of ACN were added then the solution was cooled to 0 °C. NaOH (202 mg, 5.04 mmol) was added followed by Di-tert-butyl pyrocarbonate (1430 mg, 6.55 mmol) and the reaction mixture was brought to room temperature and left to stir for 1.5 h. The reaction mixture was concentrated as possible under reduced pressure and then the water was lyophilized to give a white foam (1121 mg, 92% yield). ^{1}H NMR (400 MHz, DMSO) δ 1.37 (s, 9H), 1.64-1.71 (m, 2H), 3.64 (b, 4H), 5.98 (b, 1H). ^{13}C NMR (100 MHz, DMSO) δ 173.64, 154.59, 79.14, 59.69, 54.58, 36.45, 28.18.

Methyl (*tert*-butoxycarbonyl)-*L*-homoserinate (62)



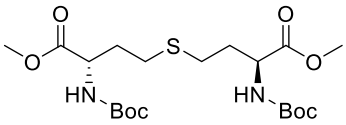
Compound **48i** (1121 mg, 4.65 mmol) was dissolved in 10 mL of anhydrous DMF. Iodomethane (350 uL, 5.6 mmol) was added at 0 °C then the reaction mixture was brought to room temperature and left to stir for 5 h. The reaction mixture was diluted with (150 mL) of EtOAc and washed with water (2 x 25 mL), sodium bicarbonate (2 x 25 mL), 0.5 N $KHSO_4$ (25 mL), and brine (15 mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to give a colorless oil (487 mg, 45% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33 (s, 9H), 1.59-1.65 (m, 1H), 1.99 (b, 1H), 3.42 (b, 1H), 3.59 (b, 2H), 3.64 (s, 3H), 4.34 (b, 1H), 5.51 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.26, 156.19, 80.12, 58.29, 52.30, 50.83, 35.44, 28.17. LC-MS (ESI$^+$) m/z 234.72 $[M + H]^+$.

Methyl *N*-(*tert*-butoxycarbonyl)-*O*-tosyl-*L*-homoserinate (64)



Compound **48** (487 mg, 2.09 mmol) was dissolved in 12 mL of dry DCM and cooled to 0 °C. Pyridine (337 uL, 4.18 mmol) was added to the chilled solution followed by p-toluene sulfonylchloride (1196 mg, 6.27 mmol) and the reaction mixture was left to stir at 0 °C for 15 m then was warmed to room temperature and left to stir for 1 h. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 30%. The solvent was evaporated under reduced pressure to give a colorless oil (561 mg, 69% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 9H), 2.08-2.24 (m, 2H), 2.43 (s, 3H), 3.70 (s, 3H), 4.07 (b, 2H), 4.31 (b, 1H), 5.12 (b, 1H), 7.34 (d, 2H, *J*= 8 Hz), 7.77 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.01, 155.53, 144.91, 132.72, 129.86, 128.00, 80.15, 66.24, 52.56, 50.49, 31.60, 28.23, 21.63. LC-MS (ESI^+) m/z 388.57 $[M + H]^+$.

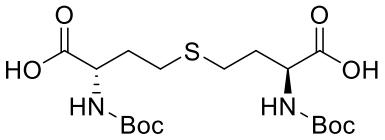
Dimethyl 4,4'-thio(2*S*,2′*S*)-bis(2-((*tert*-butoxycarbonyl)amino)butanoate) (66)



Compound **50** (550 mg, 1.42 mmol) was dissolved in 10 mL of degassed anhydrous DMF and cooled to 0 °C. Compound **47** (354 mg, 1.42 mmol) was added to the chilled solution followed by NaH (58 mg, 2.4 mmol) and the reaction mixture was left to stir at 0 °C for 30 m then was brought to room temperature and left to stir under Argon for 3 h. The mixture was quenched with (2 mL) of water and diluted with (150 mL) of EtOAc. The organic layer was washed with (2 x 50 mL) of water, (20 mL) of brine and

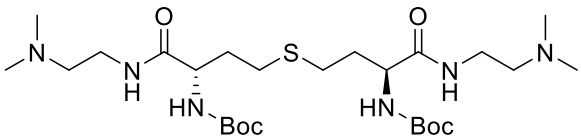
dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the brown residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 30%. The solvent was evaporated under reduced pressure to give a light yellow oil (443 mg, 67% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 18H), 1.84-1.91 (m, 2H), 2.03 (b, 2H), 2.52 (t, 4H, *J*= 8 Hz), 3.71 (s, 6H), 4.36 (b, 2H), 5.16 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.68, 155.33, 80.03, 60.33, 52.45, 52.36, 32.56, 28.27. LC-MS (ESI$^+$) m/z 465.29 [M + H]$^+$.

(2*S*,2′*S*)-4,4'-Thiobis(2-((*tert*-butoxycarbonyl)amino)butanoic acid) (68)



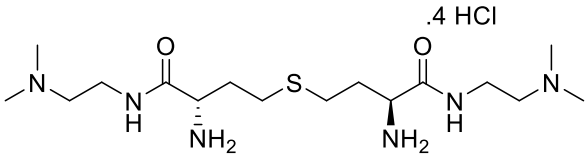
Compound **52** (443 mg, 0.95 mmol) was dissolved in 6 mL of THF and cooled to 0 °C. LiOH (48 mg, 2 mmol) was dissolved in 6 mL of water then was added to the chilled solution and the reaction mixture was left to stir at room temperature for 2 h. The reaction mixture was diluted with (20 mL) of water and washed with (3 x 20 mL) of DCM. Then the aqueous layer was acidified by 0.5 N $KHSO_4$ to pH=2 then was extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with (10 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to give a pale yellow solid (361 mg, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 18H), 2.11 (b, 4H), 2.45-2.69 (m, 4H), 4.31 (b, 2H), 5.43 (b, 1H), 7.59 (b, 1H), 10.81 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.21, 157.13, 81.95, 54.09, 32.66, 32.20, 28.32. LC-MS (ESI$^+$) m/z 437.13 [M + H]$^+$.

Di-*tert*-butyl-((7*S*,13*S*)-2,18-dimethyl-6,14-dioxo-10-thia-2,5,15,18-tetraazanona-decane-7,13-diyl)dicarbamate (72)



To a solution of compound **54** (361 mg, 0.83 mmol) in 1.5 mL of anhydrous DMF, HATU (663 mg, 1.74 mmol) and DIEA (868 uL, 4.98 mmol) were added. The reaction mixture was left to stir at room temperature for 15 m. N,N-dimethylethylenediamine (271 uL, 2.48 mmol) was added to the mixture and the reaction mixture was left to stir at room temperature for 2 h. The mixture was diluted with (120 mL) of EtOAc and washed with (2 x 20 mL) of water, (20 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the dark yellow oil was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 65%. The solvent was evaporated under reduced pressure to give a light yellow oil (208 mg, 44% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 18H), 1.84-1.89 (m, 2H), 1.99-2.03 (m, 2H), 2.20 (s, 12H), 2.40 (t, 4H, *J*= 8 Hz), 2.53 (b, 4H), 3.29 (b, 4H), 4.22 (b, 2H), 5.46 (d, 2H, *J*= 4 Hz), 6.87 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.48, 155.53, 79.88, 57.76, 53.48, 45.95, 45.10, 36.90, 32.66, 28.33, 27.80. LC-MS (ESI$^+$) m/z 577.24 [M + H]$^+$.

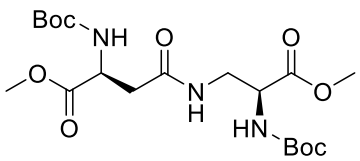
(2*S*,2'*S*)-4,4'-Thiobis(2-amino-*N*-(2-(dimethylamino)ethyl)butanamide) tetrahydro-chloride (76)



Compound **58** (190 mg, 0.33 mmol) was dissolved in 400 uL of MeOH and

cooled to 0 °C. 4 M HCl/ dioxane (989 uL, 3.96 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a white solid (104 mg, 84% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.23-230 (m, 4H), 2.77 (t, 4H, *J*= 8 Hz), 3.02 (s, 12H), 3.40-3.45 (m, 4H), 3.61-3.66 (m, 2H), 3.81-3.89 (m, 2H), 4.21-4.24 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 170.15, 56.33, 52.67, 43.44, 34.98, 30.39, 26.29. LC-MS (ESI$^+$) m/z 377.33 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{16}H_{37}N_6O_2S^+$ [M + H]$^+$ 377.2693, found 377.2724.

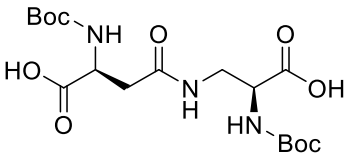
Methyl-*N*2-(*tert*-butoxycarbonyl)-*N*4-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methoxy-3-oxo-propyl)-*L*-asparaginate (79)



To a solution of Boc-Asp-OMe (866 mg, 3.5 mmol) in 3 mL of anhydrous DMF, HATU (1472 mg, 3.87 mmol) and DIEA (1.9 mL, 10.62 mmol) were added. The reaction mixture was left to stir at room temperature for 10 m. Compound **64** (769 mg, 3.5 mmol) was dissolved in 2 mL of anhydrous DMF and then was added to the mixture and the reaction mixture was left to stir at room temperature for 2 h. The mixture was diluted with (100 mL) of EtOAc and washed with (3 x 50 mL) of water, (5 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at

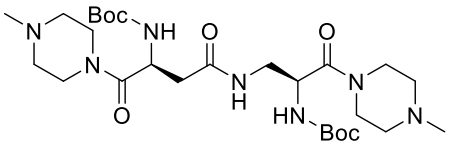
40%. The solvent was evaporated under reduced pressure to give a colorless oil (630 mg, 41% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 18H), 2.60 (b, 1H), 3.02-3.09 (m, 1H), 3.75 (s, 6H), 3.85-3.93 (m, 2H), 4.33 (b, 1H), 4.60 (b, 1H), 5.45 (b, 2H), 7.22 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.83, 174.50, 170.11, 155.64, 80.34, 52.87, 51.73, 49.77, 40.77, 36.45, 28.25. LC-MS (ESI$^+$) m/z 448.18 [M + H]$^+$.

***N*2-(*tert*-Butoxycarbonyl)-*N*4-((*S*)-2-((*tert*-butoxycarbonyl)amino)-2-carboxyethyl)-*L*-asparagine (80)**



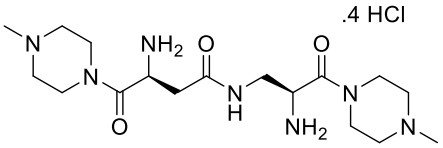
Compound **65** (600 mg, 1.34 mmol) was dissolved in 9 mL of THF and cooled to 0 °C. LiOH (81 mg, 3.35 mmol) was dissolved in 9 mL of water then was added to the chilled solution and the reaction mixture was left to stir at room temperature for 1 h. The reaction mixture was diluted with (60 mL) of water and washed with (3 x 20 mL) of DCM. Then the aqueous layer was acidified by 0.5 N $KHSO_4$ to pH=2 then was extracted with (3 x 120 mL) of EtOAc. The combined organic layers were washed with (20 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to give a pale yellow solid (439 mg, 79% yield). ^{1}H NMR (400 MHz, DMSO) δ 1.37 (s, 18H), 2.45-2.49 (m, 2H), 3.21-3.58 (m, 2H), 4.00 (b, 1H), 4.22 (b, 1H), 6.80 (b, 1H), 6.90 (b, 1H), 7.92 (b, 1H), 12.46 (b, 2H). ^{13}C NMR (100 MHz, DMSO) δ 173.15, 172.12, 169.78, 155.31, 78.23, 53.36, 50.12, 48.56, 36.85, 28.13. LC-MS (ESI$^+$) m/z 420.42 [M + H]$^+$.

tert-Butyl ((_S_)-3-((_S_)-3-((_tert_-butoxycarbonyl)amino)-4-(4-methylpiperazin-1-yl)-4-oxobutan-amido)-1-(4-methylpiperazin-1-yl)-1-oxopropan-2-yl)carbamate (85)



Compound **66** (206 mg, 0.5 mmol) was suspended in 6 mL of dry DCM. DIEA (523 uL, 3 mmol) was added and gave a clear solution. Then 1-methyl piperazine (164 uL, 1.5 mmol) and PyAOP (548 mg, 1 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 1.5 h. The mixture was diluted with (40 mL) of water and extracted with (7 x 100 mL) of DCM. The combined organic layers were washed with (20 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 60%. The solvent was evaporated under reduced pressure to give a pale yellow solid (160 mg, 55% yield). ^{1}H NMR (400 MHz, DMSO) δ 1.35 (s, 18H), 2.18 (s, 6H), 2.26 (b, 10H), 3.02-3.52 (m, 10H), 4.48 (b, 1H), 4.69 (b, 1H), 6.84 (b, 1H), 7.03 (b, 1H), 7.88 (b, 1H). ^{13}C NMR (100 MHz, DMSO) δ 169.88, 169.17, 168.32, 155.02, 79.14, 54.63, 54.15, 49.61, 46.93, 45.47, 44.54, 41.36, 37.58, 28.12. LC-MS (ESI$^+$) m/z 584.22 [M + H]$^+$.

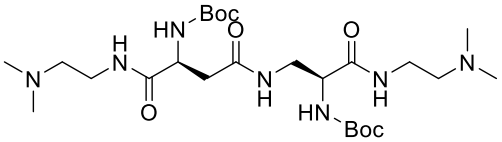
(_S_)-3-Amino-_N_-((_S_)-2-amino-3-(4-methylpiperazin-1-yl)-3-oxopropyl)-4-(4-methylpiperazin-1-yl)-4-oxobutanamide tetrahydrochloride (86)



Compound 71 (140 mg, 0.24 mmol) was dissolved in 550 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (725 uL, 2.9 mmol) in four portions was

added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (363 uL, 1.45 mmol) was added in two portions to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated five times. The residue was dried to give a yellowish white solid (125 mg, 98% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.97 (s, 6H), 3.19-3.43 (m, 8H), 3.66 (b, 12H), 4.30 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 172.97, 166.07, 52.57, 51.10, 50.07, 43.30, 42.52, 40.86, 39.57, 35.12. LC-MS (ESI$^+$) m/z 384.29 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{17}H_{34}N_7O_3^+$ [M + H]$^+$ 384.2718, found 384.2755.

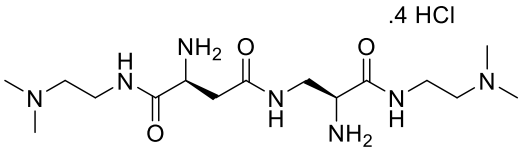
Di-*tert*-butyl ((7*S*,12*S*)-2,17-dimethyl-6,10,13-trioxo-2,5,9,14,17-pentaazaoctadecane-7,12-di-yl)dicarbamate (87)



Compound **66** (208 mg, 0.5 mmol) was suspended in 6 mL of dry DCM. DIEA (523 uL, 3 mmol) was added and gave a clear solution. Then N,N-dimethylethylenediamine (164 uL, 1.5 mmol) and PyAOP (548 mg, 1 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 45 m. The mixture was diluted with (40 mL) of water and extracted with (12 x 80 mL) of DCM. The combined organic layers were washed with (20 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the

yellow residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 75%. The solvent was evaporated under reduced pressure to give a pale yellow solid (156 mg, 56% yield). ^{1}H NMR (400 MHz, DMSO) δ 1.36 (s, 18H), 2.23 (b, 2H), 2.81 (b, 4H), 3.11 (b, 16H), 3.71 (b, 1H), 3.99 (b, 1H), 6.55 (b, 2H), 8.03 (b, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.84, 170.79, 155.10, 79.19, 55.72, 54.17, 51.45, 45.34, 42.36, 37.53, 34.12, 28.12. LC-MS (ESI$^+$) m/z 560.29 [M + H]$^+$.

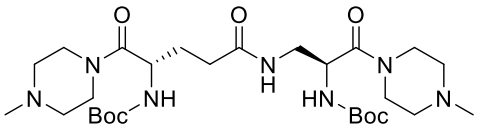
(*S*)-2-Amino-*N*4-((*S*)-2-amino-3-((2-(dimethylamino)ethyl)amino)-3-oxopropyl)-*N*1-(2-(di-methylamino)ethyl)succinamide tetrahydrochloride (88)



Compound **73** (130 mg, 0.23 mmol) was dissolved in 550 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (700 uL, 2.8 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (175 uL, 0.7 mmol) was added to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a yellowish white solid (75 mg, 65% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.97 (s, 6H), 3.19-3.43 (m, 8H), 3.66 (b, 12H), 4.30 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 171.55, 169.45, 168.47, 56.27, 53.13,

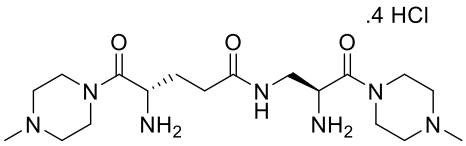
49.95, 43.24, 39.85, 35.25, 34.87. LC-MS (ESI$^+$) m/z 360.29 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{15}H_{34}N_7O_3^+$ [M + H]$^+$ 360.2718, found 360.2748.

***tert*-Butyl ((*S*)-5-(((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(4-methylpiperazin-1-yl)-3-oxopropyl)-amino)-1-(4-methylpiperazin-1-yl)-1,5-dioxopentan-2-yl)carbamate (89)**



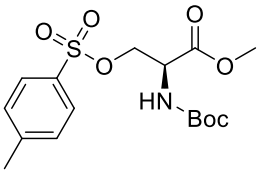
Compound **68** (50 mg, 0.12 mmol) was suspended in 1.5 mL of dry DCM. DIEA (126 uL, 0.72 mmol) was added and gave a clear solution. Then 1-methyl piperazine (40 uL, 0.36 mmol) and PyAOP (132 mg, 0.25 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 m. The mixture was diluted with (10 mL) of water and extracted with (3 x 20 mL) of DCM. The combined organic layers were washed with (5 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 60%. The solvent was evaporated under reduced pressure to give a pale yellow solid (69 mg, 97% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 18H), 1.64-1.93 (m, 2H), 2.23 (b, 2H), 2.53 (d, 6H, *J*= 12 Hz), 2.84 (b, 8H), 3.37-3.59 (m, 2H), 3.83 (b, 8H), 4.66 (b, 2H), 5.62 (b, 1H), 6.02 (b, 1H), 6.94 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.92, 170.21, 168.34, 155.61, 79.90, 54.47, 50.13, 49.36, 45.14, 44.45, 41.12, 31.91, 29.78, 28.32. LC-MS (ESI$^+$) m/z 598.31 [M + H]$^+$.

(*S*)-4-Amino-*N*-((*S*)-2-amino-3-(4-methylpiperazin-1-yl)-3-oxopropyl)-5-(4-methylpiperazin-1-yl)-5-oxopentanamide tetrahydrochloride (90)



Compound **75** (16 mg, 0.027 mmol) was dissolved in 40 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (80 uL, 0.32 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated five times. The residue was dried to give a yellowish white solid (6 mg, 55% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.97 (s, 6H), 3.19-3.43 (m, 8H), 3.66 (b, 12H), 4.30 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 175.11, 166.16, 52.66, 51.03, 50.08, 43.08, 42.51, 39.58, 29.90, 25.29. LC-MS (ESI$^+$) m/z 398.28 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{18}H_{36}N_7O_3^+$ [M + H]$^+$ 398.2874, found 398.2906.

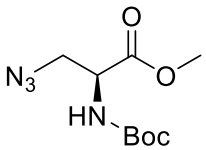
Methyl *N*-(*tert*-butoxycarbonyl)-*O*-tosyl-*L*-serinate (63)



Boc-Ser-OMe (2.5 g, 11.4 mmol) was dissolved in 50 mL of dry DCM and cooled to 0 °C. Pyridine (2 mL, 23 mmol) was added to the chilled solution followed by p-toluene sulfonylchloride (6.6 g, 34.2 mmol) and the reaction mixture was left to stir at 0 °C for 15 m then was warmed to room temperature and left to stir for 45 m. The solvent was evaporated under

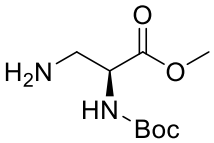
reduced pressure and the white residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 15%. The solvent was evaporated under reduced pressure to give a colorless oil (4180 mg, 98% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.39 (s, 9H), 2.43 (s, 3H), 3.67 (s, 3H), 4.25-4.39 (m, 2H), 4.49 (b, 1H), 5.29 (b, 1H), 7.33 (d, 2H, *J*= 8 Hz), 7.74 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.96, 154.95, 145.15, 132.42, 129.93, 128.00, 80.47, 69.49, 52.98, 52.86, 28.21, 21.02. LC-MS (ESI$^+$) m/z 374.49 [M + H]$^+$.

Methyl (*S*)-3-azido-2-((*tert*-butoxycarbonyl)amino)propanoate (77)



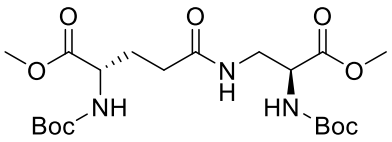
Compound **49** (4100 mg, 10.98 mmol) was dissolved in 6.5 mL of anhydrous DMF. Sodium azide (1785 mg, 27.5 mmol) was added slowly to the solution and the reaction mixture was shaken at 50 °C for 3 h. The reaction mixture was diluted with (100 mL) of cold water and extracted with (2 x 50 mL) of DCM. The combined organic layers were washed with (5 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the colorless residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 10%. The solvent was evaporated under reduced pressure to give a colorless oil (1150 mg, 43% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 9H), 3.69 (b, 2H), 3.76 (s, 3H), 4.45 (b, 1H), 5.37 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.23, 155.05, 80.47, 53.53, 52.83, 52.67, 28.25. LC-MS (ESI$^+$) m/z 245.05 [M + H]$^+$.

Methyl (*S*)-3-amino-2-((*tert*-butoxycarbonyl)amino)propanoate (78)



Compound **63** (1150 mg, 4.7 mmol) was dissolved in 15 mL of MeOH and 10% Pd on carbon (64 mg) was added and the reaction mixture was deoxygenated and stirred at room temperature under H_2 at balloon pressure for 3 h. The mixture was filtered through a celite layer and the filtrate was dried under reduced pressure to get a pale yellow oil (1024 mg, 100% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.24 (b, 2H), 1.41 (s, 9H), 3.01 (b, 2H), 3.73 (s, 3H), 4.27 (b, 1H), 5.42 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.10, 155.53, 79.96, 55.93, 52.37, 43.91, 28.30. LC-MS (ESI$^+$) m/z 219.1 [M + H]$^+$.

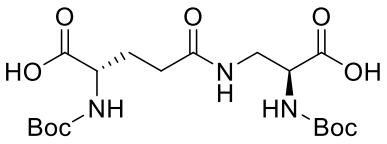
Methyl-*N*2-(*tert*-butoxycarbonyl)-*N*5-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methoxy-3-oxo-propyl)-*L*-glutaminate (81)



Compound **64** (200 mg, 0.92 mmol) was dissolved in 6 mL of dry DCM. DIEA (481 uL, 2.76 mmol) was added and gave a clear solution. Then Boc-Glu-OMe (240 mg, 0.92 mmol) and PyAOP (528 mg, 1 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 1.5 h. The mixture was diluted with (40 mL) of water and extracted with (3 x 80 mL) of DCM. The combined organic layers were washed with (10 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 50%. The solvent was evaporated under reduced pressure to give a white solid (176 mg, 42% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41

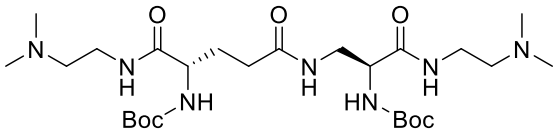
(s, 18H), 1.81 (b, 1H), 2.12-2.26 (m, 3H), 3.51 (b, 1H), 3.72 (s, 6H), 3.82 (b, 1H), 4.29 (b, 1H), 4.40 (b, 1H), 5.35 (b, 1H), 5.78 (b, 1H), 6.73 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.71, 171.22, 155.08, 80.35, 53.82, 52.58, 41.65, 32.55, 29.67, 28.30. LC-MS (ESI$^+$) m/z 462.31 [M + H]$^+$.

***N*2-(*tert*-Butoxycarbonyl)-*N*5-((*S*)-2-((*tert*-butoxycarbonyl)amino)-2-carboxyethyl)-*L*-glutamine (82)**



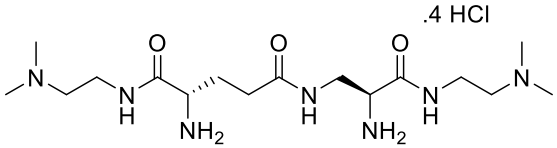
Compound **67** (170 mg, 0.37 mmol) was dissolved in 3 mL of THF and cooled to 0 °C. LiOH (23 mg, 0.93 mmol) was dissolved in 3 mL of water then was added to the chilled solution and the reaction mixture was left to stir at room temperature for 3 h. The reaction mixture was concentrated as possible under reduced pressure and then diluted with (30 mL) of water and washed with (3 x 10 mL) of DCM. Then the aqueous layer was acidified by 0.5 N $KHSO_4$ to pH=2 then was extracted with (6 x 60 mL) of EtOAc. The combined organic layers were washed with (20 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (EtOAc/ hexane + 0.1% AcOH) and the product was eluted at 60%. The solvent was evaporated under reduced pressure to give a colorless oil (146 mg, 91% yield). ^{1}H NMR (400 MHz, DMSO) δ 1.36 (s, 18H), 1.49-1.79 (m, 2H), 2.20 (t, 2H, *J*= 8 Hz), 3.16-3.48 (m, 2H), 3.95 (b, 2H), 6.87 (b, 2H), 7.84 (b, 1H), 10.81 (b, 2H). ^{13}C NMR (100 MHz, DMSO) δ 173.88, 172.10, 155.27, 89.91, 78.18, 53.62, 36.20, 28.12, 27.21. LC-MS (ESI$^+$) m/z 434.6 [M + H]$^+$.

Di-*tert*-butyl ((7*S*,13*S*)-2,18-dimethyl-6,10,14-trioxo-2,5,9,15,18-pentaazanona-decane-7,13-di-yl)dicarbamate (91)



Compound **68** (66 mg, 0.15 mmol) was suspended in 2 mL of dry DCM. DIEA (160 uL, 0.9 mmol) was added and gave a clear solution. Then N,N-dimethylethylenediamine (55 uL, 0.5 mmol) and PyAOP (167 mg, 0.32 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 2 h. The mixture was diluted with (15 mL) of water and extracted with (6 x 40 mL) of DCM. The combined organic layers were washed with (10 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 70%. The solvent was evaporated under reduced pressure to give a pale yellow solid (51 mg, 60% yield). ^{1}H NMR (400 MHz, DMSO) δ 1.39 (s, 18H), 1.72 (b, 2H), 2.10 (b, 2H), 2.51 (s, 12H), 2.71 (b, 4H), 3.17-3.59 (m, 6H), 3.85 (b, 2H), 6.84 (b, 2H), 8.17 (b, 3H). ^{13}C NMR (100 MHz, DMSO) δ 172.39, 171.88, 170.50, 155.20, 79.17, 56.88, 54.51, 54.03, 51.36, 43.65, 35.27, 31.85, 28.11, 27.87. LC-MS (ESI$^+$) m/z 574.3 $[M + H]^+$.

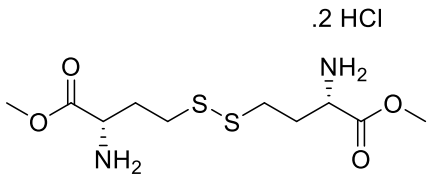
(*S*)-2-Amino-*N*5-((*S*)-2-amino-3-((2-(dimethylamino)ethyl)amino)-3-oxopropyl)-*N*1-(2-(dimethylamino)ethyl)pentanediamide tetrahydrochloride (92)



Compound **77** (41 mg, 0.071 mmol) was dissolved in 185 uL of MeOH and cooled

to 0 °C. 4 M HCl/ dioxane (215 uL, 0.86 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (215 uL, 0.86 mmol) was added to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated five times. The residue was dried to give a yellowish white solid (30 mg, 83% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.20-2.28 (m, 2H), 2.54 (t, 2H, *J*= 8 Hz), 3.02 (s, 12H), 3.37-3.44 (m, 4H), 3.64-3.69 (m, 2H), 3.77-3.91 (m, 4H), 4.14-4.29 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 175.21, 170.08, 168.59, 56.30, 53.15, 52.80, 43.28, 40.10, 34.87, 30.64, 26.16. LC-MS (ESI$^+$) m/z 374.28 $[M + H]^+$. HRMS (ESI$^+$) m/z calculated for $C_{16}H_{36}N_7O_3^+$ $[M + H]^+$ 374.2874, found 374.2903.

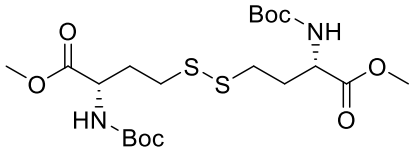
***L*-Homocystine-dimethyl ester-dihydrochloride (60i)**



L-Homocystine (500 mg, 1.86 mmol) was dissolved in 10 mL of methanol and cooled to 0 °C. Thionyl chloride (811 uL, 11.18 mmol) was added slowly to the solution then the reaction mixture was brought to room temperature and left to stir for overnight. The solvent was evaporated under reduced pressure then redissolved in DCM and evaporated again under reduced pressure which was repeated twice to help getting rid of thionyl chloride. The white foamy residue was then dried on pump for overnight to

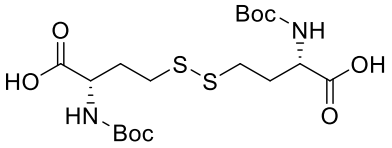
give a white solid (680 mg, 99% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.34-2.46 (m, 4H), 2.88 (b, 4H), 3.86 (s, 6H), 4.31 (t, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, D_2O) δ 170.30, 53.77, 51.49, 32.14, 28.99. LC-MS (ESI$^+$) m/z 297.21 [M + H]$^+$.

***N,N'*-Bis(*tert*-butoxycarbonyl)-*L*-homocystine-dimethyl ester (60)**



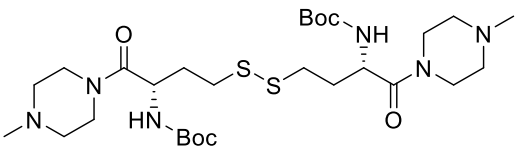
Compound **46i** (680 mg, 1.84 mmol) was dissolved in dioxane/ water (2:1, 5.25 mL). A solution of sodium carbonate (449 mg, 4.2 mmol) in 1.75 mL of water was added to the solution then the mixture was cooled to 0 °C. Di-tert-butyl pyrocarbonate (1004 mg, 4.6 mmol) was added in one portion to the chilled mixture and then the reaction mixture was brought to room temperature and left to stir for 6 h. The reaction mixture was concentrated as possible under reduced pressure and then diluted with (15 mL) of chloroform. The organic layer was washed with (2 x 5 mL) of water, (2 x 5 mL) of 0.5 N $KHSO_4$, (3 x 5 mL) of saturated $NaHCO_3$ and (3 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and a white solid was obtained (910 mg, 99% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 18H), 1.94-2.24 (m, 4H), 2.69 (t, 4H, *J*= 8 Hz), 3.73 (s, 6H), 4.38 (b, 2H), 5.12 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.62, 155.31, 80.12, 52.47, 34.52, 32.57, 28.30. LC-MS (ESI$^+$) m/z 497.35 [M + H]$^+$.

***N,N'*-Bis(*tert*-butoxycarbonyl)-*L*-homocystine (93)**



Compound **46** (905 mg, 1.8 mmol) was dissolved in 15 mL of THF and cooled to 0 °C. LiOH (110 mg, 4.6 mmol) was dissolved in 15 mL of water then was added to the chilled solution and the reaction mixture was left to stir at room temperature for 1 h. There was no progress in the reaction from the 30 minutes check so additional LiOH (110 mg, 4.6 mmol) was added at 0 °C then the reaction mixture was left to stir at room temperature for additional 30 minutes. The reaction mixture was concentrated as possible under reduced pressure and then diluted with (100 mL) of water and DCM. Then the aqueous layer was acidified by 0.5 N $KHSO_4$ to pH=2 then was extracted with (3 x 50 mL) of EtOAc. The combined organic layers were washed with (10 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and a white solid was obtained (800 mg, 95% yield). ^{1}H NMR (400 MHz, DMSO) δ 1.37 (s, 18H), 1.88-2.05 (m, 4H), 2.72 (t, 4H, *J*= 8 Hz), 4.00 (b, 2H), 7.11 (b, 2H), 12.53 (b, 2H). ^{13}C NMR (100 MHz, DMSO) δ 173.61, 155.54, 78.08, 52.18, 34.17, 30.34, 28.17. LC-MS (ESI$^+$) m/z 469.27 [M + H]$^+$.

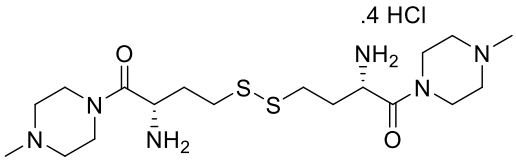
***N,N'*-Bis(*tert*-butoxycarbonyl)-*L*-homocystine bis(*N'*-methylpiperazide) (94)**



Compound **79** (70 mg, 0.14 mmol) was suspended in 2 mL of dry DCM. DIEA (151 uL, 0.86 mmol) was added and gave a clear solution. Then 1-methyl piperazine (48 uL, 0.43 mmol) and PyAOP (164 mg, 0.31 mmol)

were added to the solution and the reaction mixture was left to stir at room temperature for 30 min. The mixture was diluted with (20 mL) of water and extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with (15 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 50%. The solvent was evaporated under reduced pressure to give a light yellow solid (62 mg, 70% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 18H), 1.80-1.89 (m, 2H), 2.07-2.13 (m, 2H), 2.38 (s, 6H), 2.54 (b, 8H), 2.67 (t, 4H, *J*= 8 Hz), 3.66 (b, 8H), 4.74 (b, 2H), 5.48 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.04, 155.64, 79.89, 54.60, 48.69, 45.61, 41.63, 33.55, 32.96, 28.33. LC-MS (ESI$^+$) m/z 633.1 $[M + H]^+$.

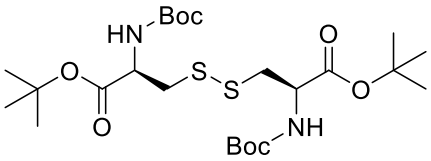
***L*-Homocystine bis(*N′*-methylpiperazide) tetrahydrochloride (95)**



Compound **80** (50 mg, 0.08 mmol) was dissolved in 200 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (240 uL, 0.95 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still some mono-deprotected product so additional 4 M HCl/ dioxane (240 uL, 0.95 mmol) was added in two portions to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 1h. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a yellowish

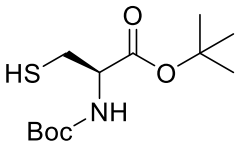
white solid (34 mg, 74% yield). ^{1}H NMR (400 MHz, D_2O) δ 2.29-2.38 (m, 4H), 2.81-2.88 (m, 4H), 2.98 (s, 6H), 3.25 (b, 7H), 3.66 (b, 7H), 4.17 (b, 2H), 4.60 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 167.86, 52.68, 49.44, 43.15, 42.65, 39.55, 30.82, 28.86. LC-MS (ESI^+) m/z 433.23 $[M + H]^+$. HRMS (ESI^+) m/z calculated for $C_{18}H_{37}N_6O_2S_2^+$ $[M + H]^+$ 433.2414, found 433.2443.

Di-*tert*-butyl-3,3'-disulfanediyl(2*R*,2'*R*)-bis(2-((*tert*-butoxycarbonyl)amino)-propanoate) (102)



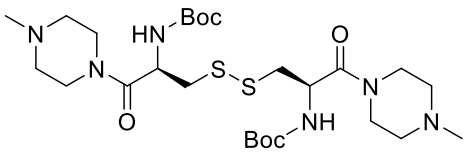
Boc-L-cystine-OH (1 g, 2.27 mmol) was suspended in 10 mL of anhydrous DCM. Tert-butyl-2,2,2-trichloroacetimidate (1.2 mL, 6.81 mmol) was added to the mixture and the reaction mixture was stirred at room temperature for 4 h. Additional tert-butyl-2,2,2-trichloroacetimidate (0.6 mL, 3.4 mmol) was added to the mixture and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was filtered and washed with DCM and 3% EtOAc/ hexane. The filtrate was evaporated under reduced pressure and the residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 20%. The solvent was evaporated under reduced pressure to give a colorless oil (760 mg, 61%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (d, 36H, *J*= 12 Hz), 3.09-3.22 (m, 4H), 4.45 (b, 2H), 5.33 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.63, 155.06, 82.70, 79.96, 53.81, 42.12, 28.33, 27.99.

***tert*-Butyl (*tert*-butoxycarbonyl)-*L*-cysteinate (103)**



Compound **88** (693 mg, 1.25 mmol), triphenyl phosphine (428 mg, 1.63 mmol), sodium acetate (41 mg, 0.7 mmol) were suspended in a mixture of 6 mL of MeOH, 3 mL of water, and 30 uL of glacial acetic acid and heated to 60 °C for 75 m. The mixture was concentrated as possible and diluted with (200 mL) of DCM and washed with (2 x 100 mL) of water, brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 10%. The solvent was evaporated under reduced pressure to give a white solid (600 mg, 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (d, 19H, *J*= 12 Hz), 2.90-2.95 (m, 2H), 4.45 (b, 1H), 5.40 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.29, 155.14, 82.67, 79.97, 55.09, 28.30, 28.01, 27.53.

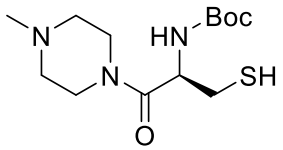
Di-*tert*-butyl ((2*R*,2′*R*)-disulfanediylbis(3-(4-methylpiperazin-1-yl)-3-oxopropane-1,2-diyl))di-carbamate (96)



Boc-L-cystine-OH (1 g, 2.27 mmol) was suspended in 25 mL of anhydrous DCM. DIEA (2.4 mL, 13.62 mmol) was added and gave a clear solution. Then 1-methyl piperazine (756 uL, 6.81 mmol) and PyAOP (2.9 g, 5.6 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 2 h. The mixture was diluted with (100 mL) of water and extracted with (2 x 200 mL) of DCM. The combined organic layers were washed with brine and dried

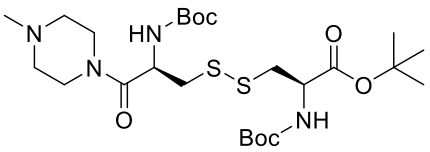
over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 90%. The solvent was evaporated under reduced pressure to give a light yellow oil (1250 mg, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 18H), 2.27 (s, 6H), 2.35-2.41 (m, 8H), 2.92-3.04 (m, 4H), 3.61 (b, 8H), 4.92 (b, 2H), 5.53 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.80, 155.02, 80.05, 54.53, 49.05, 45.74, 42.22, 41.67, 28.34.

***tert*-Butyl (*R*)-(3-mercapto-1-(4-methylpiperazin-1-yl)-1-oxopropan-2-yl)carbamate (97)**



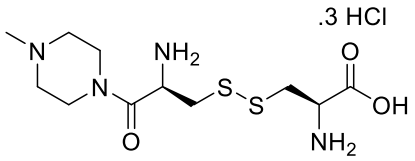
Compound **82** (1071 mg, 1.77 mmol), triphenyl phosphine (604 mg, 2.3 mmol), sodium acetate (58 mg, 0.7 mmol) were suspended in a mixture of 10 mL of MeOH, 5 mL of water, and 42 uL of glacial acetic acid and heated to 60 °C for 75 m. The mixture was concentrated as possible and diluted with (300 mL) of DCM and washed with (2 x 100 mL) of water, brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the light yellow oil was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 35%. The solvent was evaporated under reduced pressure to give a colorless oil (900 mg, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.39 (s, 9H), 1.50 (t, 1H, *J*= 8 Hz), 2.25 (s, 3H), 2.33-2.38 (m, 4H), 2.62-2.86 (m, 2H), 3.54-3.61 (m, 4H), 4.70 (b, 2H), 5.59 (d, 2H, *J*= 12 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.58, 155.10, 80.02, 54.61, 51.63, 45.70, 42.18, 28.30, 27.39.

tert-Butyl-_N_-(_tert_-butoxycarbonyl)-_S_-(((_R_)-2-((_tert_-butoxycarbonyl)amino)-3-(4-methyl-piperazin-1-yl)-3-oxopropyl)thio)-_L_-cysteinate (104)



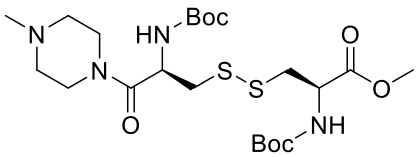
To a stirred solution of 1-chlorobenzotriazole (74 mg, 0.48 mmol) and benzotriazole (29 mg, 0.24 mmol) in 8 mL of anhydrous DCM under Argon at -78 °C was added dropwise during 10 m a solution of compound **83** (73 mg, 0.24 mmol) in 2 mL of anhydrous DCM. After 30 m, a solution of thiourea (54 mg, 0.72 mmol) in 6 mL of anhydrous THF was added and the reaction mixture was left to stir at -78 °C for further 15 m. Compound **89** (100 mg, 0.36 mmol) in 2 mL of anhydrous DCM was added slowly at -78 °C and the reaction mixture was warmed slowly to room temperature and left to stir for overnight. The reaction mixture was filtered with additional DCM washes and the filtrate was evaporated under reduced pressure. The residue was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 25%. The solvent was evaporated under reduced pressure to give a colorless oil (80 mg, 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (d, 27H, *J*= 12 Hz), 2.26 (s, 3H), 2.34-2.41 (m, 4H), 2.83-3.16 (m, 4H), 3.60 (b, 4H), 4.39 (b, 1H), 4.87-4.93 (m, 1H), 5.43 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.55, 168.91, 155.05, 82.70, 80.13, 55.02, 54.45, 53.93, 48.98, 45.80, 42.08, 28.35, 27.98.

S-(((R)-2-Amino-3-(4-methylpiperazin-1-yl)-3-oxopropyl)thio)-L-cysteine trihydrochloride (105)



Compound **90** (15 mg, 0.025 mmol) was dissolved in 100 uL of dioxane and cooled to 0 °C. 4 M HCl/ dioxane (324 uL, 1.3 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. The reaction mixture was left to shake at room temperature overnight. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a white solid (11 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 2.83 (d, 3H, *J*= 8 Hz), 3.18-3.48 (m, 8H), 3.67-3.80 (m, 4H), 4.23 (b, 1H), 4.37 (b, 1H). ^{13}C NMR (100 MHz, D_2O) δ 171.56, 166.67, 52.49, 49.52, 43.00, 42.47, 39.59, 37.09, 36.55. LC-MS (ESI$^+$) m/z 323.2 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{11}H_{23}N_4O_3S_2^+$ [M + H]$^+$ 323.1206, found 323.1233.

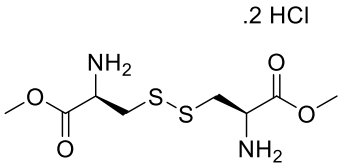
Methyl N-(*tert*-butoxycarbonyl)-S-(((R)-2-((*tert*-butoxycarbonyl)amino)-3-(4-methylpiperazin-1-yl)-3-oxopropyl)thio)-L-cysteinate (100)



To a stirred solution of 1-chlorobenzotriazole (51 mg, 0.33 mmol) and benzotriazole (20 mg, 0.16 mmol) in 6 mL of dry DCM under Argon at -78 °C was added

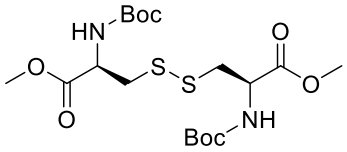
dropwise during 10 m a solution of compound **83** (50 mg, 0.16 mmol) in 1 mL of dry DCM. After 30 m, a solution of thiourea (38 mg, 0.5 mmol) in 4.5 mL of anhydrous THF was added and the reaction mixture was left to stir at -78 °C for further 15 m. Compound **85** (57 mg, 0.24 mmol) in 1 mL of dry DCM was added slowly at -78 °C and the reaction mixture was warmed slowly to room temperature and left to stir for overnight. The solvent was evaporated under reduced pressure. The residue was purified via ISCO (5% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 70%. The solvent was evaporated under reduced pressure to give a yellowish oil (55 mg, 65%). ^{1}H NMR (400 MHz, MeOD) δ 1.43 (s, 18H), 2.33 (s, 3H), 2.40-2.54 (m, 4H), 2.83-3.20 (m, 4H), 3.54-3.70 (m, 4H), 3.75 (s, 3H), 4.47 (b, 2H). ^{13}C NMR (100 MHz, MeOD) δ 173.06, 170.88, 157.78, 80.90, 56.03, 55.46, 54.41, 52.95, 51.01, 45.97, 43.06, 41.30, 28.73. LC-MS (ESI^{+}) m/z 537.37 [M + H]$^{+}$.

Dimethyl 3,3'-disulfanediyl(2*R*,2'*R*)-bis(2-aminopropanoate) dihydrochloride (98i)



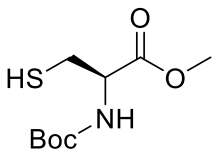
L-Cystine-OH (1 g, 4.16 mmol) was dissolved in 20 mL of methanol and cooled to 0 °C. Thionyl chloride (1.8 mL, 25 mmol) was added slowly to the solution then the reaction mixture was brought to room temperature and left to stir for overnight. The solvent was evaporated under reduced pressure then redissolved in chloroform and evaporated again under reduced pressure which was repeated twice to help getting rid of thionyl chloride. The white foamy residue was then dried on pump for overnight to give a white solid (1450 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 3.34-3.46 (m, 4H), 3.89 (s, 6H), 4.60 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 169.13, 53.97, 51.56, 35.71.

Dimethyl 3,3'-disulfanediyl(2*R*,2'*R*)-bis(2-((*tert*-butoxycarbonyl)amino)propanoate) (98)



Compound **84i** (1400 mg, 4.11 mmol) was dissolved in dioxane/ water (2:1, 12 mL). A solution of sodium carbonate (935 mg, 8.8 mmol) in 4 mL of water was added to the solution then the mixture was cooled to 0 °C. Di-tert-butyl pyrocarbonate (2243 mg, 10.3 mmol) was added in one portion to the chilled mixture and then the reaction mixture was brought to room temperature and left to stir for 4 h. The reaction mixture was concentrated as possible under reduced pressure and then diluted with (30 mL) of EtOAc. The organic layer was washed with (2 x 10 mL) of water, (2 x 10 mL) of 0.5 N $KHSO_4$, (3 x 10 mL) of saturated $NaHCO_3$ and (6 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and a white solid was obtained (1750 mg, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.35 (s, 18H), 3.06 (b, 4H), 3.66 (s, 6H), 4.49 (b, 2H), 5.43 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.91, 155.01, 80.03, 66.94, 52.46, 41.09, 28.19.

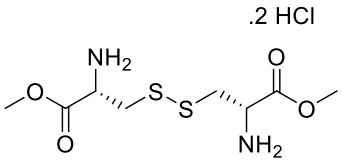
Methyl (*tert*-butoxycarbonyl)-*L*-cysteinate (99)



Compound **84** (1700 mg, 3.63 mmol), triphenyl phosphine (1047 mg, 4 mmol), sodium acetate (120 mg, 1.45 mmol) were suspended in a mixture of 16 mL of MeOH, 7 mL of water, and 124 uL of glacial acetic acid and heated to 50 °C for 3 h. The mixture was concentrated as possible

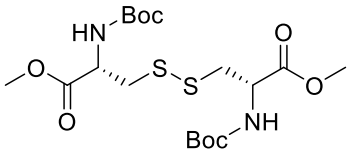
and diluted with (80 mL) of EtOAc and washed with (2 x 25 mL) of water, (10 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 30%. The solvent was evaporated under reduced pressure to give a colorless oil (1371 mg, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (t, 1H, *J*= 8 Hz), 1.43 (s, 9H), 2.92-2.96 (m, 2H), 3.75 (s, 3H), 4.57 (b, 1H), 5.42 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.81, 155.09, 80.24, 54.85, 52.65, 28.27, 27.29. LC-MS (ESI$^+$) m/z 236.24 [M + H]$^+$.

Dimethyl 3,3'-disulfanediyl(2*S*,2'*S*)-bis(2-aminopropanoate) dihydrochloride (106i)



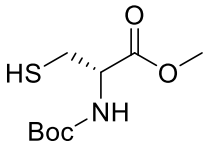
D-Cystine-OH (500 mg, 2.1 mmol) was dissolved in 10 mL of methanol and cooled to 0 °C. Thionyl chloride (1 mL, 12.5 mmol) was added slowly to the solution then the reaction mixture was brought to room temperature and left to stir for overnight. The solvent was evaporated under reduced pressure then redissolved in chloroform and evaporated again under reduced pressure which was repeated twice to help getting rid of thionyl chloride. The white foamy residue was then dried on pump for overnight to give a white solid (701 mg, 98%). ^{1}H NMR (400 MHz, D_2O) δ 3.34-3.45 (m, 4H), 3.88 (s, 6H), 4.61 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 169.13, 53.97, 51.56, 35.70.

Dimethyl 3,3'-disulfanediyl(2*S*,2'*S*)-bis(2-((*tert*-butoxycarbonyl)amino)propanoate) (106)



Compound **92i** (D-cystine-OMe) (670 mg, 1.96 mmol) was dissolved in dioxane/ water (2:1, 6 mL). A solution of sodium carbonate (445 mg, 4.2 mmol) in 2 mL of water was added to the solution then the mixture was cooled to 0 °C. Di-tert-butyl pyrocarbonate (1075 mg, 4.93 mmol) was added in one portion to the chilled mixture and then the reaction mixture was brought to room temperature and left to stir for 5 h. The reaction mixture was concentrated as possible under reduced pressure and then diluted with (15 mL) of EtOAc. The organic layer was washed with (2 x 5 mL) of water, (2 x 5 mL) of 0.5 N $KHSO_4$, (3 x 5 mL) of saturated $NaHCO_3$ and (3 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and a white solid was obtained (850 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.35 (s, 18H), 3.07 (b, 4H), 3.67 (s, 6H), 4.51 (b, 2H), 5.42 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.94, 155.02, 80.08, 66.96, 52.49, 41.13, 28.21.

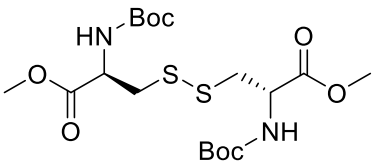
Methyl (*tert*-butoxycarbonyl)-*D*-cysteinate (107)



Compound **92** (800 mg, 1.7 mmol), triphenyl phosphine (493 mg, 1.88 mmol), sodium acetate (56 mg, 0.68 mmol) were suspended in a mixture of 8 mL of MeOH, 3.5 mL of water, and 58 uL of glacial acetic acid and heated to 50 °C for 3 h. The mixture was concentrated as possible and diluted with (60 mL) of EtOAc and washed with (2 x 20 mL) of water, (10 mL) of brine

and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 30%. The solvent was evaporated under reduced pressure to give a colorless oil (447 mg, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (t, 1H, *J*= 8 Hz), 1.44 (s, 9H), 2.94-2.99 (m, 2H), 3.77 (s, 3H), 4.61 (b, 1H), 5.42 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.82, 155.10, 80.27, 54.84, 52.68, 28.28, 27.32.

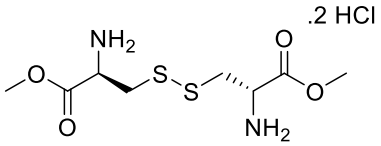
Methyl-*N*-(*tert*-butoxycarbonyl)-*S*-(((*R*)-2-((*tert*-butoxycarbonyl)amino)-3-methoxy-3-oxo-propyl)thio)-*D*-cysteinate (108)



To a stirred solution of 1-chlorobenzotriazole (262 mg, 1.7 mmol) and benzotriazole (102 mg, 0.85 mmol) in 22 mL of dry DCM under Argon at -78 °C was added dropwise during 10 m a solution of compound **93** (200 mg, 0.85 mmol) in 4 mL of dry DCM. After 15 m, a solution of thiourea (195 mg, 2.6 mmol) in 30 mL of anhydrous THF was added and the reaction mixture was left to stir at -78 °C for further 15 m. Compound **85** (200 mg, 0.85 mmol) in 4 mL of dry DCM was added slowly at -78 °C and the reaction mixture was warmed slowly to room temperature and left to stir for overnight. The reaction mixture was evaporated under reduced pressure and the residue was dissolved in (200 mL) of EtOAc and washed with (4 x 50 mL) of water, (20 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the pale white residue was purified via ISCO (50% EtOAc/ hexane) and the product was eluted at 40%. The solvent was evaporated under reduced pressure to give a colorless oil (250 mg, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 18H), 3.10-3.26 (m, 4H), 3.76 (s,

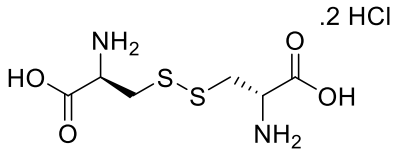
6H), 4.61 (b, 2H), 5.38 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.20, 155.07, 80.35, 52.66, 41.25, 28.30. LC-MS (ESI$^+$) m/z 469.45 [M + H]$^+$.

Methyl *S*-(((*R*)-2-amino-3-methoxy-3-oxopropyl)thio)-*D*-cysteinate dihydrochloride (109)



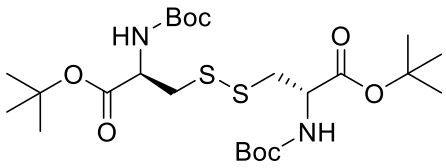
Compound **94** (7 mg, 0.015 mmol) was dissolved in 50 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (68 uL, 0.27 mmol) in four portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. Additional 4 M HCl/ dioxane (17 uL, 0.07 mmol) was added to the chilled solution and the mixture was warmed to room temperature and left to shake for 30 m. The solvent was evaporated via N_2 and the residue was dried on pump. The yellow residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a yellowish white solid (4 mg, 80%). ^{1}H NMR (400 MHz, D_2O) δ 3.39 (b, 4H), 3.86 (s, 6H), 4.58 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 169.12, 53.88, 51.43, 35.59. LC-MS (ESI$^+$) m/z 269.1 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_8H_{17}N_2O_4S_2^+$ [M + H]$^+$ 269.0624, found 269.0643.

_S_-(((_R_)-2-Amino-2-carboxyethyl)thio)-_D_-cysteine dihydrochloride (113)



Compound **98** (19 mg, 0.034 mmol) was dissolved in 150 uL of dioxane and cooled to 0 °C. 4 M HCl/ dioxane (860 uL, 3.44 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition and the reaction mixture was left to shake for overnight. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a yellowish white solid (11 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 3.30-3.51 (m, 4H), 4.41-4.47 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 170.76, 51.94, 36.47. LC-MS (ESI$^+$) m/z 241.1 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_6H_{13}N_2O_4S_2^+$ [M + H]$^+$ 241.0311, found 241.0331.

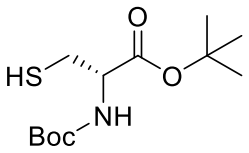
***tert*-Butyl-_S_-(((_R_)-3-(*tert*-butoxy)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)thio)-_N_-(*tert*-butoxycarbonyl)-_D_-cysteinate (112)**



To a stirred solution of 1-chlorobenzotriazole (66 mg, 0.43 mmol) and benzotriazole (25 mg, 0.21 mmol) in 4 mL of anhydrous DCM under Argon at -78 °C was added dropwise during 10 m a solution of compound **97** (60 mg, 0.21 mmol) in 2 mL of anhydrous DCM. After 15 m, a solution of thiourea (48 mg, 0.63 mmol) in 4 mL of anhydrous THF was added and the reaction mixture was left to stir at -78 °C for

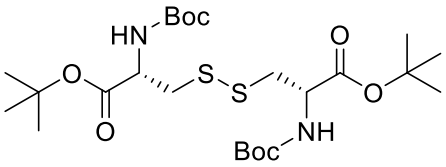
further 15 m. Compound **89** (60 mg, 0.21 mmol) in 2 mL of anhydrous DCM was added slowly at -78 °C and the reaction mixture was warmed slowly to room temperature and left to stir for overnight. The reaction mixture was filtered with additional DCM washes and the filtrate was evaporated under reduced pressure. The residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 20%. The solvent was evaporated under reduced pressure to give a colorless oil (75 mg, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (d, 36H, *J*= 12 Hz), 3.09-3.20 (m, 4H), 4.43 (b, 2H), 5.33 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.62, 155.04, 82.68, 79.95, 53.72, 42.02, 28.32, 27.98.

***tert*-Butyl (*tert*-butoxycarbonyl)-*D*-cysteinate (111)**



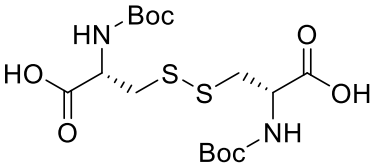
Compound **96** (76 mg, 0.137 mmol), triphenyl phosphine (44 mg, 0.165 mmol), sodium acetate (5 mg, 0.054 mmol) were suspended in a mixture of 1 mL of MeOH, 0.5 mL of water, and 4 uL of glacial acetic acid and heated to 60 °C for 1.5 h. The mixture was concentrated as possible and diluted with (20 mL) of EtOAc and washed with (2 x 10 mL) of water, (3 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 10%. The solvent was evaporated under reduced pressure to give a white solid (60 mg, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (d, 19H, *J*= 12 Hz), 2.90-2.96 (m, 2H), 4.44 (b, 1H), 5.38 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.28, 155.13, 82.66, 79.96, 55.09, 28.29, 28.01, 27.50.

Di-*tert*-butyl-3,3'-disulfanediyl(2*S*,2'*S*)-bis(2-((*tert*-butoxycarbonyl)amino)-propanoate) (110)



Compound **96i** (313 mg, 0.71 mmol) was suspended in 3 mL of anhydrous DCM. Tert-butyl-2,2,2-trichloroacetimidate (764 uL, 4.26 mmol) was added to the mixture and the reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure and the white residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 20%. The solvent was evaporated under reduced pressure to give a white solid (245 mg, 62%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (d, 36H, *J*= 12 Hz), 3.07-3.20 (m, 4H), 4.43 (b, 2H), 5.34 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.64, 155.07, 82.70, 79.96, 53.80, 42.08, 28.32, 27.98.

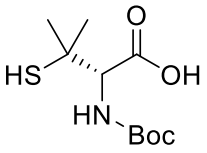
(2*S*,2'*S*)-3,3'-Disulfanediylbis(2-((*tert*-butoxycarbonyl)amino)propanoic acid) (110i)



D-Cystine-OH (500 mg, 2.1 mmol) was dissolved in dioxane/ water (2:1, 6 mL). A solution of sodium carbonate (463 mg, 4.3 mmol) in 2 mL of water was added to the solution then the mixture was cooled to 0 °C. Di-*tert*-butyl pyrocarbonate (1135 mg, 5.2 mmol) was added in one portion to the chilled mixture and then the reaction mixture was brought to room temperature and left to stir for 5 h. The reaction mixture was concentrated as possible under reduced pressure and then diluted with (30 mL) of water. The aqueous layer was washed with (3 x 60 mL) of DCM and then was

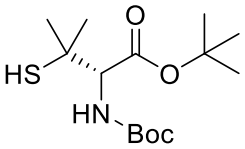
acidified using 0.5 N $KHSO_4$ to pH 2 and extracted with (5 x 60 mL) of EtOAc. The combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and a white solid was obtained (450 mg, 49%). ^{1}H NMR (400 MHz, DMSO) δ 1.38 (s, 18H), 2.85-3.19 (m, 4H), 4.43 (b, 2H), 7.20 (b, 2H), 8.23 (b, 2H). ^{13}C NMR (100 MHz, DMSO) δ 172.35, 155.33, 78.25, 65.14, 52.68, 28.12.

(*R*)-2-((*tert*-Butoxycarbonyl)amino)-3-mercapto-3-methylbutanoic acid (114)



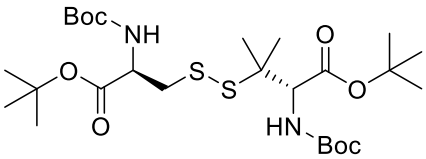
A flask was charged with *D*-Pen-OH (200 mg, 5.04 mmol) and 20 mL of THF was added followed by sodium carbonate (160 mg, 1.5 mmol) and water (2 mL) then the mixture was cooled to 0 °C. Di-*tert*-butyl pyrocarbonate (328 mg, 1.5 mmol) was added and the reaction mixture was brought to room temperature and left to stir for 3 h. The reaction mixture was concentrated as possible under reduced pressure and then was diluted with (50 mL) of water. The aqueous layer was extracted with (3 x 25 mL) of EtOAc then was acidified to pH 2 using 0.5 N $KHSO_4$ then was extracted with (3 x 100 mL) of EtOAc. The combined organic layers were washed with (20 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to give a white solid (340 mg, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 3H), 1.46 (s, 9H), 1.52 (s, 3H), 3.49 (s, 1H), 4.32 (d, 1H, *J*= 8 Hz), 5.32 (d, 1H, *J*= 8 Hz), 10.00 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.97, 155.72, 80.61, 62.35, 46.27, 30.81, 29.64, 28.28.

***tert*-Butyl (*R*)-2-((*tert*-butoxycarbonyl)amino)-3-mercapto-3-methylbutanoate (115)**



Compound **100** (320 mg, 1.28 mmol) was suspended in 10 mL of anhydrous DCM. *tert*-Butyl-2,2,2-trichloroacetimidate (931 uL, 5.2 mmol) was added to the mixture and the reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure and the white residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 30%. The solvent was evaporated under reduced pressure to give a colorless oil (345 mg, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.28-1.39 (m, 24H), 1.86 (s, 1H), 4.06 (d, 1H, *J*= 12 Hz), 5.34 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.62, 155.45, 82.04, 79.73, 62.67, 46.65, 32.95, 30.96, 29.71, 28.23.

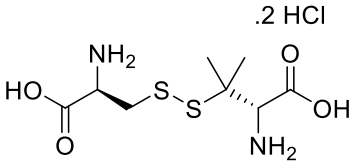
***tert*-butyl (*R*)-3-(((*R*)-3-(*tert*-butoxy)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)-disulfaneyl)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanoate (116)**



To a stirred solution of 1-chlorobenzotriazole (74 mg, 0.48 mmol) and benzotriazole (29 mg, 0.24 mmol) in 8 mL of anhydrous DCM under Argon at -78 °C was added dropwise during 10 m a solution of compound **89** (66 mg, 0.24 mmol) in 2 mL of anhydrous DCM. After 20 m, a solution of thiourea (55 mg, 0.72 mmol) in 6 mL of anhydrous THF was added and the reaction mixture was left to stir at -78 °C for further 15 m. Compound **101** (80 mg, 0.26 mmol) in 2 mL of anhydrous DCM was added slowly at -78 °C and the reaction mixture was warmed slowly to room temperature and left to stir for overnight. The reaction mixture was filtered with additional DCM washes and the

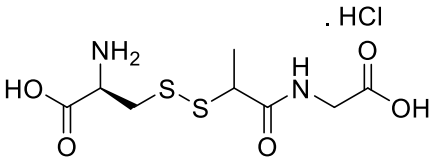
filtrate was evaporated under reduced pressure. The residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 18%. The solvent was evaporated under reduced pressure to give a colorless oil (80 mg, 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (d, 6H, *J*= 12 Hz), 1.42 (d, 36H, *J*= 12 Hz), 3.08-3.21 (m, 2H), 4.27 (b, 1H), 4.42 (b, 1H), 5.19 (b, 1H), 5.27 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.49, 155.31, 82.46, 79.93, 60.50, 54.05, 52.71, 43.46, 28.29, 25.40.

(*R*)-2-Amino-3-(((*R*)-2-amino-2-carboxyethyl)disulfaneyl)-3-methylbutanoic acid di-hydro-chloride (117)



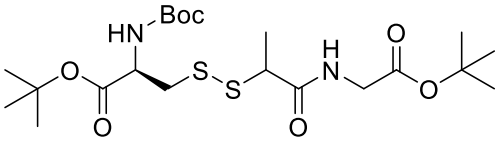
Compound **102** (18 mg, 0.031 mmol) was dissolved in 200 uL of dioxane and cooled to 0 °C. 4 M HCl/ dioxane (774 uL, 3.1 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition and the reaction mixture was left to stir for overnight. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a white solid (12 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 1.39 (s, 3H), 1.49 (s, 3H), 3.30-3.39 (m, 2H), 4.00 (s, 1H), 4.26 (t, 1H, *J*= 4 Hz). ^{13}C NMR (100 MHz, D_2O) δ 169.95, 60.56, 53.10, 51.64, 39.14, 21.50. LC-MS (ESI$^+$) m/z 269.1 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_8H_{17}N_2O_4S_2^+$ [M + H]$^+$ 269.0624, found 269.0648.

_S_-((1-((Carboxymethyl)amino)-1-oxopropan-2-yl)thio)-_L_-cysteine hydrochloride (128)



Compound **113** (18 mg, 0.036 mmol) was dissolved in 150 uL of dioxane and cooled to 0 °C. 4 M HCl/ dioxane (910 uL, 3.64 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition and the reaction mixture was left to stir for overnight. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a white solid (12 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 1.49 (d, 3H, *J*= 8 Hz), 3.13-3.47 (m, 1H), 3.75-3.89 (m, 2H), 4.07-4.13 (m, 2H), 4.26-4.38 (m, 1H). ^{13}C NMR (100 MHz, D_2O) δ 175.12, 173.06, 170.94, 70.82, 68.25, 64.57, 52.18, 48.21, 47.18, 43.28, 41.37, 37.31, 15.68. LC-MS (ESI$^+$) m/z 283.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_8H_{15}N_2O_5S_2^+$ [M + H]$^+$ 283.0417, found 283.0444.

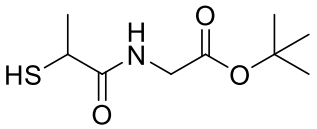
***tert*-Butyl-*S*-((1-((2-(*tert*-butoxy)-2-oxoethyl)amino)-1-oxopropan-2-yl)thio)-*N*-(*tert*-butoxy-carbonyl)-*L*-cysteinate (127)**



To a stirred solution of 1-chlorobenzotriazole (166 mg, 1.08 mmol) and benzotriazole (65 mg, 0.54 mmol) in 14 mL of anhydrous DCM under Argon at -78 °C was added dropwise during 10 m a solution of compound **89** (150

mg, 0.54 mmol) in 3 mL of anhydrous DCM. After 15 m, a solution of thiourea (123 mg, 1.62 mmol) in 10 mL of anhydrous THF was added and the reaction mixture was left to stir at -78 °C for further 15 m. Compound **112** (166 mg, 0.75 mmol) in 3 mL of anhydrous DCM was added slowly at -78 °C and the reaction mixture was warmed slowly to room temperature and left to stir for overnight. The reaction mixture was diluted with DCM and filtered with additional DCM washes. The filtrate was evaporated under reduced pressure and the yellowish white residue was purified via ISCO (50% EtOAc/ hexane) and the product was eluted at 40%. The solvent was evaporated under reduced pressure to give a colorless oil (145 mg, 55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (d, 30H, *J*= 8 Hz), 3.03-3.22 (m, 2H), 3.49-3.59 (m, 1H), 3.87-4.12 (m, 2H), 4.44 (b, 1H), 5.43 (b, 1H), 6.83 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.52, 169.71, 168.70, 155.25, 82.92, 82.30, 80.16, 53.77, 49.44, 42.34, 28.33, 28.04, 27.97, 17.17.

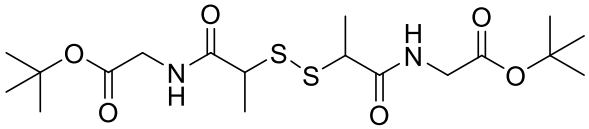
***tert*-Butyl (2-mercaptopropanoyl)glycinate (126)**



Compound **111** (524 mg, 1.2 mmol), triphenyl phosphine (378 mg, 1.44 mmol), sodium acetate (40 mg, 0.48 mmol) were suspended in a mixture of 5 mL of MeOH, 2.5 mL of water, and 32 uL of glacial acetic acid and heated to 80 °C for 6 h. The mixture was concentrated as possible and diluted with (200 mL) of EtOAc and washed with (2 x 50 mL) of water, (30 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 45%. The solvent was evaporated under reduced pressure to give a white solid (420 mg, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 9H),

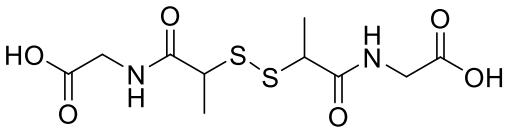
1.51 (d, 3H, *J*= 8 Hz), 2.05 (d, 1H, *J*= 8 Hz), 3.42-3.46 (m, 1H), 3.87-3.89 (m, 2H), 6.91 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.94, 168.81, 82.44, 42.33, 37.89, 28.03, 22.05.

Di-*tert*-butyl 2,2'-((2,2'-disulfanediylbis(propanoyl))bis(azanediyl))diacetate (125)



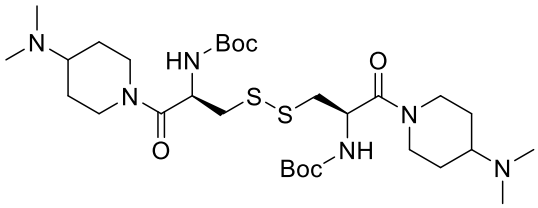
Compound **110** (750 mg, 2.31 mmol) was suspended in 10 mL of anhydrous DCM. *tert*-Butyl-2,2,2-trichloroacetimidate (1.2 mL, 6.94 mmol) was added to the mixture and the reaction mixture was stirred at room temperature for 1 h. Additional *tert*-butyl-2,2,2-trichloroacetimidate (1.2 mL, 6.94 mmol) was added to the mixture and the reaction mixture was stirred at room temperature for 3 h. The solvent was evaporated under reduced pressure and the white residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 30%. The solvent was evaporated under reduced pressure to give a colorless oil (674 mg, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40-1.48 (m, 24H), 3.57-3.65 (m, 2H), 3.76-3.82 (m, 1H), 3.95-3.98 (m, 2H), 4.07-4.13 (m, 1H), 7.04 (b, 1H), 7.16 (b, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.11, 169.59, 82.44, 49.88, 48.23, 42.28, 28.04, 17.33.

2,2'-((2,2'-Disulfanediylbis(propanoyl))bis(azanediyl))diacetic acid (124)



Tiopronin (1 g, 6.12 mmol) was suspended in 85 mL of *tert*-BuOH and a solution of iodine (311 mg, 1.22 mmol) in 15 mL of *tert*-BuOH was added and the reaction mixture was left to stir at room temperature for 7 h. Additional iodine (311 mg, 1.22 mmol) was added and the reaction mixture was left to stir at room temperature for overnight. The reaction was not complete so additional iodine (311 mg, 1.22 mmol) was added and the reaction mixture was left to stir at room temperature for 5 h. The reaction mixture was quenched with (10 mL) of 0.01 N sodium thiosulfate solution and concentrated as possible under reduced pressure. The mixture was diluted with (100 mL) of 0.01 N sodium thiosulfate solution and was acidified using 0.5 N $KHSO_4$ the pH 2 and the solution was extracted with large volumes of EtOAc. The combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to give a yellow oil (760 mg, 77%). ^{1}H NMR (400 MHz, DMSO) δ 1.34 (d, 6H, *J*= 8 Hz), 3.65-3.85 (m, 6H), 8.25-8.33 (m, 2H), 12.33 (b, 2H). ^{13}C NMR (100 MHz, DMSO) δ 171.94, 170.92, 47.93, 20.71, 17.09.

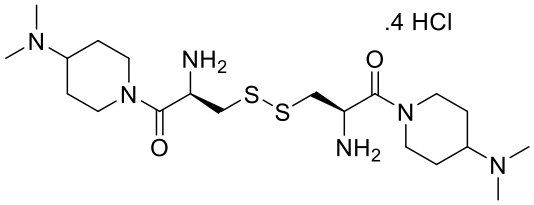
***N,N'*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(4'-(dimethylamino)-piperidineamide) (46)**



Boc-L-cystine-OH (50 mg, 0.11 mmol) was suspended in 1 mL of dry DCM. DIEA (119 uL, 0.68 mmol) was added

and gave a clear solution. Then 4-(dimethylamino)piperidine (40 uL, 0.28 mmol) and PyAOP (125 mg, 0.24 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 m. The mixture was diluted with (15 mL) of water and extracted with (3 x 30 mL) of DCM. The combined organic layers were washed with (10 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 35%. The solvent was evaporated under reduced pressure to give a colorless oil (68 mg, 94% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 22H), 1.85 (b, 4H), 2.24 (s, 12H), 2.34 (b, 2H), 2.58-2.67 (m, 2H), 2.92-3.09 (m, 6H), 4.07 (b, 2H), 4.54 (b, 2H), 4.93 (b, 2H), 5.46 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.61, 155.00, 80.02, 74.76, 61.83, 53.42, 49.10, 44.83, 41.60, 29.01, 28.33. LC-MS (ESI^+) m/z 633.1 $[M + H]^+$.

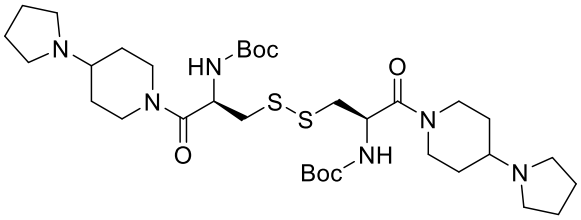
***L*-Cystine bis(4'-(dimethylamino)-piperidineamide) tetrahydrochloride (49)**



Compound **35A** (24 mg, 0.036 mmol) was dissolved in 500 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (270 uL, 1.08 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still mono-deprotected product so additional 4 M HCl/ dioxane (270 uL, 1.08 mmol) was added to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 3 h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount

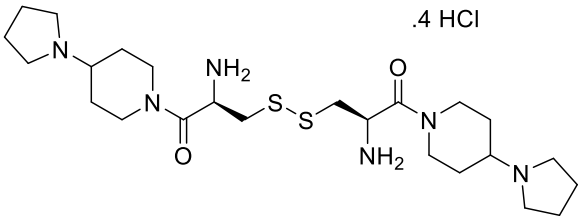
of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was dried to give a white solid (22 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 1.71-1.88 (m, 4H), 2.25-2.36 (m, 4H), 2.92 (d, 12H, *J*= 4 Hz), 3.24-3.41 (m, 6H), 3.62 (b, 2H), 4.18 (b, 2H), 4.64 (b, 2H), 4.89-4.99 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 166.16, 62.56, 49.91, 44.13, 41.34, 39.77, 26.25, 25.46. LC-MS (ESI$^+$) m/z 461.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{20}H_{41}N_6O_2S_2^+$ [M + H]$^+$ 461.2727, found 461.2757.

***N,N'*-Bis(*tert*-butoxycarbonyl)-*L*-cystine bis(4'-(1''-Pyrrolidinyl)piperidine) (47)**



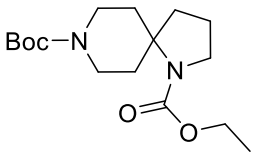
Boc-L-cystine-OH (50 mg, 0.11 mmol) was suspended in 1 mL of dry DCM. DIEA (119 uL, 0.68 mmol) was added and gave a clear solution. Then 4-(1-pyrrolidinyl)-piperidine (44 mg, 0.28 mmol) and PyAOP (125 mg, 0.24 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 30 m. The mixture was diluted with (15 mL) of water and extracted with (3 x 30 mL) of DCM. The combined organic layers were washed with (10 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 30%. The solvent was evaporated under reduced pressure to give a colorless oil (70 mg, 90% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 20H), 1.76 (b, 8H), 1.92 (b, 4H), 2.25 (b, 4H), 2.55 (b, 8H), 2.76-3.15 (m, 8H), 4.01 (b, 2H), 4.40 (b, 2H), 4.94 (b, 2H), 5.50 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.59, 155.11, 80.03, 74.76, 61.36, 51.39, 49.10, 44.24, 41.14, 31.93, 30.97, 28.34, 23.24. LC-MS (ESI$^+$) m/z 633.1 [M + H]$^+$.

_L_-Cystine bis(4'-(1''-Pyrrolidinyl)piperidine) tetrahydrochloride (50)



Compound **36A** (22 mg, 0.03 mmol) was dissolved in 500 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (232 uL, 0.93 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. There was still mono-deprotected product so additional 4 M HCl/ dioxane (232 uL, 0.93 mmol) was added to the solution at 0 °C and the solution was warmed to room temperature and left to stir for 3 h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was dried to give a white solid (20 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 1.69-1.87 (m, 4H), 2.02 (b, 4H), 2.19 (b, 4H), 2.35-2.43 (m, 4H), 2.85-2.96 (m, 2H), 3.20-3.46 (m, 10H), 3.55 (b, 2H), 3.71 (b, 4H), 4.13 (b, 2H), 4.59 (b, 2H), 4.91-4.99 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 165.86, 60.88, 51.76, 49.87, 43.90, 41.18, 28.52, 27.72, 22.48. LC-MS (ESI$^+$) m/z 513.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{24}H_{45}N_6O_2S_2^+$ [M + H]$^+$ 513.3040, found 513.3070.

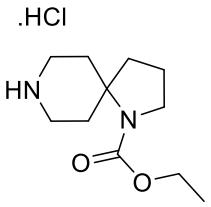
8-(_tert_-Butyl) 1-ethyl 1,8-diazaspiro[4.5]decane-1,8-dicarboxylate (52)



Sodium carbonate (133 mg, 1.248 mmol) was added to a mixture of 1,8-Diazaspiro[4.5]decane-8-carboxylic acid *tert*-butyl ester

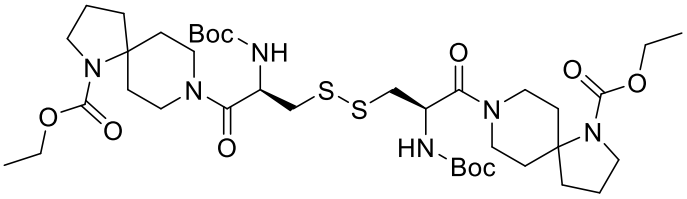
(100 mg, 0.416 mmol) in 3 mL of dry DCM. Ethyl chloroformate (119 µL, 1.248 mmol) was added to the mixture then the reaction mixture was left to stir at room temperature for overnight. The mixture was diluted with (10 mL) of water and extracted with (3 x 20 mL) of DCM. The combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 40%. The solvent was evaporated under reduced pressure to give a colorless oil (100 mg, 77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (b, 3H), 1.42 (s, 9H), 1.73-179 (m, 2H), 1.85 (b, 4H), 2.42 (b, 2H), 2.69 (b, 2H), 3.44 (b, 2H), 4.30 (b, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.55, 153.88, 79.34, 65.94, 62.52, 60.23, 47.55, 42.09, 34.09, 28.43, 22.10, 14.70.

Ethyl 1,8-diazaspiro[4.5]decane-1-carboxylate hydrochloride (53)



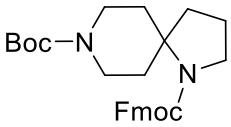
Compound **39** (90 mg, 0.29 mmol) was dissolved in 2 mL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (1.45 mL, 5.8 mmol) was added to the chilled solution and the solution was warmed to room temperature and left to stir for 3 h. The solvent was evaporated under reduced pressure and the white residue was dried on pump to give a white solid (73 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 1.26 (b, 3H), 1.71 (b, 2H), 1.83-1.89 (m, 2H), 2.09 (b, 2H), 2.76 (b, 2H), 3.12 (b, 2H), 3.48 (b, 4H), 4.12 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 155.77, 61.65, 60.85, 48.03, 42.55, 34.88, 29.39, 21.60, 13.97.

Diethyl-8,8'-((2*R*,2'*R*)-3,3'-disulfanediylbis(2-((*tert*-butoxycarbonyl)amino)-propanoyl))bis(1,8-diazaspiro[4.5]decane-1-carboxylate) (54)



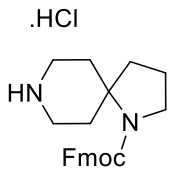
Boc-L-cystine-OH (20 mg, 0.045 mmol) was suspended in 600 µL of dry DCM. DIEA (94 uL, 0.54 mmol) was added and gave a clear solution. Then compound **40** (56 mg, 0.227 mmol) and PyAOP (58 mg, 0.11 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 1 h. The mixture was diluted with (5 mL) of water and extracted with (3 x 10 mL) of DCM. The combined organic layers were washed with (10 mL) of 0.5 N $KHSO_4$, (3 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow oil was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 14%. The solvent was evaporated under reduced pressure to give a colorless oil (33 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (t, 6H, *J*= 8 Hz), 1.41 (s, 26H), 1.73-1.82 (m, 4H), 1.94 (b, 4H), 2.61 (b, 2H), 2.82-3.15 (m, 6H), 3.45 (b, 4H), 4.04 (b, 6H), 4.54 (b, 2H), 4.92 (b, 2H), 5.45 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.86, 154.88, 153.93, 79.96, 62.27, 60.35, 49.23, 47.58, 44.04, 42.02, 40.53, 35.48, 33.50, 28.34, 21.70, 14.66.

1-((9*H*-Fluoren-9-yl)methyl) 8-(*tert*-butyl) 1,8-diazaspiro[4.5]decane-1,8-di-carboxylate (57)



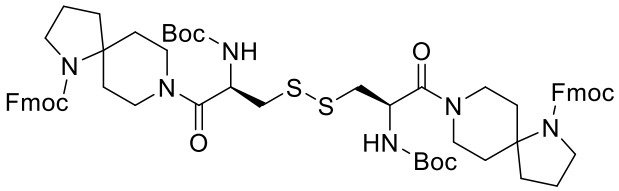
Sodium carbonate (331 mg, 3.12 mmol) in 3 mL of water was added to a mixture of 1,8-Diazaspiro[4.5]decane-8-carboxylic acid *tert*-butyl ester (375 mg, 1.56 mmol) in 9 mL of dioxane and cooled to 0 °C. Fmoc-Cl (605 mg, 2.34 mmol) was added to the chilled mixture then the reaction mixture was brought to room temperature and left to stir for 2 h then the mixture was kept in fridge for overnight. The mixture was concentrated as possible and diluted with (30 mL) of water and extracted with (3 x 60 mL) of DCM. The combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow oil was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 30%. The solvent was evaporated under reduced pressure to give a colorless oil (676 mg, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.81 (b, 2H), 1.22 (b, 2H), 1.39 (s, 9H), 1.82 (b, 4H), 2.63 (b, 2H), 3.32-3.45 (m, 4H), 4.20 (b, 2H), 4.61 (b, 1H), 7.25 (t, 2H, *J*= 8 Hz), 7.34 (t, 2H, *J*= 8 Hz), 7.54 (d, 2H, *J*= 8 Hz), 7.71 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.58, 153.57, 144.24, 141.35, 127.61, 126.99, 125.08, 119.94, 79.40, 66.34, 62.84, 47.48, 42.09, 34.91, 32.91, 31.59, 28.47, 22.19. LC-MS (ESI$^+$) m/z 633.1 $[M + H]^+$.

(9*H*-Fluoren-9-yl)methyl 1,8-diazaspiro[4.5]decane-1-carboxylate hydrochloride (58)



Compound **43** (324 mg, 0.7 mmol) was dissolved in 3 mL of dioxane and cooled to 0 °C. 4 M HCl/ dioxane (4.7 mL, 18.92 mmol) was added to the chilled solution and the solution was warmed to room temperature and left to stir for 1.5 h. The solvent was evaporated under reduced pressure and the white residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a white solid (280 mg, quantitative yield). ^{1}H NMR (400 MHz, MeOD) δ 1.53 (d, 2H, *J*= 12 Hz), 1.76 (t, 2H, *J*= 8 Hz), 1.99 (t, 2H, *J*= 8 Hz), 2.79 (t, 2H, *J*= 12 Hz), 3.02 (t, 2H, *J*= 12 Hz), 3.62-3.77 (m, 3H), 4.20 (dd, 2H, *J*= 4, 8 Hz), 4.41 (d, 2H, *J*= 8 Hz), 7.30 (t, 2H, *J*= 8 Hz), 7.38 (t, 2H, *J*= 8 Hz), 7.58 (d, 2H, *J*= 8 Hz), 7.78 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, MeOD) δ 145.39, 142.75, 128.82, 128.16, 126.01, 120.94, 67.62, 61.92, 47.40, 43.66, 36.22, 30.85, 23.00.

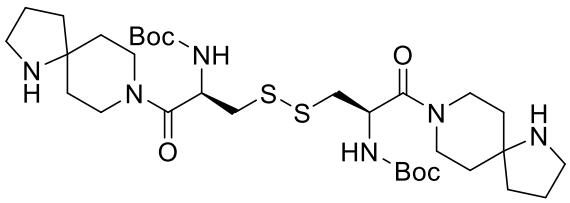
Bis((9*H*-fluoren-9-yl)methyl)-8,8'-((2*R*,2'*R*)-3,3'-disulfanediylbis(2-((*tert*-butoxy-carbonyl)-amino)propanoyl))bis(1,8-diazaspiro[4.5]decane-1-carboxylate) (59)



Boc-L-cystine-OH (97 mg, 0.22 mmol) was suspended in 3 mL of dry DCM. DIEA (460 uL, 2.64 mmol) was added and gave a clear solution. Then compound **44** (263 mg, 0.66 mmol) and PyAOP (286 mg, 0.55 mmol) were added to the

solution and the reaction mixture was left to stir at room temperature for 3 h. The mixture was quenched with water and concentrated as possible under reduced pressure and the residue was dissolved in (50 mL) of EtOAc and washed with (2 x 25 mL) of water, (3 x 25 mL) of 0.5 N $KHSO_4$, (25 mL) of water, (5 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 70%. The solvent was evaporated under reduced pressure to give a white solid (248 mg, quantitative yield). ^{1}H NMR (400 MHz, DMSO) δ 1.33 (d, 22H, *J*= 12 Hz), 1.73 (b, 4H), 1.92 (b, 4H), 2.34 (b, 4H), 2.50-3.08 (m, 8H), 3.20 (b, 4H), 3.99 (b, 2H), 4.24 (b, 2H), 7.25 (b, 2H), 7.32 (t, 4H, *J*= 8 Hz), 7.40 (t, 4H, *J*= 8 Hz), 7.60 (b, 4H), 7.88 (d, 4H, *J*= 8 Hz). ^{13}C NMR (100 MHz, DMSO) δ 168.12, 154.73, 152.55, 143.97, 140.75, 127.58, 127.06, 125.02, 120.05, 78.30, 65.58, 62.26, 49.50, 47.20, 46.76, 42.68, 34.40, 32.93, 32.07, 28.09, 21.59.

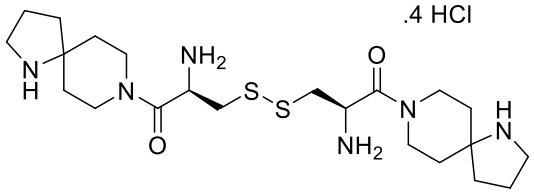
Di-*tert*-butyl ((2*R*,2′*R*)-disulfanediylbis(3-oxo-3-(1,8-diazaspiro[4.5]decan-8-yl)-propane-1,2-di-yl))dicarbamate (55)



Compound **45** (177 mg, 0.15 mmol) was dissolved in 9 mL of anhydrous DCM and cooled to 0 °C. Piperidine (155 uL, 1.57 mmol) was added to the chilled solution and the reaction mixture was left to stir at room temperature for 7 h. The reaction mixture was cooled to 0 °C and additional piperidine (155 uL, 1.57 mmol) was added to the chilled solution and the reaction mixture was left to stir at room temperature for overnight. The solvent was evaporated and the yellow residue was purified via ISCO

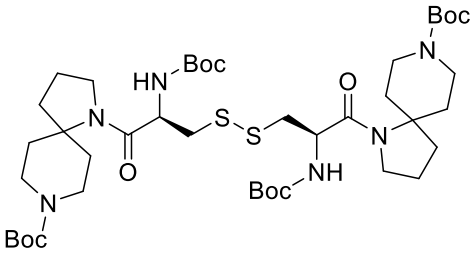
(10% MeOH/ DCM) and the product was eluted at 90%. The solvent was evaporated under reduced pressure to give a colorless oil (70 mg, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 18H), 1.73 (b, 2H), 1.89 (m, 2H), 2.17 (m, 12H), 2.81 (m, 2H), 3.00 (dd, 2H, *J*= 4, 12 Hz), 3.17 (m, 2H), 3.27 (m, 2H), 3.45 (m, 2H), 3.50 (m, 2H), 4.02 (b, 2H), 4.46 (b, 2H), 5.05 (b, 2H), 5.38 (b, 2H), 9.98 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.54, 154.77, 80.18, 65.56, 47.78, 43.76, 43.30, 39.04, 38.74, 35.30, 33.82, 32.70, 28.32, 22.55.

***L*-Cystine bis(1,8-Diazaspiro[4.5]decan-8-yl) tetrahydrochloride (56)**



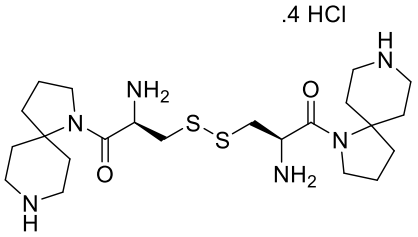
Compound **42** (7 mg, 0.01 mmol) was dissolved in 50 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (76 uL, 0.3 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was dried to give a white solid (5 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 1.95-2.20 (m, 12H), 3.19 (t, 2H, *J*= 12 Hz), 3.25-3.47 (m, 10H), 3.51-3.62 (m, 2H), 3.90-3.98 (m, 2H), 4.08 (b, 2H), 4.29 (b, 2H), 4.91 (b,2H). ^{13}C NMR (100 MHz, D_2O) δ 165.99, 65.81, 49.80, 44.40, 42.99, 39.98, 37.61, 33.46, 32.55, 22.30. LC-MS (ESI$^+$) m/z 485.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{22}H_{41}N_6O_2S_2^+$ [M + H]$^+$ 485.2727, found 485.2761.

Di-*tert*-butyl 1,1'-((2*R*,2′*R*)-3,3'-disulfanediylbis(2-((*tert*-butoxycarbonyl)amino)-propanoyl))-bis(1,8-diazaspiro[4.5]decane-8-carboxylate) (48)



Boc-L-cystine-OH (120 mg, 0.272 mmol) was suspended in 4 mL of dry DCM. DIEA (285 uL, 1.63 mmol) was added and gave a clear solution. Then 1,8-Diazaspiro[4.5]decane-8-carboxylic acid *tert*-butyl ester (327 mg, 1.36 mmol) and PyAOP (355 mg, 0.68 mmol) were added to the solution and the reaction mixture was left to stir at room temperature for 3 h. The mixture was diluted with (10 mL) of water and extracted with (3 x 25 mL) of DCM and the combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 25%. The solvent was evaporated under reduced pressure to give a colorless oil (80 mg, 33%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.21-1.25 (m, 4H), 1.41 (d, 36H, *J*= 8 Hz), 1.86-2.05 (m, 8H), 2.70-2.98 (m, 12H), 3.61 (b, 2H), 3.70 (b, 2H), 4.01 (b, 2H), 4.10 (b, 2H), 4.65 (b, 2H), 5.34 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.87, 155.21, 154.52, 79.96, 79.42, 65.18, 52.42, 48.72, 41.83, 40.98, 34.96, 32.43, 28.45, 28.33, 22.70.

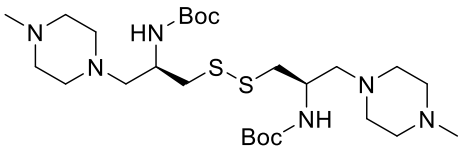
***L*-Cystine bis(1,8-Diazaspiro[4.5]decan-1-yl) tetrahydrochloride (51)**



Compound **37A** (8 mg, 0.009 mmol) was dissolved in 50 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (76 uL, 0.3 mmol) in two portions was added

to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a white solid (6 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 1.75 (b, 4H), 1.97-2.10 (m, 6H), 2.15-2.22 (m, 2H), 2.90-2.96 (m, 4H), 3.07-3.18 (m, 6H), 3.38-3.47 (m, 6H), 3.66-3.72 (m, 2H), 3.77-3.82 (m, 2H), 4.57-4.60 (m,2H). ^{13}C NMR (100 MHz, D_2O) δ 165.92, 64.06, 51.94, 49.04, 42.18, 36.78, 34.57, 28.72, 28.08, 22.32. LC-MS (ESI^+) m/z 485.3 $[M + H]^+$. HRMS (ESI^+) m/z calculated for $C_{22}H_{41}N_6O_2S_2^+$ $[M + H]^+$ 485.2727, found 485. 2761.

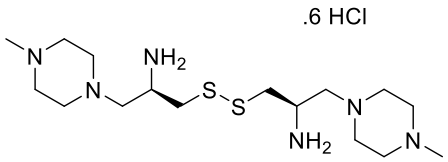
Di-*tert*-butyl((2*R*,2′*R*)-disulfanediylbis(3-(4-methylpiperazin-1-yl)propane-1,2-diyl))di-carbamate (132)



Compound **116** (60 mg, 0.147 mmol) was dissolved in 2 mL of dry DCM and N-methyl piperazine (49 uL, 0.441 mmol) was added and the reaction mixture was left to stir at room temperature for 30 m. sodium triacetoxy borohydride (94 mg, 0.441 mmol) was added and the mixture was left to stir at room temperature for 3 h. The reaction mixture was diluted with (50 mL) of DCM and washed with saturated sodium bicarbonate (3 x 20 mL), brine (10 mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 35%. The

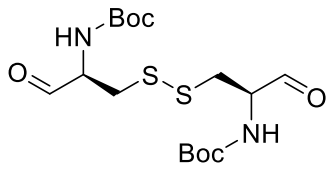
solvent was evaporated under reduced pressure to give a colorless oil (35 mg, 42%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 18H), 2.26 (s, 6H), 2.41-2.55 (m, 20H), 2.89-3.26 (m, 4H), 3.98 (b, 2H), 4.96 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.50, 79.55, 60.30, 55.14, 53.38, 47.98, 46.02, 42.78, 28.45.

1-Piperazinepropane, 3,3'-dithiobis(2-amino),4-methyl,(2*R*,2'*R*) hexahydrochloride (134)



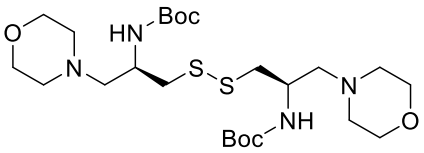
Compound **118** (9 mg, 0.016 mmol) was dissolved in 200 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (108 uL, 0.432 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. Additional 4 M HCl/ dioxane (54 uL, 0. 216 mmol) was added to the chilled solution in two portions and the mixture was warmed to room temperature and left stir for 2 h. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a light brown solid (9.5 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 2.61 (b, 2H), 2.80 (s, 6H), 2.81-3.02 (m, 6H), 3.11-3.30 (m, 12H), 3.55 (b, 4H), 3.85 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 57.09, 53.12, 50.47, 48.94, 47.86, 42.76. LC-MS (ESI$^+$) m/z 377.3 $[M + H]^+$. HRMS (ESI$^+$) m/z calculated for $C_{16}H_{37}N_6S_2^+$ $[M + H]^+$ 377.2516, found 377.2544.

Di-*tert*-butyl ((2*R*,2′*R*)-disulfanediylbis(3-oxopropane-1,2-diyl))dicarbamate (130)



Compound **115** (45 mg, 0.11 mmol) was suspended in 1.5 mL of dry DCM and cooled to 0 °C. Dess-Martin periodinane (94 mg, 0.22 mmol) was added and the reaction was brought to room temperature then (5 uL, 1.76 mmol) of water dissolved in 5 mL of dry DCM was added dropwise to the mixture during 15 m and the mixture was left to stir for 30 m. The reaction mixture was diluted with (4 mL) of DCM and then a solution of $Na_2S_2O_3$ (175 mg) in saturated $NaHCO_3$ (1 mL) was added and the mixture was left to stir vigorously for 10 m. The layers were separated and the aqueous layer was washed with additional DCM (2 x 10 mL). The combined organic layers were washed with saturated sodium bicarbonate, water, brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the white residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 45%. The solvent was evaporated under reduced pressure to give a white solid (41 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 18H), 3.10-3.18 (m, 4H), 4.41 (b, 2H), 5.63 (b, 2H), 9.70 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.01, 155.39, 80.72, 59.05, 38.27, 28.15.

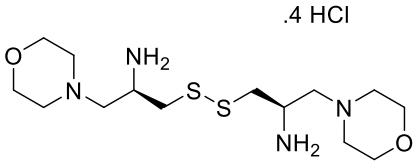
Di-*tert*-butyl ((2*R*,2′*R*)-disulfanediylbis(3-morpholinopropane-1,2-diyl))dicarbamate (131)



Compound **116** (53 mg, 0.13 mmol) was dissolved in 2 mL of dry DCM and morpholine (34 uL, 0.39 mmol) was added and the reaction mixture was left to stir at room temperature for 30 m. sodium triacetoxy borohydride (83 mg, 0.39 mmol)

was added and the mixture was left to stir at room temperature for 3 h. The reaction mixture was diluted with (50 mL) of DCM and washed with saturated sodium bicarbonate (3 x 20 mL), brine (10 mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 15%. The solvent was evaporated under reduced pressure to give a colorless oil (40 mg, 56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 18H), 2.45 (b, 12H), 2.88-3.18 (m, 4H), 3.66 (b, 8H), 3.97 (b, 2H), 5.10 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.58, 79.53, 66.97, 60.70, 53.87, 47.56, 43.00, 28.45.

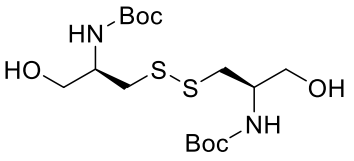
***N*-Morpholinepropane, 3,3'-dithiobis(2-amino),(2*R*,2'*R*) tetrahydrochloride (133)**



Compound **117** (19 mg, 0.035 mmol) was dissolved in 400 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (236 uL, 0.945 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. Additional 4 M HCl/ dioxane (236 uL, 0.945 mmol) was added to the chilled solution in two portions and the mixture was warmed to room temperature and left stir for 3 h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether then the vial was centrifuged and the solvent was decanted; this purification step was repeated three times. The residue was dried to give a light brown solid (17.5 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 3.16-3.33 (m, 4H), 3.48 (b, 8H), 3.62-3.75 (m, 4H), 4.00 (b, 8H), 4.22 (b, 2H).

^{13}C NMR (100 MHz, D_2O) δ 63.79, 57.57, 52.98, 45.49, 37.11. LC-MS (ESI$^+$) m/z 351.2 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{14}H_{31}N_4O_2S_2^+$ [M + H]$^+$ 351.1883, found 351.1906.

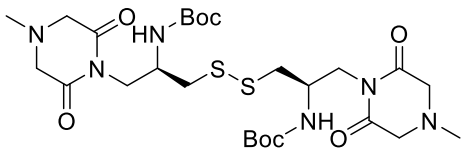
Di-*tert*-butyl ((2*R*,2′*R*)-disulfanediylbis(3-hydroxypropane-1,2-diyl))dicarbamate (129)



A flask was charged with Boc-L-cystine-OH (2 g, 4.54 mmol) and 30 mL of anhydrous THF was added then was cooled to -20 °C. N-methyl morpholine (1.1 mL, 10 mmol) was added and left to stir at -20 °C for 10 m then iso-butylchloroformate (1.3 mL, 10 mmol) was added slowly then the reaction mixture was warmed slowly to 10 °C and left to stir for 30 m. The reaction mixture was then filtered through a glass fritted funnel topped with a filter paper and vacuum was applied via water aspirator and THF washes were used (3 x 10 mL). The filtrate was cooled to -30 °C and $NaBH_4$ (2063 mg, 54.5 mmol) was added and the mixture was left to stir for 30 m then water (10 mL) was added slowly and after stirring for 1 h additional (30 mL) of water was added followed by (60 mL) of saturated NH_4Cl then the mixture was left to stir at room temperature then was left in fridge for overnight. The reaction mixture was concentrated under reduced pressure then extracted with (3 x 100 mL) of EtOAc and the combined organic layers were washed with (30 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the colorless oil was purified via ISCO (EtOAc/ hexane) and the product was eluted at 60%. The solvent was evaporated under reduced pressure to give a white solid (1 g, 53%). ^{1}H NMR (400 MHz, MeOD) δ 1.43 (s, 18H), 2.79-2.98 (m,

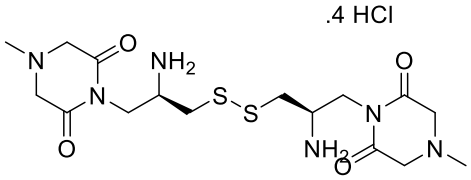
4H), 3.53-3.63 (m, 4H), 3.80-3.86 (m, 2H). ^{13}C NMR (100 MHz, MeOD) δ 157.98, 80.28, 63.92, 53.36, 41.55, 28.86.

Di-*tert*-butyl((2*R*,2′*R*)-disulfanediylbis(3-(4-methyl-2,6-dioxopiperazin-1-yl)propane-1,2-diyl))-dicarbamate (135)



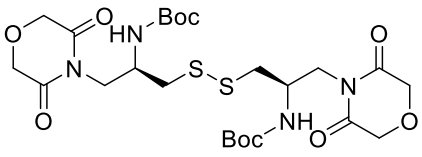
Triphenyl phosphine (140 mg, 0.53 mmol) was dissolved in 1 mL of anhydrous THF and cooled to 0 °C and then DIAD (105 uL, 0.53 mmol) was added and the mixture was left to stir at 0 °C for 10 m where it gave a yellowish white suspension. Compound **115** (100 mg, 0.24 mmol) followed by 4-methyl piperazine-2,6-dione (68.5 mg, 0.53 mmol) were added and the reaction mixture was left to stir at 0 °C for 15 m then was brought to room temperature and left to stir for 2.5 h. The solvent was evaporated under reduced pressure and the dark yellow oil was purified via ISCO (20% MeOH: DCM + 0.05% TEA/ DCM) and the product was eluted at 18%. The solvent was evaporated under reduced pressure to give a white solid (100 mg, 66%). ^{1}H NMR (400 MHz, Pyridine-d5) δ 1.20 (s, 18H), 1.94 (s, 6H), 2.93-3.01 (m, 4H), 3.17 (s, 8H), 3.89-4.08 (m, 4H), 4.49 (b, 2H), 7.38 (b, 2H). ^{13}C NMR (100 MHz, Pyridine-d5) δ 172.54, 158.58, 80.44, 60.48, 51.31, 45.50, 44.73, 44.38, 30.50.

2,6-Piperazinedione-1-propane, 3,3'-dithiobis(2-amino),4-methyl,(2*R*,2'*R*) tetra-hydrochloride (137)



Compound **121** (30 mg, 0.048 mmol) was dissolved in 500 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (326 uL, 1.3 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. Additional 4 M HCl/ dioxane (163 uL, 0.65 mmol) was added to the chilled solution and the mixture was warmed to room temperature and left to shake for 30 m then was left in fridge for overnight. The solvent was evaporated under reduced pressure and the residue was dried on pump. The yellow residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a white solid (27 mg, 98%). ^{1}H NMR (400 MHz, D_2O) δ 3.04 (s, 6H), 3.05-3.09 (m, 2H), 3.19-3.24 (m, 2H), 3.93-3.99 (m, 2H), 4.27 (b, 4H), 4.34 (s, 8H). ^{13}C NMR (100 MHz, D_2O) δ 166.88, 54.54, 49.49, 42.98, 40.78, 37.15. LC-MS (ESI$^+$) m/z 433.2 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{16}H_{29}N_6O_4S_2^+$ [M + H]$^+$ 433.1686, found 433.1736.

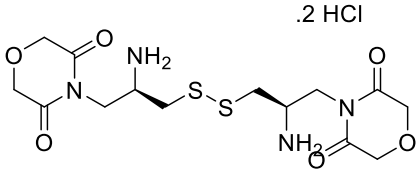
Di-*tert*-butyl ((2*R*,2'*R*)-disulfanediylbis(3-(3,5-dioxomorpholino)propane-1,2-diyl))-dicarbamate (136)



Triphenyl phosphine (140 mg, 0.53 mmol) was dissolved in 1 mL of anhydrous THF and cooled to 0 °C and then DIAD (105 uL, 0.53 mmol) was added

and the mixture was left to stir at 0 °C for 10 m where it gave a yellowish white suspension. Compound **115** (100 mg, 0.24 mmol) followed by morpholine-3,5-dione (62 mg, 0.53 mmol) were added and the reaction mixture was left to stir at 0 °C for 15 m then was brought to room temperature and left to stir for 2 h. The solvent was evaporated under reduced pressure and the colorless oil was purified via ISCO (EtOAc/ hexane) and the product was eluted at 50%. The solvent was evaporated under reduced pressure to give a white solid (90 mg, 62%). ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 1.39 (s, 18H), 2.96-3.07 (m, 4H), 3.88-4.01 (m, 4H), 4.19 (b, 2H), 4.36 (b, 8H), 5.83 (b, 2H). ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ 170.51, 156.45, 79.09, 68.14, 49.47, 42.40, 42.20, 28.65.

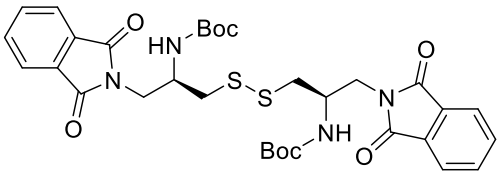
3,5-Morpholinedione-4-propane, 3,3'-dithiobis(2-amino),(2*R*,2'*R*) dihydrochloride (138)



Compound **122** (30 mg, 0.05 mmol) was dissolved in 500 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (374 uL, 1.5 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. Additional 4 M HCl/ dioxane (374 uL, 1.5 mmol) was added to the chilled solution and the mixture was warmed to room temperature and left to shake for 1 h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a white solid (22 mg, 96%). ^{1}H NMR (400 MHz, D_2O) δ 3.04-3.09 (m, 2H), 3.19-3.24 (m, 2H), 3.94-4.01 (m, 2H), 4.22-4.28 (m, 4H), 4.57 (s, 8H). ^{13}C NMR (100

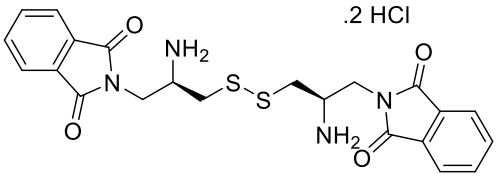
MHz, D_2O) δ 172.27, 67.15, 49.76, 39.51, 37.21. LC-MS (ESI^+) m/z 407.2 $[M + H]^+$. HRMS (ESI^+) m/z calculated for $C_{14}H_{23}N_4O_6S_2^+$ $[M + H]^+$ 407.1054, found 407.1090.

Di-*tert*-butyl((2*R*,2′*R*)-disulfanediylbis(3-(1,3-dioxoisoindolin-2-yl)propane-1,2-diyl))di-carbamate (139)



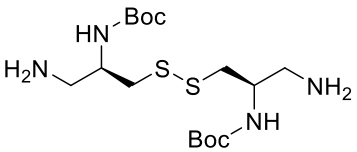
Triphenyl phosphine (66 mg, 0.25 mmol) was dissolved in 0.5 mL of anhydrous THF and cooled to 0 °C and then DIAD (50 uL, 0.25 mmol) was added and the mixture was left to stir at 0 °C for 15 m where it gave a yellowish white suspension. Compound **115** (47 mg, 0.11 mmol) followed by phthalimide (37 mg, 0.25 mmol) were added and the reaction mixture was left to stir at 0 °C for 15 m then was brought to room temperature and left to stir for 7 h. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (EtOAc/ hexane) and the product was eluted at 48%. The solvent was evaporated under reduced pressure to give a white solid (63 mg, 86%). ^1H NMR (400 MHz, DMSO) δ 1.14 (s, 18H), 2.77-3.02 (m, 4H), 3.58-3.77 (m, 4H), 4.02 (b, 2H), 6.81 (d, 2H, *J*= 8 Hz), 7.81 (b, 8H). ^{13}C NMR (100 MHz, DMSO) δ 167.86, 155.23, 134.13, 131.74, 122.86, 77.72, 48.45, 46.55, 41.12, 27.89.

Phthalimide-*N*-propane, 3,3'-dithiobis(2-amino),(2*R*,2'*R*) dihydrochloride (140)



Compound **125** (34 mg, 0.051 mmol) was dissolved in 500 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (350 uL, 1.4 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. Additional 4 M HCl/ dioxane (175 uL, 0.7 mmol) was added to the chilled solution and the mixture was warmed to room temperature and left in fridge for overnight. Additional 4 M HCl/ dioxane (350 uL, 1.4 mmol) was added in two portions at 0 °C to the solution and the mixture was warmed to room temperature and left to stir for 1 h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a white solid (25 mg, 92%). ^{1}H NMR (400 MHz, DMSOd6) δ 2.99−3.05 (m, 2H), 3.20-3.25 (m, 2H), 3.72 (b, 2H), 3.94 (b, 4H), 7.84-7.88 (m, 8H), 8.56 (b, 4H). ^{13}C NMR (100 MHz, DMSOd6) δ 168.18, 134.35, 131.94, 123.06, 48.81, 38.36, 36.57. LC-MS (ESI^{+}) m/z 471.2 [M + H]$^{+}$. HRMS (ESI^{+}) m/z calculated for $C_{22}H_{23}N_4O_4S_2^{+}$ [M + H]$^{+}$ 471.1155, found 471.1196.

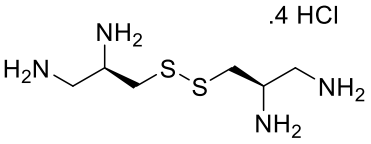
Di-*tert*-butyl ((2*R*,2'*R*)-disulfanediylbis(3-aminopropane-1,2-diyl))dicarbamate (141)



To a suspension of compound **125** (142 mg, 0.21 mmol) in EtOH (6 mL) was added hydrazine monohydrate (205 uL, 4.22 mmol) and the reaction mixture was left to stir at

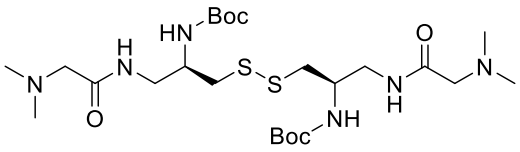
room temperature for overnight. The solvent was evaporated under reduced pressure and then was diluted with DCM and filtered with additional DCM washes and the filtrate was collected and evaporated under reduced pressure to give a yellow oil (90 mg, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 18H), 1.88 (b, 4H), 2.83-2.98 (m, 8H), 3.82 (b, 2H), 5.26 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.70, 79.56, 52.16, 43.65, 41.57, 28.44.

(2*R*,2′*R*)-3,3'-Disulfanediylbis(propane-1,2-diamine) tetrahydrochloride (142)



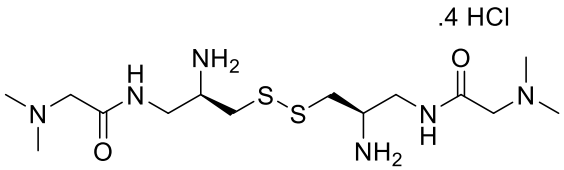
Compound **127** (3 mg, 0.007 mmol) was dissolved in 50 uL of dioxane and cooled to 0 °C. 4 M HCl/ dioxane (38 uL, 0.146 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. The solvent was evaporated via N_2 and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a white solid (2.5 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 3.11-3.16 (m, 2H), 3.29-3.34 (m, 2H), 3.46-3.58 (m, 4H), 4.04-4.10 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 48.42, 40.25, 36.62. LC-MS (ESI$^+$) m/z 211.2 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_6H_{29}N_6O_2S_2^+$ [M + H]$^+$ 211.1046, found 211.1063.

Di-*tert*-butyl((7*R*,12*R*)-2,17-dimethyl-4,15-dioxo-9,10-dithia-2,5,14,17-tetraaza-octadecane-7,12-diyl)dicarbamate (143)



Compound **127** (50 mg, 0.12 mmol) was dissolved in 1 mL of dry DCM and DIEA (128 uL, 0.73 mmol) followed by N,N-dimethylglycine (25 mg, 0.25 mmol) were added. PyAOP (130 mg, 0.25 mmol) was added to the stirred solution and the reaction mixture was left to stir at room temperature for 1.5 h. The reaction mixture was diluted with (15 mL) of water and extracted with (3 x 30 mL) of DCM. The combined organic layers were washed with (10 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the colorless oil was purified via ISCO (20% MeOH: DCM/ DCM) and the product was eluted at 17%. The solvent was evaporated under reduced pressure to give a white solid (45 mg, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 18H), 2.27 (s, 12H), 2.71-2.78 (m, 2H), 2.95 (s, 4H), 3.05 (b, 2H), 3.47-3.58 (m, 4H), 3.97 (b, 2H), 5.82 (b, 2H), 7.51 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.05, 155.77, 79.52, 63.01, 51.12, 45.99, 41.06, 28.40, 19.61.

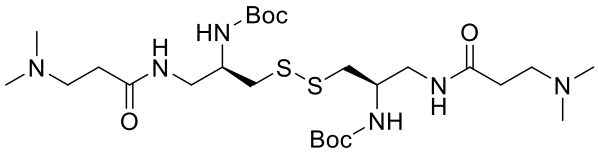
***N,N'*-((2*R*,2'*R*)-Disulfanediylbis(2-aminopropane-3,1-diyl))bis(2-(dimethylamino)-acetamide) tetrahydrochloride (147)**



Compound **129A** (10 mg, 0.017 mmol) was dissolved in 200 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (130 uL, 0.517 mmol) in two portions was added to the chilled solution every half an hour and the

solution was warmed to room temperature after each addition. Additional 4 M HCl/ dioxane (130 uL, 0.517 mmol) was added to the chilled solution and the mixture was warmed to room temperature and left stir for 3 h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a white solid (9 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 2.93 (s, 12H), 2.94-3.14 (m, 4H), 3.56-3.61 (m, 2H), 3.70-3.75 (m, 2H), 3.81 (b, 2H), 4.05 (s, 4H). ^{13}C NMR (100 MHz, D_2O) δ 166.58, 58.07, 50.16, 43.84, 40.27, 36.42. LC-MS (ESI$^+$) m/z 381.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{14}H_{33}N_6O_2S_2^+$ [M + H]$^+$ 381.2101, found 381.2129.

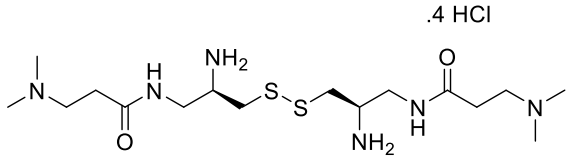
Di-*tert*-butyl((8*R*,13*R*)-2,19-dimethyl-5,16-dioxo-10,11-dithia-2,6,15,19-tetra-azaicosane-8,13-diyl)dicarbamate (144)



Compound **127** (40 mg, 0.097 mmol) was dissolved in 1 mL of dry DCM and DIEA (203 uL, 1.16 mmol) followed by 3-(dimethylamino)propionic acid hydrochloride (31 mg, 0.2 mmol) were added. PyAOP (104 mg, 0.2 mmol) was added to the stirred solution and the reaction mixture was left to stir at room temperature for 2 h. The reaction mixture was diluted with (15 mL) of water and extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with (25 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow oil was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 50%. The solvent was evaporated under reduced

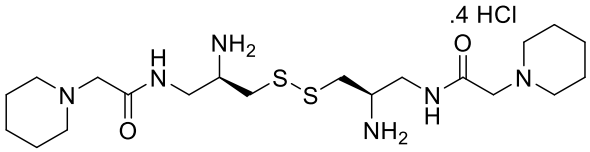
pressure to give a white solid (48 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 18H), 2.25 (s, 12H), 2.37 (t, 4H, *J*= 8 Hz), 2.55 (t, 4H, *J*= 8 Hz), 2.73 (b, 2H), 3.03 (b, 2H), 3.39-3.55 (m, 4H), 3.92 (b, 2H), 5.86 (b, 2H), 8.56 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.81, 155.76, 79.38, 55.17, 51.13, 44.65, 41.09, 40.99, 32.99, 28.44.

***N,N′*-((2*R*,2′*R*)-Disulfanediylbis(2-aminopropane-3,1-diyl))bis(3-(dimethylamino)propanamide) tetrahydrochloride (148)**



Compound **130A** (22 mg, 0.036 mmol) was dissolved in 500 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (244 uL, 0.976 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. Additional 4 M HCl/ dioxane (244 uL, 0.976 mmol) was added to the chilled solution in two portions and the mixture was warmed to room temperature and left stir for 3 h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a yellowish white solid (20 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 2.83-2.87 (m, 4H), 2.91 (s, 12H), 2.92-3.01 (m, 2H), 3.12-3.18 (m, 2H), 3.42-3.47 (m, 4H), 3.53-3.59 (m, 2H), 3.68-3.74 (m, 2H), 3.84 (b, 2H). ^{13}C NMR (100 MHz, D_2O) δ 172.95, 53.50, 50.48, 42.97, 40.30, 36.54, 29.64. LC-MS (ESI$^+$) m/z 409.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{16}H_{37}N_6O_2S_2^+$ [M + H]$^+$ 409.2414, found 409.2443.

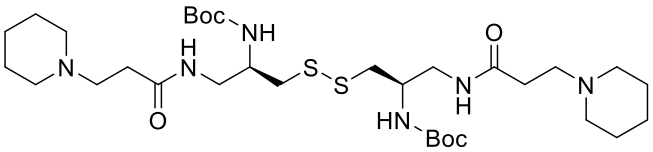
***N,N'*-((2*R*,2'*R*)-Disulfanediylbis(2-aminopropane-3,1-diyl))bis(2-(piperidin-1-yl)acetamide) tetrahydrochloride (149)**



Compound **127** (50 mg, 0.12 mmol) was dissolved in 1 mL of dry DCM and DIEA (128 uL, 0.73 mmol) followed by 2-(piperidin-1-yl)acetic acid hydrochloride (45 mg, 0.25 mmol) were added. PyAOP (130 mg, 0.25 mmol) was added to the stirred solution and the reaction mixture was left to stir at room temperature for 1.5 h. The reaction mixture was diluted with (15 mL) of water and extracted with (3 x 30 mL) of DCM. The combined organic layers were washed with (10 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 12%. The solvent was evaporated under reduced pressure to give a colorless oil (55 mg, crude 69%). Amberlyst A-15 was first soaked in MeOH for 24 h, washed with MeOH and then neutralized with 4 M ammonia in MeOH. The neutralized resin was acidified with 3 M HCl in 50% MeOH and rinsed with MeOH, THF, and DCM successively. The acidic capacity of the resin was calculated to be 3.5 mequiv/g. Compound 131A (30 mg, 0.045 mmol) crude was dissolved in 1 mL of dry DCM and cleaned Amberlyst A-15 (100 mg, 0.36 mmol) was added and the reaction mixture was left to stir at room temperature for 5 h. The resin was filtered and washed with DCM and MeOH excessively. The amine-bound resin was transferred to 1 mL of 3.5 N ammonia methanolic solution and was gently stirred for overnight. The resin was then filtered, and the filtrate was evaporated under reduced

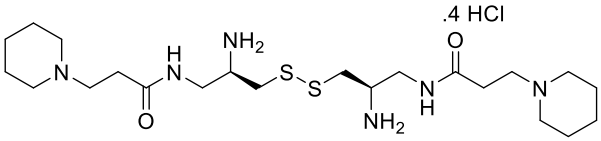
pressure then the residue was diluted with water and HCl and lyophilized to give a white solid (17 mg, 85%). ^{1}H NMR (400 MHz, D_2O) δ 1.40-1.52 (m, 2H), 1.72-1.93 (m, 10H), 2.93-3.16 (m, 8H), 3.52-3.63 (m, 6H), 3.71-3.76 (m, 2H), 3.83 (b, 2H), 4.00 (s, 4H). ^{13}C NMR (100 MHz, D_2O) δ 166.49, 56.94, 54.37, 50.21, 40.28, 36.47, 22.48, 20.78. LC-MS (ESI$^+$) m/z 461.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{20}H_{41}N_6O_2S_2^+$ [M + H]$^+$ 461.2727, found 461.2759.

Di-*tert*-butyl((2*R*,2′*R*)-disulfanediylbis(3-(3-(piperidin-1-yl)propanamido)propane-1,2-diyl))di-carbamate (146)



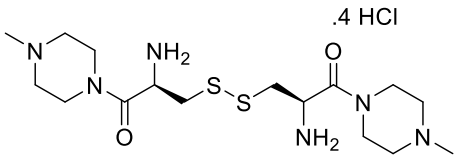
Compound **127** (45 mg, 0.11 mmol) was dissolved in 1 mL of dry DCM and DIEA (115 uL, 0.66 mmol) followed by 3-(piperidin-1-yl)propanoic acid (36 mg, 0.23 mmol) were added. PyAOP (121 mg, 0.23 mmol) was added to the stirred solution and the reaction mixture was left to stir at room temperature for 1.5 h. The reaction mixture was diluted with (15 mL) of water and extracted with (3 x 40 mL) of DCM. The combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.3 N NH_3/ DCM) and the product was eluted at 40%. The solvent was evaporated under reduced pressure to give a white solid (58 mg, 77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.39 (s, 22H), 1.53-1.58 (m, 8H), 2.33-2.38 (m, 12H), 2.51 (t, 4H, *J*= 4 Hz), 2.69 (b, 2H), 3.06 (b, 2H), 3.40-3.54 (m, 4H), 3.90 (b, 2H), 5.95 (b, 2H), 8.92 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.11, 155.65, 79.28, 54.52, 53.82, 50.98, 40.63, 40.34, 32.02, 28.42, 26.08, 24.24.

***N,N'*-((2*R*,2'*R*)-Disulfanediylbis(2-aminopropane-3,1-diyl))bis(3-(piperidin-1-yl)propanamide) tetrahydrochloride (150)**



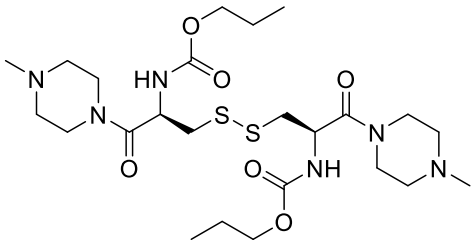
Compound **132A** (25 mg, 0.036 mmol) was dissolved in 500 uL of MeOH and cooled to 0 °C. 4 M HCl/ dioxane (244 uL, 0.98 mmol) in two portions was added to the chilled solution every half an hour and the solution was warmed to room temperature after each addition. Additional 4 M HCl/ dioxane (244 uL, 0.976 mmol) was added to the chilled solution in two portions and the mixture was warmed to room temperature and left stir for 2 h. The solvent was evaporated under reduced pressure and the residue was dried on pump. The residue then was dissolved in a minimal amount of methanol and recrystallized with an excess of diethyl ether and filtered. The residue was collected and dried to give a yellowish white solid (23 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O) δ 1.44-1.54 (m, 2H), 1.67-1.83 (m, 6H), 1.95 (b, 4H), 2.84 (t, 4H, *J*= 8 Hz), 2.94-3.01 (m, 6H), 3.14 (dd, 2H, *J*= 4, 16 Hz), 3.41 (t, 4H, *J*= 8 Hz), 3.53-3.58 (m, 6H), 3.68-3.73 (dd, 2H, *J*= 4, 16 Hz), 3.81-3.87 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 172.89, 53.46, 52.34, 50.43, 40.33, 36.56, 29.55, 22.79, 21.04. LC-MS (ESI$^+$) m/z 489.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{22}H_{45}N_6O_2S_2^+$ [M + H]$^+$ 489.3040, found 489.3072.

_L_-Cystine bis(_N′_-methylpiperazide) tetrahydrochloride (9)



To a solution of *N*,*N′*-bis(*tert*-butoxycarbonyl)-*L*-cystine bis(*N′*-methylpiperazide) (5.2 g, 8.60 mmol) in 50 mL of dichloromethane at 0 °C was added 4 M HCl in 1,4-dioxane (12.90 mL, 51.6 mmol). Solid precipitate formed. The reaction mixture was stirred at room temperature for 2 h. The solid was collected by filtration, rinsed with cold ether, and dried in a vacuum oven at 50 °C to give 2.45 g (71% yield) of the desired product. ^{1}H NMR (500 MHz, DMSOd6, 100 °C) δ 2.80 (s, 6H), 3.31 (b, 4H), 3.31−3.46 (m, 8H), 3.98 (b, 8H), 4.7 (t, 2H), 9.5 (b, 6H). ^{13}C NMR (500 MHz, CD_3OD) δ 167.36, 68.20, 54.88, 53.92, 51.01, 43.73, 40.66. LC-MS (ESI$^+$) m/z 404.9 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{16}H_{33}N_6O_2S_2^+$ [M + H]$^+$ 405.2101, found 405.2095.

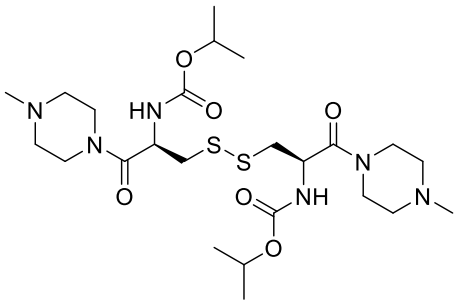
_N,N′_-Bis(propoxycarbonyl)-_L_-cystine bis(_N′_-methylpiperazide) (151)



Compound **9** (50 mg, 0.09 mmol) was suspended in 1 mL of dry DCM and cooled to 0 °C. DIEA (142 uL, 0.81 mmol) was added and gave a yellow clear solution then propylchloroformate (35 uL, 0.31 mmol) was added to the chilled solution and the reaction mixture was brought to room temperature and left to stir for 1 h. The reaction mixture was diluted with (20 mL) of cold water and extracted with (2 x 50 mL) of DCM. The combined organic layers were washed with (20 mL) of brine and dried over Na_2SO_4.

The solvent was evaporated under reduced pressure and the residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 60%. The solvent was evaporated under reduced pressure to give a colorless oil (50 mg, 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, 6H, *J*= 8 Hz), 1.56-1.63 (m, 4H), 2.38 (s, 6H), 2.56 (b, 8H), 2.95-3.05 (m, 4H), 3.70 (b, 8H), 3.97 (t, 4H, *J*= 4 Hz), 4.94 (m, 2H), 5.77 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.70, 156.11, 67.06, 54.70, 54.19, 49.48, 45.34, 45.09, 41.59, 22.26, 10.27. LC-MS (ESI$^+$) m/z 577.37 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{24}H_{45}N_6O_6S_2^+$ [M + H]$^+$ 577.2837, found 577.2872.

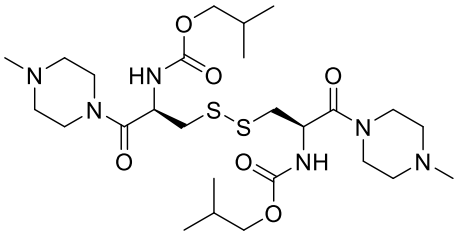
***N,N′*-Bis(isopropoxycarbonyl)-*L*-cystine bis(*N′*-methylpiperazide) (152)**



Compound **9** (50 mg, 0.09 mmol) was suspended in 1 mL of dry DCM and cooled to 0 °C. DIEA (142 uL, 0.81 mmol) was added and gave a yellow clear solution then isopropylchloroformate (112 uL, 0.81 mmol) was added to the chilled solution and the reaction mixture was brought to room temperature and left to stir for 1 h. There was still starting material and intermediate so additional DIEA (142 uL, 0.81 mmol) and isopropylchloroformate (112 uL, 0.81 mmol) were added to the mixture at 0 °C and the reaction mixture was brought to room temperature and left to stir for 1 h. The reaction mixture was diluted with (20 mL) of cold water and extracted with (2 x 50 mL) of DCM. The combined organic layers were washed with (20 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 70%.

The solvent was evaporated under reduced pressure to give a colorless oil (51 mg, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.14 (t, 12H, *J*= 8 Hz), 2.30 (s, 6H), 2.45 (b, 8H), 2.90-3.01 (m, 4H), 3.58 (b, 8H), 4.75-4.83 (m, 2H), 4.88 (m, 2H), 5.73 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.66, 155.55, 68.79, 54.80, 54.28, 53.63, 49.34, 45.54, 41.91, 22.08. LC-MS (ESI$^+$) m/z 577.4 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{24}H_{44}N_6O_6S_2^+$ [M + H]$^+$ 577.2836, found 577.2874.

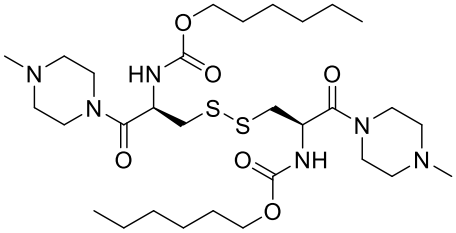
***N,N′*-Bis(isobutoxycarbonyl)-*L*-cystine bis(*N′*-methylpiperazide) (153)**



Compounnd **9** (50 mg, 0.09 mmol) was suspended in 1 mL of dry DCM and cooled to 0 °C. DIEA (142 uL, 0.81 mmol) was added and gave a yellow clear solution then isobutylchloroformate (41 uL, 0.31 mmol) was added to the chilled solution and the reaction mixture was brought to room temperature and left to stir for 1 h. The reaction mixture was diluted with (20 mL) of cold water and extracted with (2 x 50 mL) of DCM. The combined organic layers were washed with (20 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow residue was purified via ISCO (10% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 70%. The solvent was evaporated under reduced pressure to give a colorless oil (50 mg, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (d, 12H, *J*= 8 Hz), 1.83-1.92 (m, 2H), 2.32 (s, 6H), 2.43 (b, 8H), 2.99-3.08 (m, 4H), 3.65 (b, 8H), 3.83 (b, 4H), 4.98 (m, 2H), 5.70 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.61, 156.09, 71.53, 55.01, 54.44, 49.46, 45.70, 42.14, 41.63, 27.95, 19.02.

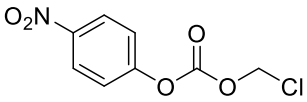
LC-MS (ESI$^+$) m/z 605.31 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{26}H_{49}N_6O_6S_2^+$ [M + H]$^+$ 605.3150, found 605.3185.

***N,N′*-Bis(hexoxycarbonyl)-*L*-cystine bis(*N′*-methylpiperazide) (154)**



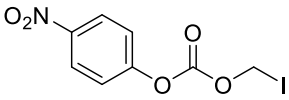
Compounnd **9** (50 mg, 0.09 mmol) was suspended in 1 mL of dry DCM and cooled to 0 °C. DIEA (142 uL, 0.81 mmol) was added and gave a yellow clear solution then N-hexylchloroformate (52 uL, 0.31 mmol) was added to the chilled solution and after 15 m the reaction mixture was brought to room temperature and left to stir for 4 h. The reaction mixture was diluted with (20 mL) of cold water and extracted with (3 x 50 mL) of DCM. The combined organic layers were washed with (30 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow oil was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 60%. The solvent was evaporated under reduced pressure to give a colorless oil (55 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (t, 6H, *J*= 8 Hz), 1.25 (s, 12H), 1.53-1.56 (m, 4H), 2.34 (s, 6H), 2.50 (b, 8H), 2.97-3.09 (m, 4H), 3.66 (b, 8H), 4.00 (t, 4H, *J*= 4 Hz), 4.94 (t, 2H, *J*= 4 Hz), 5.73 (b, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.58, 156.04, 65.58, 54.82, 54.27, 49.43, 45.55, 41.59, 41.14, 31.42, 28.88, 25.45, 22.51, 13.97. LC-MS (ESI$^+$) m/z 661.4 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{30}H_{57}N_6O_6S_2^+$ [M + H]$^+$ 661.3776, found 661.3820.

Chloromethyl (4-nitrophenyl) carbonate (155)



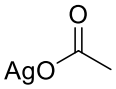
4-nitrophenol (1564 mg, 11.24 mmol) was suspended in 8 mL of dry DCM and cooled to 0 °C. Pyridine (909 uL, 11.24 mmol) was added and gave a yellow solution and then the solution was added dropwise to a chilled solution of chloromethyl chloroformate (1 mL, 11.24 mmol) in (8 mL) of dry DCM and was left to stir at 0 °C for 1 h. The mixture was brought to room temperature and left to stir for overnight. The reaction mixture was diluted with water and extracted with DCM and the organic layer was washed with water, brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 12%. The solvent was evaporated under reduced pressure to give a white solid (2110 mg, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.82 (s, 2H), 7.41 (dd, 2H, *J*= 4, 8 Hz,), 8.26 (dd, 2H, *J*= 4, 8 Hz,). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.90, 150.99, 145.80, 125.42, 121.66, 72.74.

Iodomethyl (4-nitrophenyl) carbonate (156)



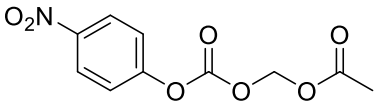
Compound **137** (200 mg, 0.9 mmol) and potassium iodide (3 g, 18 mmol) were added to 13 mL of dry acetone then the mixture was stirred at 50 °C for overnight. The reaction mixture was diluted with acetone and filtered. The filtrate was evaporated under reduced pressure to give a brown oil (291, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.06 (s, 2H), 7.41 (dd, 2H, *J*= 4, 8 Hz), 8.29 (dd, 2H, *J*= 4, 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.91, 150.74, 145.76, 125.44, 121.64, 33.28.

Acetoxysilver (157)



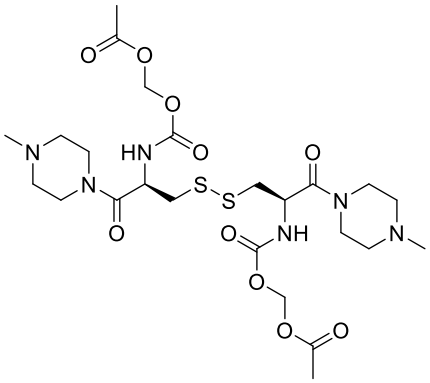
An aqueous solution of silver nitrate (170 mg, 1 mmol) in 1 mL of water was added to an equal volume of aqueous sodium acetate (99 mg, 1.2 mmol) in 1 mL of water. The reaction mixture was stirred at room temperature in dark for 1 h. The reaction mixture was filtered and washed with small portion of H_2O, MeOH, and hexane. A white solid was obtained (168 mg, quantitative yield). ^{1}H NMR (400 MHz, DMSO) δ 1.80 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 174.78, 23.50.

(((4-Nitrophenoxy)carbonyl)oxy)methyl acetate (159)



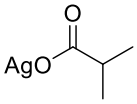
A mixture of compound **138** (145 mg, 0.45 mmol) and compound **139** (150 mg, 0.9 mmol) in 5.5 mL of anhydrous toluene was stirred at room temperature in dark for overnight. The reaction mixture was filtered with DCM washes and the filtrate was evaporated under reduced pressure and the yellow oil was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 20%. The solvent was evaporated under reduced pressure to give a light yellow oil (70 mg, 61%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.18 (s, 3H), 5.87 (s, 2H), 7.41 (d, 2H, *J*= 8 Hz), 8.29 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.38, 155.21, 151.62, 145.83, 125.53, 121.83, 82.64, 20.72.

_N_,_N′_-Bis(acetoxymethyloxycarbonyl)-_L_-cystine bis(_N′_-methylpiperazide) (161)



Compound **9** (41 mg, 0.07 mmol) was suspended in 0.5 mL of dry DCM and cooled to 0 °C. DIEA (120 uL, 0.68 mmol) was added and gave a yellow clear solution then a solution of compound **141** (57 mg, 0.22 mmol) in 0.5 mL of dry DCM was added slowly to the chilled solution and the reaction mixture was brought to room temperature and left to stir for 5 h. The reaction mixture was diluted with (20 mL) of cold water and extracted with (3 x 50 mL) of DCM. The combined organic layers were washed with (30 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow oil was purified via ISCO (20% MeOH: DCM + 0.1% TEA/ DCM) and the product was eluted at 85%. The solvent was evaporated under reduced pressure to give a colorless oil (40 mg, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.06 (s, 6H), 2.37 (s, 6H), 2.53 (b, 8H), 3.00 (m, 4H), 3.66 (b, 8H), 4.93 (m, 2H), 5.68 (dd, 4H, *J*= 8, 12 Hz), 6.17 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.70, 168.03, 154.15, 80.07, 54.67, 54.16, 49.68, 45.84, 45.21, 41.55, 20.77. LC-MS (ESI^+) m/z 637.3 $[M + H]^+$. HRMS (ESI^+) m/z calculated for $C_{24}H_{41}N_6O_{10}S_2^+$ $[M + H]^+$ 637.2320, found 637.2368.

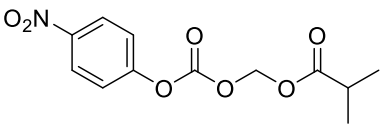
(Isobutyryloxy)silver (158)



An aqueous solution of NaOH (1034 mg, 25.87 mmol) in 40 mL of water was mixed with isobutyric acid (2 mL, 21.56 mmol) at 0 °C. Silver nitrate

(3662 mg, 21.56 mmol) in 40 mL of water was cooled to 0 °C and added dropwise to the chilled solution. The reaction mixture was stirred at room temperature in dark for 1 h. The reaction mixture was filtered and washed with small portion of H_2O, MeOH, and hexane. A greyish white solid was obtained (3600 mg, quantitative yield). ^{1}H NMR (400 MHz, DMSO) δ 1.05 (d, 6H, *J*= 8 Hz), 2.34-2.41 (m, 1H). ^{13}C NMR (100 MHz, DMSO) δ 180.45, 35.63, 20.66.

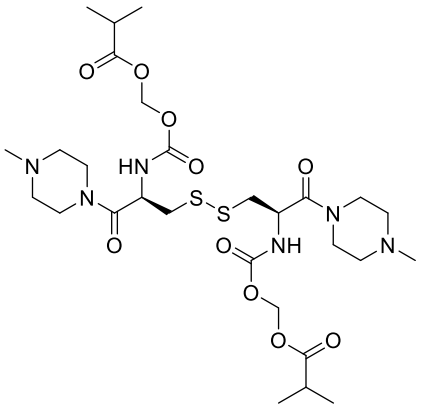
(((4-Nitrophenoxy)carbonyl)oxy)methyl isobutyrate (160)



A mixture of compound **138** (2874 mg, 8.9 mmol) and compound **140** (3 g, 17.8 mmol) in 60 mL of anhydrous toluene was stirred at room temperature in dark for overnight. The reaction mixture was filtered with DCM washes and the filtrate was evaporated under reduced pressure and the brown oil was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 10%. The solvent was evaporated under reduced pressure to give a light yellow oil (2 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (d, 6H, *J*= 8 Hz), 2.61-2.72 (m, 1H), 5.90 (s, 2H), 7.42 (d, 2H, *J*= 8 Hz), 8.29 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.33, 155.07, 151.42, 145.66, 125.43, 121.69, 82.67, 33.74, 18.58.

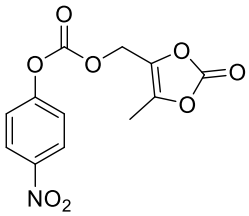
***N*,*N′*-Bis(isobutanoyloxymethyloxycarbonyl)-L-cystine bis(*N′*-methylpiperazide) (162)**

Compound **9** (600 mg, 1.09 mmol) was suspended in 20 mL of anhydrous DCM and cooled to 0 °C. DIEA (1.7 mL, 9.81 mmol) was added and gave a yellow clear solution



then a solution of compound **160** (926 mg, 3.27 mmol) in 5 mL of anhydrous DCM was added slowly to the chilled solution and the reaction mixture was brought to room temperature and left to stir for 3 h. The reaction mixture was diluted with (50 mL) of cold water and extracted with (3 x 100 mL) of DCM. The combined organic layers were washed with (30 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow oil was purified via ISCO (20% MeOH: DCM / DCM) and the product was eluted at 55%. The solvent was evaporated under reduced pressure and dried on pump to give a white solid (704 mg, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.15 (d, 12H, *J*= 8 Hz), 2.28 (s, 6H), 2.38 (m, 8H), 2.56 (m, 2H), 2.98 (m, 4H), 3.59 (m, 8H), 4.96 (m, 2H), 5.71 (dd, 4H, *J*= 4, 12 Hz), 6.08 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.82, 167.93, 154.12, 80.14, 55.04, 54.44, 52.84, 49.61, 45.84, 42.34, 41.52, 33.74, 18.67. LC-MS (ESI$^+$) m/z 693.3 [M + H]$^+$. HRMS (ESI$^+$) m/z calculated for $C_{28}H_{49}N_6O_{10}S_2^+$ [M + H]$^+$ 693.2946, found 693.2991.

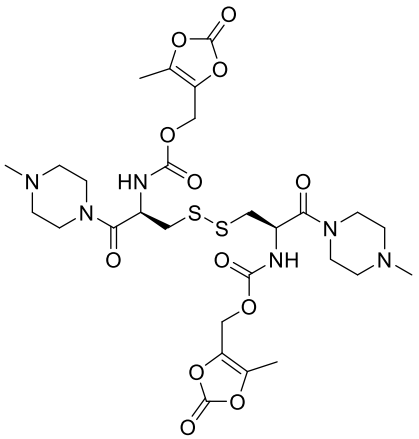
(5-Methyl-2-oxo-1,3-dioxol-4-yl)methyl (4-nitrophenyl) carbonate (163)



4-(hydroxymethyl)-5-methyl-1,3-dioxolenone (335 mg, 2.57 mmol) in 3 mL of anhydrous DCM was cooled to 0 °C. pyridine (229 uL, 2.83 mmol) was added then the solution was added dropwise to a chilled solution of 4-nitrophenylchloroformate (571 mg, 2.83 mmol) in 3 mL of anhydrous DCM and was left to stir at 0 °C for 1 h. The

mixture was brought to room temperature and left to stir for overnight. The reaction mixture was diluted with cold water and extracted with DCM and the organic layer was washed with water, brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified via ISCO (50% EtOAc: hexane/ hexane) and the product was eluted at 12%. The solvent was evaporated under reduced pressure to give a white solid (500 mg, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.18 (s, 3H), 5.02 (s, 2H), 7.36 (d, 2H, *J*= 8 Hz), 8.23 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.18, 152.20, 149.27, 145.59, 141.52, 132.29, 125.35, 121.77, 58.17, 9.39.

_N,N′_-Bis[(5-methyl-2-oxo-1,3-dioxol-4-yl)methoxycarbonyl]-_L_-cystine bis(_N′_-methyl-piperazide) (164)



Compound **9** (100 mg, 0.18 mmol) was suspended in 1 mL of anhydrous DCM and DIEA (286 uL, 1.64 mmol) was added and gave a yellow clear solution then was cooled to 0 °C. A solution of compound **145** (268 mg, 0.91 mmol) in 1 mL of anhydrous DCM was added slowly to the chilled solution and the reaction mixture was brought to room temperature and left to stir for 5 h. The reaction mixture was diluted with (20 mL) of cold water and extracted with (3 x 50 mL) of DCM. The combined organic layers were washed with (30 mL) of brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the yellow oil was purified via ISCO (20% MeOH: DCM/ DCM) and the product was eluted at 30%. The solvent was evaporated under reduced pressure to give a colorless

oil (100 mg, 77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.17 (s, 6H), 2.31 (s, 6H), 2.40 (b, 8H), 2.86-3.11 (m, 4H), 3.62 (b, 8H), 4.83 (s, 4H), 4.95 (b, 2H), 5.96 (d, 2H, *J*= 8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.90, 154.94, 152.11, 140.01, 133.69, 55.02, 54.44, 49.99, 45.86, 42.35, 41.82, 9.40. LC-MS (ESI^+) m/z 717.3 $[M + H]^+$. HRMS (ESI^+) m/z calculated for $C_{28}H_{41}N_6O_{12}S_2^+$ $[M + H]^+$ 717.2218, found 717.2277.

4.2 Effect on the Metastable Supersaturation Range of L-Cystine

For the preparation of L-cystine supersaturated solution 70 mg of L-cystine was dissolved in 100 mL of Millipore water (~3 mM) under reflux at 120 °C for 30 min until the L-cystine was completely dissolved. The supersaturated solution was then allowed to cool slowly with stirring until the oil bath temperature reached 40 °C take about 70 min depending on the lab atmosphere. To determine the L-cystine concentration all test compounds were dissolved in water to form 10 mM stock solution. Five µL of each solution was added to 495 µL L-cystine supersaturated solution. The mixtures were allowed to stand at 25 °C for 72 h. At the end of incubation, the mixtures were centrifuged at 13,000 rpm for 5 min and the supernatants were diluted 10-fold for concentration measurement. To Construct the standard curves L-Cystine (2.1 mg) was dissolved in 0.1 M Na_2CO_3 solution (9 mL) to form a 1 mM solution as a stock solution. Then, 50 µL L-cystine stock solution was diluted to 0.6 mM with 0.1 M Na_2CO_3 (33.3 µL) solution and to 0.4, 0.2, 0.1, 0.05 mM solution using Millipore water 75, 200, 450 and 950 µL, respectively.

4.3 Fluorescence Assay for the Inhibition of L-Cystine Crystal Formation

10 µL of each L-cystine solutions or test compound diluted mixtures, 100 µL of DTT solution (12.5 mM) in 0.1 M dibasic sodium phosphate solution (1:9), were mixed at r.t. for 10 min, before the addition of 10 µL of iodoacetic acid (100 mM) and continued incubation at r.t. for an additional 15 min. This was then followed by the addition of 20 µL of OPA (100 mM in methanol)/NBC (100 mM in methanol) (1:1). The derivatization was allowed to proceed for 3 min before 3 x 40 µL of the mixture were placed in a 384-well plate and read at Ex 355 nm/Em 460 nm. The standard curve was repeated for each set of experiments and used to calculate the concentration of L-cystine in each sample.

4.4 Cyanide-nitroprusside Colorimetric Assay

3 x 40 µL of each L-cystine solutions or test compound diluted mixtures were placed in a 96-well plate (black, clear bottom). 80 µL of aqueous solution of potassium cyanide (30% w/v) in PBS (pH= 7.4) (1:7) was added to each well then the plate was left to shake at 550 rpm for 25 min. 10 µL of aqueous solution of sodium nitroprusside (20% w/v) was added to each well using automated dispenser then the plate was centrifuged immediately at 1500 rpm for 20 sec. Then the UV absorbance was measured at 530 nm using Victor 3 V plate reader. The standard curve was repeated for each plate and used to calculate the concentration of L-cystine in each sample.

4.5 Chemical Stability Measurement

Solutions of 100 µM test compounds in phosphate buffered saline were incubated at 37 °C. Aliquots (20 µL) were taken at different time intervals and frozen at -20 °C. The aliquots were diluted 5-fold with water to 100 µL prior to analysis with LC-MS/MS in positive MRM mode in the case of L-CDME (269.5-134.4) and **8** (379.5-189.6) or acetylation in the case of **9** with 1 mM Ac_2O at r.t for 30 min followed by analysis by LC-MS/MS in positive MRM mode (489.2-101.0) on an API 3000 (AB Sciex, Framingham, MA). Chromatographic separation was achieved with a 2x20 mm, 5 µm, C4 reversed phase column using a water/methanol gradient containing 0.1% formic acid (5-90%) at a flowrate of 250 µL/min. The standard curves were constructed with standards of L-CDME, **8** and **9**, respectively.

4.6 Pharmacokinetic-blood

The blood samples were centrifuged at 3000 rpm for 10 min then 10 µL of plasma was collected. 10 µL of TCEP (10 mM) was then added and the mixture was vortexed and incubated for 30 min. Then 10 µL of NH_4OH (100 mM) was added followed by 20 µL of iodoacetamide (20 mM) and the mixture was vortexed and incubated for 30 min. This was followed by adding 150 µL of cold ACN and the mixture was vortexed and incubated for 30 min on ice for protein precipitation. Then the sample would be centrifuged at 13000 rpm for 5 min and 80 µL of supernatant was injected to LC-MS/MS in positive MRM mode (261.2-101.0) on an API 3000 (AB Sciex, Framingham, MA).

Chromatographic separation was achieved with a 20x2.1 mm, 5 µm, PFP column using a 10 mM NH_4HCO/H_2O and 10 mM NH_4HCO/90% ACN/H_2O gradient containing 0.125% formic acid (80-60%B) at a flowrate of 250 µL/min. The standard curves were constructed with standards of derivatized **9** as well.

4.7 Pharmacokinetic-urine

25 µL of urine samples was mixed with 25 µL of TCEP (10 mM) the mixture was then vortexed and incubated for 30 min. Then 25 µL of NH_4OH (100 mM) was added followed by 50 µL of iodoacetamide (20 mM) and the mixture was vortexed and incubated for 30 min. This was followed by adding 375 µL of cold ACN and the mixture was vortexed and incubated for 30 min on ice for protein precipitation. Then the samples were centrifuged at 13000 rpm for 5 min and 150 µL of supernatant was injected to LC-MS/MS in positive MRM mode (261.2-101.0) on an API 3000 (AB Sciex, Framingham, MA). Chromatographic separation was achieved with a 20x2.1 mm, 5 µm, PFP column using a 10 mM NH_4HCO/H_2O and 10 mM NH_4HCO/90% ACN/H_2O gradient containing 0.125% formic acid (80-60%B) at a flowrate of 250 µL/min. The standard curves were constructed with standards of derivatized **9** as well.

4.8 Prodrug Activation in Plasma

100 µL of each prodrug (2 mM in 10% DMSO/H_2O) was added to 400 µL of 50 mM phosphate buffer containing 1 mM EDTA at pH 7.4, mixed and incubated at 37 °C for 5 min, centrifuges and the supernatant were split into two 230 µL fractions. The fractions

were diluted with equal volume of water or human serum and the mixture were incubated at 37 °C. 20 µL aliquots were taken starting from 5 min and quenched with 40 µL of cold ACN/MeOH (2:1), vortexed and stored on ice for 30 min. The samples were then centrifuged at 9000 rpm for 5 min and 40 µL of supernatant was diluted with 40 µL of water then the samples were stored frozen before LC/MS analysis.

4.9 Prodrug Activation in Liver Microsomes

100 µL of each prodrug (2 mM in 10% DMSO/H_2O) was added to 875 µL of 50 mM phosphate buffer containing 1 mM EDTA at pH 7.4, mixed and incubated at 37 °C for 5 min, centrifuges and the supernatant were split into three 292.5 µL fractions. Water, mouse liver microsomes or human liver microsomes (7.5 µL) were added to the fractions and the mixtures were incubated at 37 °C. 20 µL aliquots were taken starting from 5 min and quenched with 40 µL of cold ACN/MeOH (2:1), vortexed and stored on ice for 30 min. The samples were then centrifuged at 9000 rpm for 5 min and 40 µL of supernatant was diluted with 40 µL of water then the samples were stored frozen before LC/MS analysis.

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