UNIVERSITY OF
EASTERN FINLAND
Faculty of Science and Forestry

SYNTHESIS AND USE OF POLYAMINE DERIVATIVES AS INTERNAL MASS SPECTROMETRY STANDARDS

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PREFACE

The present study was carried out in the laboratory of Professor Jouko Vepsäläinen, Department of Biosciences, Kuopio campus, Snellmania building, university of Eastern Finland during the session 2011 and 2012. After my keen work in laboratory and research I am finally done with my writing.

I have obtained a good deal of knowledge about Polyamines especially TETA, MAT, DAT and some learned to use some analytical techniques like NMR and mass spectrometry. The knowledge about different methods and techniques that I have used in laboratory is valuable for my future in the research field.

I would like to present my hearty thanks to my supervisor Janne Weisell whose constant guidance enabled me to full fill all the research and writing work of my thesis. I would also like to thank Professor Jouko Vepsäläinen, who was always there in every kind of problem during my synthesis work to assist me with his vast experience. I also want to thank Marita Salminkoski for her technical assistance during my thesis work. My special thanks to Tapani Pakkanen and Mari Heiskanen for their support.

I am really great full for the prayers of my parents, brothers and sisters as they are the real source of inspiration for me.

A bundle of thanks to all my friends without you this work could have been impossible.

ABSTRACT

UNIVERSITY OF EASTERN FINLAND, Faculty of Science and Forestry, M.Sc. program in Research Chemistry, Medicinal Chemistry

DAR, SAMI, ULLAH: SYNTHESIS AND USE OF POLYAMINE DERIVATIVES AS INTERNAL MASS SPECTROMETRY STANDARDS

M.Sc. Thesis 46 pages

Supervisors: M.Sc. Researcher Janne Weisell, School of Pharmacy

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Key words: Polyamines, deuterated analogues, TETA, MAT, DAT.

Systemic and metabolic studies reveal that many biogenic polyamines play a pivotal role in different functions of the cells like growth, interaction and proliferation and hence can serve as potential targets for drug discovery and development. TETA has played a significant role in treatment of various diseases like Wilson´s disease as a synthetic drug molecule in clinics. The purpose of the project is to prepare deuterated TETA and its deuterated metabolites MAT and DAT as they also play important role in different biological processes.

The first novel synthetic chemical route was initiated by using N, N- dibenzylethylenediamine as a starting reagent. After getting the product from the first step, the synthesis was continued by applying the chemical methods of reduction and acetylation to get the desired product. The different synthesis steps which were applied for the deprotection have not been successful in removing aromatic groups of the penultimate product hence this synthetic route did not work.

The second synthetic route was initiated by using ethylenediamine as a starting reagent. After getting the product from the first step in dinitrile form, the synthesis was continued by introducing protective group BOC to the first product. Then methods of reduction and acetylation were applied to get the desired products. Reduction was also performed with the deuterated reducing agents in order to get the deuterated product. The last step of deprotection was performed successfully by using a mixture of 60% TFA in DCM to give one of our desired products DAT.

ABBREVIATIONS

ACN	Acetonitrile
AdoMetDC	*S*-adenosylmethionine decarboxylase
AGEs	Advanced glycation end products
AMD	Age-related molecular degeneration
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AZ	Antizyme
Boc	Tert-butoxycarbonyl
DCM	Dichloromethylene
DEAD	Diethylazodicarboxylate
dcSAM	Decarboxylase *S*-adenosylmethionine decarboxylase
DMF	Dimethyl formamide
DNA	Deoxy ribonucleic acid
DFMO	Difluoromethylornithine
DAT	N^1, N^{10}-diacetyltriethylenetetramine
ERα	Eestrogen receptor α
Fmoc	Fluorenylmethyloxycarbonyl
FGF-1	Fibroblast growth factor-1
HPLC	High performance liquid chromatography
IF5A	Initiation factor 5A
IL	Interleukin
KIR	Inwardly rectifying K+
LV	Left ventricular
LC	Liquid chromatography
mRNA	Messenger ribonucleic acid
MAT	N^1-acetyltriethylenetetramine
MTA	5´-methylthioadenosine
MAPK	Mitogen-activated protein kinase
MS	Mass spectrometry
MeOH	Methanol
NMDA	*N*-methyl-D-aspartate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NMR	Nuclear magnetic resonance
ODC	Ornithine decarboxylase
Put	Putrescine
PAE	Polyaspartic ester
PARP	Poly (ADP-ribose) polymerase
Spd	Spermidine
Spm	Spermine
SAM	*S*-adenosylmethionine decarboxylase
SSAT	Spermidine/spermine N (1)-acetyltransferase
SMO	Spermine oxidase
TETA	Triethylenetetraamine
THF	Tetrahydrofuran
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TOF-MS	Time of flight Mass spectrometer
Ub	Ubiquitination
UV	Ultra violet
VEGF-1	Vascular endothelial growth factor-1

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1. Introduction

Systemic and metabolic studies always play a very significant and vital role in drug discovery as they present the fundamental background which is essentially required before the clinical usage of drugs by giving information on the drug safety and efficacy. These studies are also pivotal in identifying new targets of therapeutic importance hence they play very important function in the development, testing and approval of the drugs.

The main goal in this thesis work is to prepare deuterated triethylenetetraamine (TETA) and its terminally mono- and diacetylated deuterated metabolites, N^1-acetyltriethylenetetramine (MAT) and N^1, N^{10}-diacetyltriethylenetetramine (DAT) respectively. After successful preparation of deuterated TETA and its metabolites, they will be used as internal standard in mass spectrometry (MS) for their analytical investigation. Due to very polar structure of TETA it elutes very inefficiently from conventional high performance liquid chromatography (HPLC) columns. It also absorbs poorly at accessible ultra violet (UV) detection wavelength. There are several methods which have been applied recently. One of them is fluorescent-labelling reagents method which is very helpful to derivatize TETA and its derivatives. The fluorescent-labelling method is very challenging and highly demanding as it requires a high level of accuracy while labelling analyte and also the detection of peaks weather they are separated from their respective metabolites or from other metabolites or not. An HPLC conductivity method has also been developed but it renders poor sensitivity due to high detection limit. TETA is spermidine (Spd) analogue and it is used for many clinical purposes nowadays. The detection of its two major metabolites MAT and DAT from biological samples matrix is quite difficult as these metabolites have the ability of undergoing structural rearrangement during their separation from the samples. In order to overcome all these analytical problems, one of the best way of detection of these molecules is to use quantitative mass spectrometry but for that purpose we require labelled derivatives of the original molecules.

In order to prepare these molecules in laboratory we developed two synthesis routes starting from different starting materials and reagents. In first method our aim is to test a different synthetic route to prepare TETA, MAT and DAT starting from the N, N´- dibenzylethylenediamine. This synthetic route consists of four different steps of dinitrile formation, reduction, acetylation and deprotection of protective groups. The description of each synthetic step is given in experimental section in detail. The synthesis is first tested with non deuterated derivatives. Every time a new deuterated molecule is synthesized, the synthetic route is first tested with the non-deuterated compounds.

In second method our aim is to test another different synthetic route to prepare TETA, MAT and DAT starting from the diethyleneamine. This synthetic route is also consists of five different steps including dinitrile formation, Boc protection, reduction, acetylation and deprotection. Step six reduction was performed with both deuterated and non- deuterated reagents. This scheme along with the description of each synthetic step is given ahead in experimental section in detail.

A. Literature Review

2. Polyamines

The term polyamines clearly indicates that these are a group of compounds containing more than one (-NH) amine group in their structure at different positions in the molecules. There has been an ever increasing interest during the last two decades in naturally occurring polyamines, putrescine (Put: 1,4-diaminobutane), spermidine (Spd: N-(3 aminopropyl)-1,4-butadiamine), (4-azaoctane-1,8-diamine) and spermine (Spm: N,N'- bis (3-aminopropyl-1,4-butanediamine), (4,9-diazadodecane-1,12-diamine)(Figure 1).[1] The fact behind this ever growing interest is that, their specific functions in cells is yet not much clear and still needs further investigation. Their unique nature and ubiquitous distribution, their peculiar behaviour in cells and increase in concentration in rapidly growing cells make this group of compounds an area of modern research for scientists.

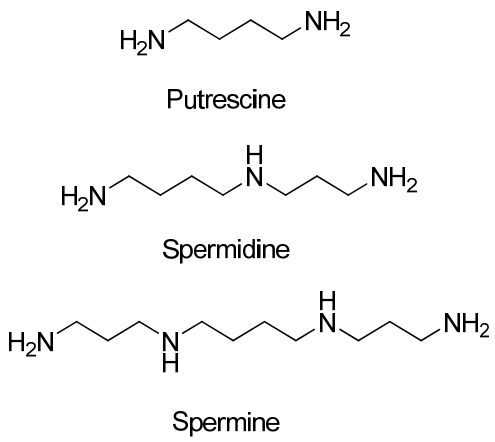


Figure 1 Structure of polyamines

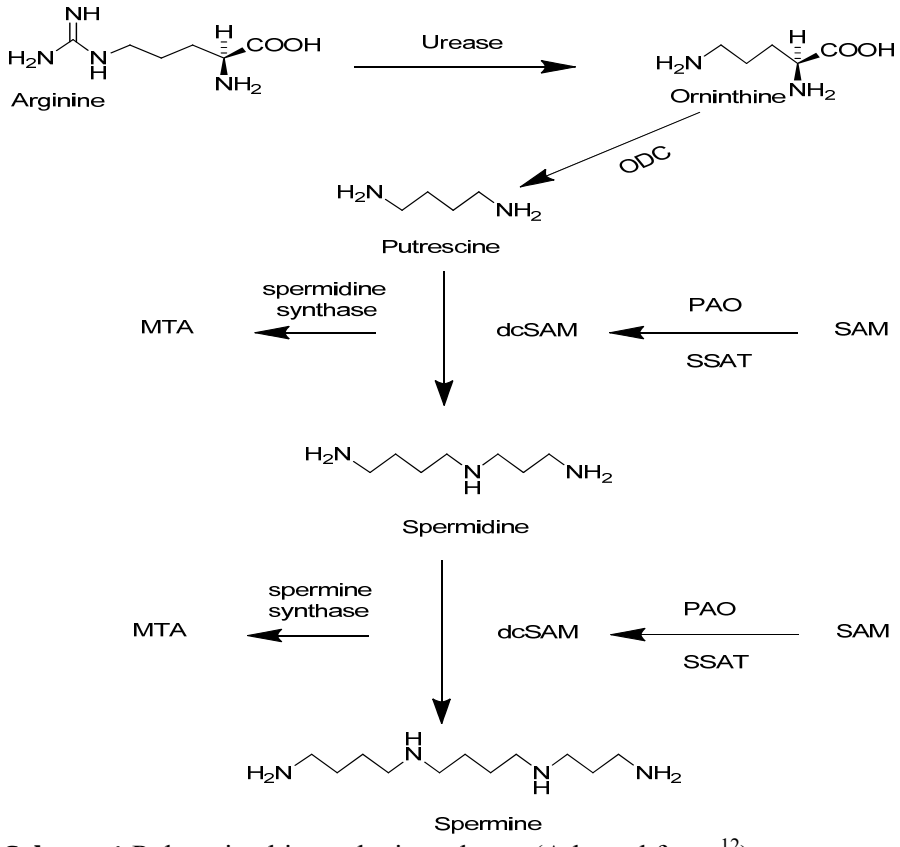
The first ever discovery of the polyamines was in 1678 when Antonie van Leeuwenhoek isolated some 'tri-sided' crystals from human semen.[2] The empirical formula of these crystals was deduced in 1924[3] and it took further two years to synthesize these newly discovered products chemically.[4] Therefore the names Spd and Spm reflect the original discovery as they have been isolated from human semen. Put was first isolated from a bacterium *Vibrio cholerae*, but its common name was derived from the large quantities found in putrefying flesh.[5] Polyamines are found almost in every living species, except two orders of Archaea, Methanobacteriales and Halobacterials.[6] This strange behaviour of their presence in almost every species across evolution is a peculiar feature in that it argues for their importance in cellular survival, but it can also be a short coming as it implies as a lack of specific function.[7]

Polycationic polyamines and their diamine precursor Put are apparently simple in structure, but they have very strong role to play as growth factors in eukaryotic cells.[8]The total intracellular concentration of polyamines is in the millimolar range; however, freely available amount of polyamines are considerably lower. This is partly because of the reason that they are having ionic interactions to various anions in the cell including nucleic acids, proteins and phospholipids. Polyamines play their role in the support of cell growth and survival. They are associated with the nucleic acids and maintenance of chromatin conformation. They regulate specific gene expressions and ion-channel functions.[9]The maintenance of membrane stability, provision of a precursor in the synthesis of eukaryotic translation initiation factor 5A (IF5A), free-radical scavenging and antioxidant activity is also come under the area of their influence .[10, 11]

3. Synthesis of Polyamines

3.1 In *Vivo*

Biogenic polyamines are widely distributed in both animal and plant kingdom. Due to their immense impact in number of biological processes and variety of pharmacological attributes their biosynthetic pathway is of great importance, depicted in (Scheme 1).



Scheme 1 Polyamine biosynthetic pathway (Adopted from [12])

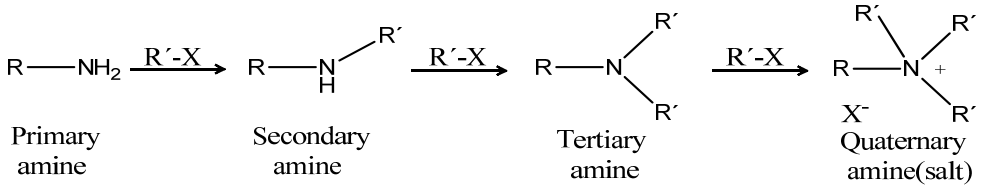
There is a lot of data available including different articles and reviews describing the biosynthetic pathway of polyamines metabolism.[18] By considering the importance of most vital steps which take place in most organisms, this pathway is briefly illustrated in this literature review. In first step an enzyme urease converts arginine to ornithine. In second step ortnihine is decarboxylated by the enzyme ornithine decarboxylase (ODC) to convert it to Put, the simplest polyamine which is the rate limiting step of the biosynthetic pathway. The aminopropyl groups in Spm and Spd are derived from decarboxylase *S*-adenosylmethionine decarboxylase (dcSAM), which is formed from *S*-adenosylmethionine decarboxylase (SAM) in a reaction catalysed by *S*-adenosylmethionine decarboxylase (AdoMetDC). The aminopropyl transfer reactions are catalysed by spermine and spermidine synthases generating 5´-methylthioadenosine (MTA) in addition to Spm and Spd.

3.2 In *Vitro*

Beside this biosynthetic pathway which produces polyamines in living species, scientists have developed some synthetic pathways for the synthesis of the polyamines in laboratories to study their different biochemical properties in detail. The general synthetic pathways used in polyamines chemistry can be divided in to five main groups.

3.2.1 Alkylation Reactions

These are the most widely applied reactions for the synthesis of the polyamines. The extension of polyamine chains can be readily achieved by this process hence this method is progressively used for the synthesis of secondary, tertiary and quaternary amines from the primary amine groups (Scheme 2).

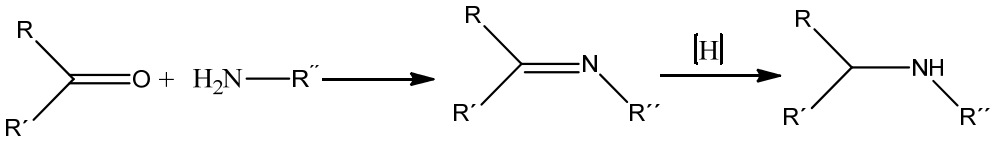


Scheme 2 Alkylation of amines (Adopted from [12])

As an example of the alkylation reaction in polyamines synthesis, the low cost corrosion inhibitors of steel can be prepared by reacting different polyamines with oleic acid at 1:1 molar ratio and then this amide mixture is made to react with benzyl chloride to get alkylated. This alkylated amide can be used as an active component for the inhibitory action against corrosion of steel in acidic and water-saline media.[13]

3.2.2 Reduction Reactions

Reduction of variety of functional groups to the amino group using a range of reducing agents is one of the simplest methods for the synthesis of the polyamines. The functional groups which are mostly used in the preparation of polyamines are amides, nitriles, nitro groups and azides. The synthesis of polyamines has also been applied in polyamine chemistry by reduction of the Schiff´s bases. The general reaction first involves the condensation reaction followed by the reduction to give the substituted amines (Scheme 3).



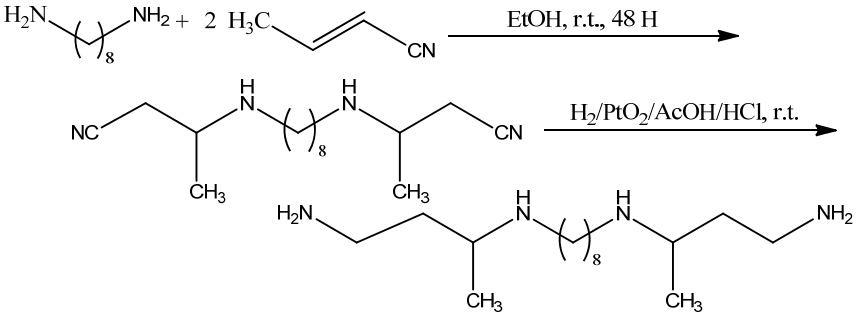
R, R´, R´´= H, alkyl, aryl, etc.

Scheme 3 Formation of amines by reduction of Schiff´s bases (Adopted from [12])

As an example of reduction reaction in polyamines synthesis, a novel macrobicyclic polyamine compound is synthesized through "tripod-tripod coupling" method using Schiff base condensation reaction. This formed complex is further reduced by sodium borohydride. This macrobicyclic compound is used in recognition of the oxalate.[14]

3.2.3 Michael Addition Reactions

This is also a general method for the synthesis of polyamines involving addition of amines to alkenes. In this method stepwise process of polyamine chains extension is usually applied to get the large sized polyamine chains. Tetraamines have been prepared by the addition of diamines with acrylonitrile giving the product by subsequent reduction (scheme 4).



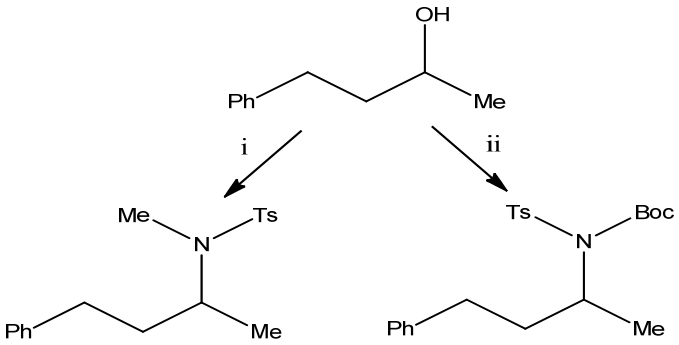
Scheme 4 An example of Michael addition reaction (Adopted from [12])

As an example of Michael addition reaction in polyamines synthesis, a novel type of polyaspartic ester (PAE) chain extender having name PAE-f was prepared by Michael addition

reaction. The reagents 4,4´-methylenebis (2-Me cyclohexyl amine) is made to react with excess amount of dialkyl maleates. Then this residual dialkyl meleate is reacted with polyester polyamine Jeffamine D230. This two step synthesis considerably reduces the synthesis time in industry and gives the product a great tensile strength and module and hardness.[15]

3.2.4 Mitsunobu Reactions

It is kind of condensation reaction involving a primary or secondary alcohol and compound containing an acidic hydrogen (e.g. sulphonamide) in the presence of triphenylphosphine and diethylazodicarboxylate (DEAD). This reaction has been of great success in the production of substituted polyamines (scheme 5).



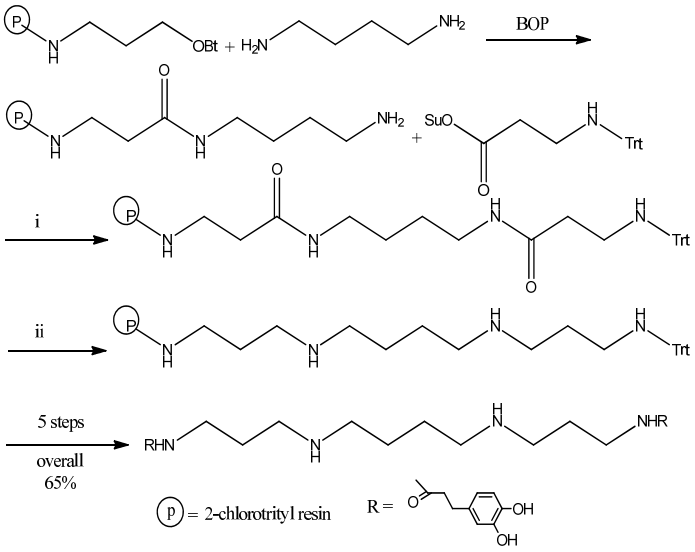
Scheme 5 An example of the Mitsunobu reaction (Adopted from [12]) (Reagents and Conditions: i) N-methyl-p-toluene sulfonamide:DEAD/THF/Ph3P, r.t; ii) N-Boc-p-toluene sulfonamide:DEAD/THF/Ph3P, r.t;)

As an example of Mitsunobu reaction in polyamines synthesis, a compound 3,24-Bis(6-chloropyridin-2-yloxy)cholane has been prepared by Mitsunobu reaction using a reagent cholane-3,24-diol. This reaction proved successful in synthesizing various polyaza macrocycles via palladium catalyzed amination with linear polyamines. Cyclic oligomers are produced as side product in these reactions. The polyamine nature of the reagent has been found responsible for the side formation of the cyclic oligomers.[16]

3.2.5 Solid Phase Synthesis of Polyamines

The increasing high demand for more complex polyamines, monofuntionalized polyamines, unsymmetrical polyamines and polyamines chemical libraries has laid greater stress to the application of the solid phase approach to the synthesis of these complex compounds. This method has many advantages over the traditional solution phase synthesis especially in the simplification of the reaction procedures, ease of purification steps and application to the

automated system. These reactions either involve the use of different resins as solid support for the reactants or commercially available substrate during the reactions (Scheme 6).



Scheme 6 An example of solid phase synthesis of polyamines (Adopted from [12]) (Reagents and conditions: i) Et3N; ii) B2H6, THF, 2 d, heat)

For example, a selective protection of symmetrical oligoethylenimine precursors with Fluorenylmethyloxycarbonyl (Fmoc) followed by introduction of a carboxylic acid handle using cyclic anhydride has been used for the production of four different Fmoc-polyamino acid building blocks. This is a versatile solid-phase approach to develop sequence-defined polyamidoamines.[17]This simple method of building polyamine blocks along with the high efficiency of the solid-phase coupling reaction permit the unique synthesis of defined polycations.

4. Charge in Polyamines

Charge is the fundamental property of any chemical entity that gives it peculiar properties in terms of its reactivity in different chemical and biological systems. The same implication of charge uniqueness can be applied on the polyamines. Polyamines contain positive charge on each nitrogen atom in their structure at physiological pH. So it has been suggested that polyamines as simply 'supercations', equivalent to one or two calcium or magnesium molecules. The characteristic feature which makes polyamines unique and distinct from the point charges of the cellular bivalent cations is that the charge on them is distributed along the entire length of the carbon chain.[18] The positive charge on polyamines enables their interaction electrostatically with polyanionic bio-macromolecules within the cells and tissues.

Spd and Spm have ability to bridge up the gap between major and minor grooves of the deoxyribonucleic acid (DNA). They can act as a clamp holding together either two different DNA chains or the distinct part of the same molecule. The studies regarding interaction of polyamines have shown that they prefer to interact with individual chains of DNA rather than multiple DNA molecules.[19] Crystallographic studies with polyamines indicate that they bind selectivity to the secondary structure of DNA.[20] It also indicates polyamines preference for binding with pyrimidine residues, particularly thymidine. This might be influenced by the neighbouring nucleotides and the nature of the secondary structure of DNA. Analogues of polyamines such as bis(ethyl) homospermine ('BEHSpm'; 'BE-4-4-4') have an effect to alter the DNA-nuclear matrix interaction owing to their unique charge. Due to this polyamines create an impact in changing the structure of DNA and also influence its different functions.[21] In nucleosome, deficiency of polyamines can results in partial unwinding of the DNA chains. They can also unmask the complementary sequences previously hidden in the nucleosome.[22] These newly discovered sequences are potential sites for transcription regulating factors. The ability of polyamines to favour the formation of triplex DNA at neutral pH, may provide a mechanism by which polyamines regulate the transcription of the growth regulatory genes such as *c-myc*.[23]

Polyamines can also interact with acidic phospholipids in cellular membranes in addition to their interaction with nucleic acids.[24] Interaction of polyamines with phospholipids and proteins increase the rigidity of membranes by forming complexes with them.[25] They may also play the role of antioxidant, preventing lipid per oxidation. Polyamines have been implicated in the regulation of many membrane bound enzymes, including tissues transglutaminase, adenylate cyclise, and some

ion channels such as *N*-methyl-D-aspartate (NMDA), inwardly rectifying K+(KIR) and voltage-activated Ca^{2+} channels. [26]

Transport of polyamines across the mitochondria membrane in different cells occurs through controlled electrostatic interactions by a specific uniporter system. Although different catalytic rate constants values do not argue the occurrence of changes with of number of positive charges of the polyamine backbone, but Michaelis-Menten constant values highly support the dependence of electrostatic forces controlling the docking of substrate into polyamine channel. By considering this kinetic data in terms of Gibbs equation also proves the contribution of positive charges of polyamines in their transport.[27]

Assuming the charge as an integral defining feature of the polyamines, then definitely single polycation would be sufficient enough to bring about the biochemical changes in the cells but this is not yet proven with the experimental facts. The most obvious choice for different reactions, synthesis and analysis would be Spm, due to its greatest charge, longest length and most flexibility. The sheer complexity and unique nature of polyamine regulation and metabolism compels that they, or their associated enzyme activities, have other critical functions within the cells, not based on solely on direct charge-charge interaction.[18]

5. Polyamines Analogues

Chemical structural analogues are the compounds which are structurally very similar to the other compounds differing only by a certain component or a functional group or an atom but can be distinguished from each other on the basis of physical, chemical, biological and pharmaceutical properties. Analogues of polyamines are very important chemical species as they can be used in different studies due to their pharmacological and biological importance in drug discovery, development, design, analysis and synthesis.

The targeted inhibition of specific biosynthetic enzymes yet not made substantial clinical success in the treatment of cancer in the polyamine metabolic pathway. But there is a huge amount of data that can determine polyamines function and metabolism as new rational targets for anti-neoplastic drug development and discovery. Therefore, the more logical and recently applied strategy has been to use the self-regulatory nature of the polyamine metabolism through the help of polyamine analogues. The main reasons to adopt this strategy were to overcome the limitations of specific enzyme inhibitors. The compensatory effect of up-regulation in biosynthesis and the uptake induced when one of the blocked biosynthetic enzymes was also considered.[28]

The properties of best analogues which can act as inhibitors or anti metabolites would be to use the polyamine transporter to get entry into the cells. They should compete with natural polyamines for uptake and down-regulate multiple polyamine biosynthetic enzymes in order to prevent the potential increase in untargeted enzymes and up-regulate polyamine catabolism. This would cause greater deficiency of intracellular polyamine than would be afforded by simply decreasing synthesis. They are not substitute for the natural polyamines in growth-related functions but do exhibit tumour-selective activity. On the other hand, there are also some polyamine analogues which can replace the function of natural polyamines. For example, β-methylated Spd is capable of restoring the growth of *Leishmania donovani* and α-methylated Spm is used as an antidote in host-parasite system where inhibitors of the polyamine biosynthesis are used for the therapy of leishmaniasis.[29]

Different classes of the antimetabolic compounds based on the way that how the polyamine backbone in any parent molecule has been substituted, can be discussed as under. Namely these are symmetrically substituted, asymmetrically substituted, conformation ally restricted, oligoamines

and macrocyclic polyamines. All the above mentioned polyamines analogues only act polyamines antimetabolites.

5.1. Symmetrically substituted bis(alkyl)

The first group of compounds which meet the properties of best analogues criteria were synthesized and reported by Bergeron and his co-workers named as bis(alkyl) polyamines.[30]The elegance of these compounds lies in their simplicity. Although polyamines have got self regulating metabolism but due to the presence of several amine oxidases, the addition of natural polyamines and similar compounds having primary amines is dangerous. These enzymes can metabolize the free amino group to toxic species which can cause several side effects.[31] In order to solve this problem alkyl groups were symmetrically introduced to the primary amines of the general polyamine structure to create various polyamine analogues.

Despite of their own unique cytotoxic activity, in the early stages Spm and Spd analogues provided the similar results as were obtained by using difluoromethylornithine (DFMO). There was observed a general decrease in growth rate. These initial studies also revealed that Spm analogues can inhibit growth in more viable way in nature than Spd analogues.[32]This new discovery leads to the designing of subsequent generations of the analogues.

The first sign of cytotoxic effects of this class of compounds came from the response of DFMO-resistant human non-small-cell lung cancer line, NCI H157. It responded in rapidly cytotoxic manner to the treatment with the analogue N^1,N^8-bis(ethyl)spermidine [33] (figure 2).

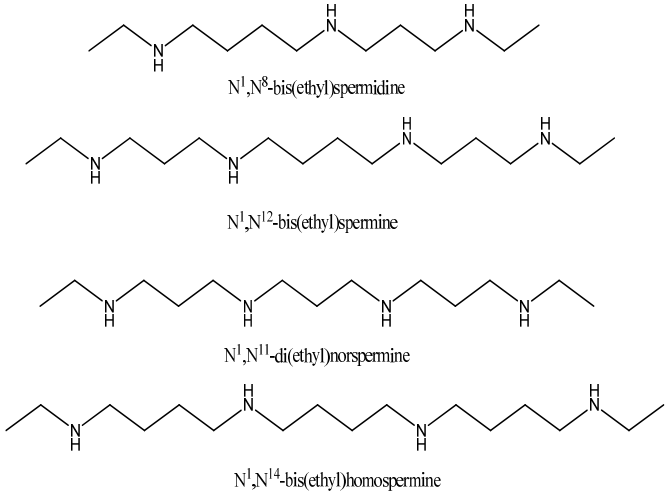


Figure 2 Examples of symmetrically substituted polyamine analogues (adopted from [28])

However, more significant about this demonstration was that the response to these agents was specific to the particular phenotype of the cells. When a DFMO-sensitive human small-cell-lung-cancer line, NCI H82, was compared with the DFMO- resistant human non-small-cell lung cancer line, NCI H157, with respect to their response to the bis(ethyl)polyamines analogues, it was concluded that the NCI H82 cells were considerably more resistant to the bis(ethyl)polyamines than the NCI H157 cells.[34]

5.2. Asymmetrically substituted

Symmetrically substituted analogues might still have their clinical importance but their utility is limited. The reason behind this is that, Bergeron and his colleagues modified only symmetrical end groups rather than to change the number of carbons between the amines that could have proved clinically more useful and potent. The new strategy which has been applied recently to extend this idea of the bis(alkyl) polyamine analogues to alter the basic polyamine backbone structure by asymmetric substitution. The analogues of Spm and norSpm were the most active of the symmetrically substituted analogue. Hence primary importance was given to Spm and norSpm backbone.

The early members of this class showed quite significant antiproliferative activity (figure 3).The results obtained from N^1-propargyl-N^{11}-ethylnorspermine and N^1-cyclopropyl-methyl-N^{11}-ethylnorspermine were demonstrating cell-type-specific cytotoxity. This property is linked with the induction of SSAT. These findings match closely to the results observed for the symmetrically substituted analogues.[35] Clinical investigations of the N^1-cycloheptylmethyl-N^{11}-ethylnorspermine and (*S*)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8-diazaundecane have proved that slight alterations in analogues structure can cause a fruitful effect on different cellular reaction pathways. The analogue N^1-propargyl-N^{11}-ethylnorspermine rapidly induces apoptosis in non-small-cell lung cancer in comparison with the other symmetrically and asymmetrically substituted analogues which induce SSAT.[36]The analysis of Cell cycle revealed that, treatment with analogue produces profound G2/M block coincident with interference with normal tubulin polymerization. It is of great worth to note that, although (*S*)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8-diazaundecane has significantly induce SSAT and effects a G2/M block, the other asymmetrically substituted analogues have very minute effect on cell cycle with respect to G2/M transit. N^1-cycloheptylmethyl-N^{11}-ethylnorspermine and (*S*)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8-diazaundecane can also induce SMO, the activity which is supposed to contribute to their antiproliferative action.[37]

A large series of asymmetrically synthesized analogues have been reported to confirm anti-tumour activity when examined against different types of tumour cells. The synthesis of these asymmetrically substituted analogues showed that slight changes in the structure can lead to significant biological results based on their anti-tumour activity. The most important characteristic revealed by this synthesis is that, multiple functional groups can be added to the same polyamine backbone, which could potentially increase targeting capacity of different compounds.

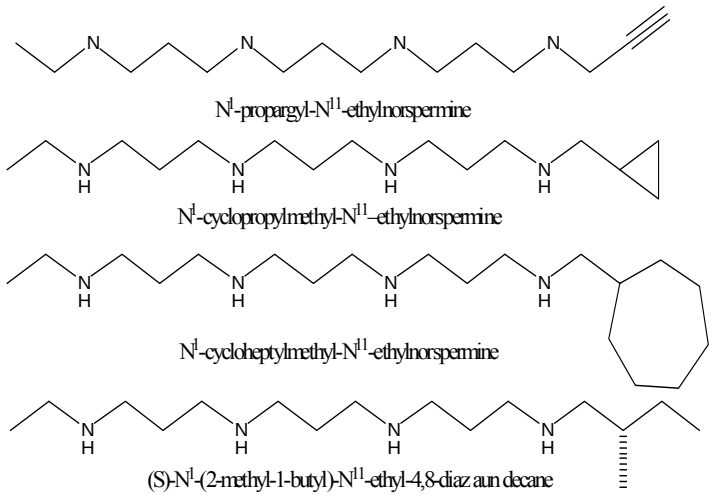


Figure 3 Examples of asymmetrically substituted polyamine analogues (adopted from [28])

5.3. Conformationally restricted

Third approach which was used to expand the area of influence of the original polyamine analogues was to restrict the conformation of different analogues. It was done by introducing modifications into the methylene chains between the amines. The molecular flexibility of the natural polyamines is due to their alkylamine structure. This flexibility in the molecular structure helps to facilitate their interaction with multiple cellular anions. Frydman and his co-workers pointed out that by restricting the free rotation of the carbon-to-carbon bonds, the resulting compounds would have altered and potentially therapeutic activity (figure 4).[38] Addition of unsaturated linkages and cyclic functional groups into bis(ethyl)tetramines and bis(ethyl)pentamines resulted in compounds with characteristic antiproliferative activities in various experimental models.

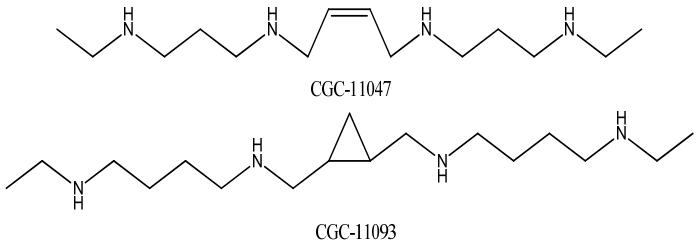


Figure 4 Examples of conformationally restricted polyamine analogues (adopted from [28])

Two main representative of this class that have shown efficient antiproliferative preclinical results are CGC-11047 and CGC-11093. The CGC-11047 is based on N^1,N^{12}-bis(ethyl)spermine, with a double bond in the central 4-carbon methylene bridge while CGC-11093 is based on N^1,N^{14}-bis(ethyl)homospermine, with cyclopropyl bond in the central 4-carbon methylene bridge. The addition of double bond into N^1,N^{12}-bis(ethyl)spermine resulted in a compound with significantly increased antiproliferative activity compared with its original form, but with minimal toxicity. The similar behaviour is observed in the activity and toxicity when cyclopropyl bond is introduced into N^1,N^{14}-bis(ethyl)homospermine. This introduction of conformational restrictions into the synthetic processes of analogues of polyamines has allowed for the creation of several novel compounds with characteristic and distinct ranges of activities and toxicities.[39]

Both CGC-11047 and CGC-11093 are in clinical trails. The CGC-11093 is in phase I trail regarding their impact on SSAT and SMO expression. The CGC-11047 is not only in phase I trails as a single agent but also in a phase Ib trail in combination with other pharmaceutically important agent that would increase its targeting specificity. Additionally, CGC-11047 is also under phase II clinical trails and enrolling patients with diseases like hormone non-responsive metastatic prostate cancer and pancreatic cancer. Beside a great similarity in structure, only CGC-11047significantly induces SSAT and SMO as compared to CGC-11093.[40] This induction does play an important role in tumour-cell response to CGC-11047. But this induction is not a property which is must require for pharmacological activity. It is only because of the reason that CGC-11093 and other important analogues do have a slight impact on SSAT and SMO expression, but are nevertheless active compounds.

A series of studies on these two analogues and others revealed their potency against a disease known as age-related molecular degeneration (AMD). It is an ailment characterized by vascular proliferation, as is diabetic retinopathy. In several mouse models of this disease it has been represented that these two analogues not only prevent the disease but can also reverse the situation of vascular lesions. These studies have led to the initiation of phase I clinical trial for AMD.[41] These two polyamine analogues are also anti-angiogenic in these mouse models, an activity related to antineoplastic activity. Another important fact is that, those polyamine analogues which are actively transported, can be administrated subconjunctivally to the eye. Their this characteristic property is in contrast to the recently approved drugs for AMD which must be injected indirectly to the eye.[42]

5.4. Oligoamines

The antiproliferative activity of the specific polyamine analogues has been demonstrated on the basis of a mechanism which explains their ability to interact with nucleic acids and other intracellular polyamine-binding sites. A large series of studies have shown the interaction of natural polyamines with DNA and chromatin material. This phenomenon led to the importance of selection of the specific polyamine analogue for targeting a specific site. By considering these important studies, scientists introduced the strategy of increasing the number of protonatable imines so that the resulting compounds would have increased affinity for DNA. This will turn them to be more potent antiproliferative agents.[43]They synthesized a series of oligoamines in both saturated and conformationally restricted unsaturated forms containing 8-14 amines (figure 5). In this way a fourth class of analogues have been created with a potential of pharmacological action.

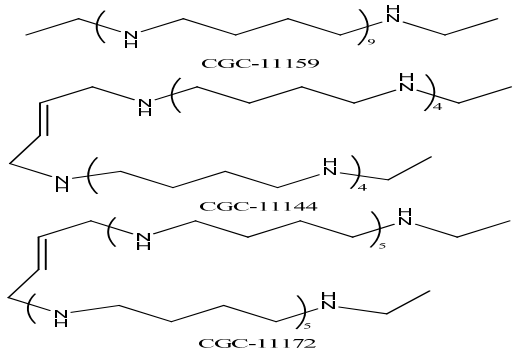


Figure 5 Examples of important oligoamines (adopted from [28])

These compounds demonstrated potent (submicromolar) antiproliferative activity in human prostate cell lines.[44] This characteristic behaviour can be correlated well with their ability to aggregate DNA *in vitro.* A representative oligoamine, CGC-11144, showed significant antitumour activity against human breast-cancer cells both *in vitro and in vivo*. It exhibited a great antiproliferative effect when treated with established MDA-MB-231-tumour-bearing nude mice, using a twice a week schedule. CGC-11144 induces many significant biological phenomenon including apoptotic cell death, caspase 3 activation, mitochondrial cytochrome *c* release, increased expression of the pro-apoptotic BCL2-assoiciated X (BAX) protein, poly (ADP-ribose) polymerase (PARP) cleavage and decreased the release of anti-apoptotic B-cell CLL/lymphoma 2 (BCL-2) protein. CGC-11144 treatment also lessens ODC activity. It also has very minute impact on the expression of the polyamine catabolic enzymes SSAT and SMO.[45] This decrease in the ODC activity is most probably because of the increase ODC antizyme protein production in the cells exposed to the oligoamines. A recently demonstrated study showed that alogoamines can change the expression of oestrogen receptor-α (ERα) in a highly selective way. Most specifically, the oligoamines CGC-

11144, CGC-11159 and CGC-11172 downregulate the transcription of the Erα through apparent disruption of Sp-1 transcription factor (SP1) family member binding to the Erα promotor.[46] These findings proposed a novel anti-oestrogen effect by the oligoamines that might provide another strategy for the best cancer prevention or at least a therapy for it.

5.5. Macrocyclic polyamines

This is a unique class of polyamine analogues that possess various multiple functions with the cells including DNA binding, complexation of the transition metals, DNA cleavage and depletion in the rate ATP production. This interesting class of analogues has recently been reviewed by Liang and co-workers.[47] It is in the early stages of its development. Beside this fact they have shown a great promise to be considered as a potent anti-viral and anti-tumour agents (figure 6).[48]

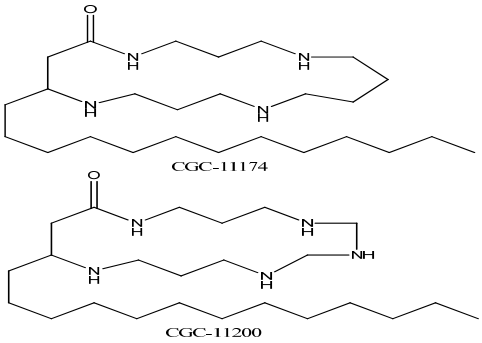


Figure 6 Examples of macrocyclic polyamine analogues (adopted from [28])

6. Potential targets of the polyamine analogues

Polyamines analogues are involved in competing with natural polyamines for uptake by the polyamine transporters. They are also playing a key role in several biochemical processes in cells and hence are having many potential targets. The potential targets of polyamine analogues revised by according to a review[28] can be listed as under.

a) They can induce the necessary alterations to go through the reading of regulatory stop of antizyme (AZ) mRNA.
b) AZ is known to bind carboxyl terminus of ornithine decarboxylase (ODC) monomers which leads to the breakdown of ODC by the 26S proteosome.
c) AZ also have the ability to down regulate the polyamine transporter by a mechanism which is currently unknown.[49]
d) polyamine analogues can increase the rate of ODC degeneration that actually decrease the rate of SSAT protein degrading by blocking the ubiquitination (Ub) of SSAT and its subsequent destruction by the 26S proteosome.
e) Natural polyamines and their analogues present in excess quantities can reduce the efficiency of ODC and S-adenosylmethionine decarboxylase (AdoMetDC) mRNA translation.[50]
f) The analogues of polyamines not only increase the efficiency at which SSAT mRNA is translated but can also increase the stability of mRNA.
g) The analogue treatment and interference with polyamine metabolism do have direct effects on the transcription of the several important growth regulatory genes as well as on genes that are involved in polyamine metabolism.
h) Polyamine analogues also lead to an increase in efflux of the polyamines and their acetyl derivatives through the diamine transporter.
i) The primary function of natural polyamines is through their ionic interactions with important cellular anions. Hence it can be concluded that there is a huge possibility that some of the antiproliferative effects of polyamine analogues and treatment that reduce intracellular polyamine concentrations are a result of either direct competition for binding to these critical sites or a displacement of natural polyamines from these critical sites.[51]
j) All of these above mentioned process can profoundly affect cellular proliferative capacity.

7. Triethylenetetraamine (TETA)

Triethylenetetraamine (TETA), Trien, is a modern alternative of D-penicillamine for the treatment of the patients suffering from Wilson´s disease which is characterized by the excess accumulation of copper metal in human brain. TETA is a 219.2 MW ligand with six CH_2 groups which are arranged as three ethylene segments (figure 13). It is a polar hydrophilic chelator that binds to excess free body copper and competes with albumin for binding copper.[52] It is excreted from the body after binding as TETA-Cu^{II} complex as TETA mobilizes copper in the kidneys and helping it pass it out through urine. It is bipositively charged molecule at physiological pH and structurally isosteric charge-deficient analogue of spermidine.[53] Its laboratory preparation was first initiated in Berlin, Germany and was made as dihydrochloric salt in 1861 and 1896 respectively. Its ability to chelate Cu^{II} was first investigated at Cambridge University in 1925. Cu^{II} prefers nitrogen as ligand over oxygen and as TETA has four nitrogen groups. These nitrogen groups can occupy four coordination sites of Cu^{II} to form a strongly bound TETA-Cu^{II} complex that helps them to make a square-planar geometry in which Cu^{II} is most stable (Figure 7). Therefore, it binds Cu^{II} very firmly having dissociation constant of 10^{-15} mol/L at Ph 7.0.[54] By increasing the urinary excretion of copper, TETA tends to have the ability of decreasing intestinal absorption of copper showing its two fold ability to act on copper metabolism.[55]

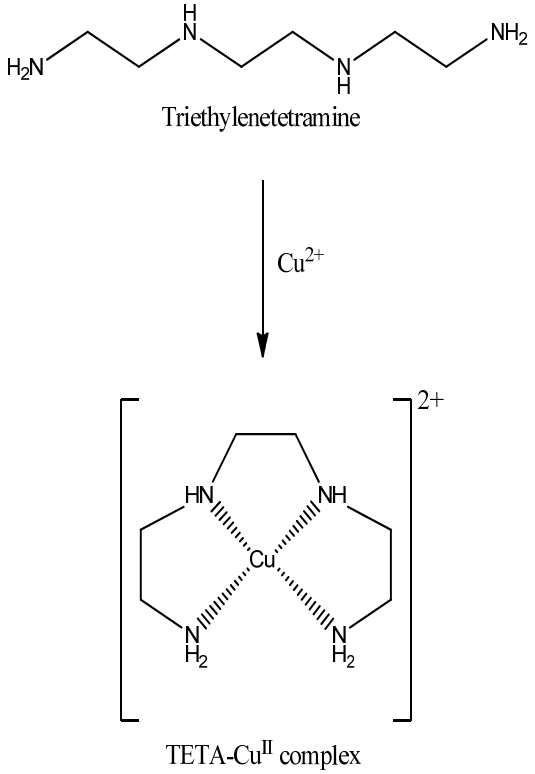


Figure 7 Structure of TETA and TETA-Cu^{II} complex

8. Biological Importance of TETA

TETA and different physiological polyamines (e.g. Spm and Spd) share the similar intestinal uptake mechanism in human bodies. In this mechanism, there occurs a charge-interaction between the negative-charge of the inner membrane layer and the polycation. In human bodies, the transporting agent for the transport of polyamines is glypican-1[56], so, as TETA and other physiological polyamines share the same mechanism of uptake, it seems that they use the same transporter glypican-1 to cross the biological membranes in different tissues and cells. By using this transporter, polyamines are instantaneously transported into the mitochondria of the cells electrophoretically.[57] After the absorption of TETA into the mitochondria, it is quickly metabolized to its two most important metabolites named as N^1-acetyltriethylenetetramine (MAT) and N^1, N^{10}-diacetyltriethylenetetramine (DAT) [58] (figure 14).

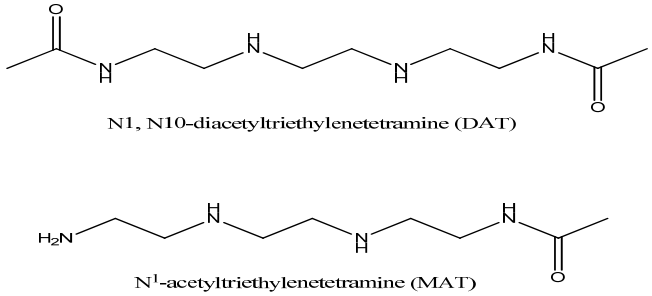


Figure14 Structure of DAT and MAT

The rate of excretion of TETA and its metabolites in their free and Cu-bound forms is much faster in other mammals like rodents as compared to humans but the mechanism of excretion is not much clear. The Na^+/spermine antiporter which present in renal brush-border is thought to be responsible for the active secretion of the all the straight-chain polyamine compounds that contain more than four amino groups and thus is regarded as mediator for the excretion of TETA and its metabolites as well.[59] The total rate of absorption TETA is extremely low and it varies a lot in different patients suffering from Wilson´s disease. The rate of recovery of TETA from excretion in human is only 1-2% which is extremely low but acetylated forms of TETA such as its metabolites MAT and DAT are recovered at significantly good rate that is 8-11% of the orally administered dose. The rate of recovery of TETA and its metabolites MAT and DAT in Cu-bound form or as free molecules after excretion is still unclear but it can be concluded that it is excreted predominantly in its acetylated forms. The pattern of excretion also varies significantly among different human beings ranging from 6 to 26 hours in the case of unmodified form and acetylated forms respectively. The major side effect which can arise with the treatment from TETA is the iron deficiency anemia in children

and pregnant women. However, this problem can be comprehended successfully by giving iron supplementation along with treatment of TETA considering the dosage time appropriately.[60]

Metabolic changes in diabetes, especially those that results in accumulation of advanced glycation end products (AGEs) in the extracellular matrix, are the reason of accumulation of excess extra cellular Cu^{II} in the extracellular matrix, resulting tissue-Cu imbalance that can cause heart failure. TETA´s ability to remove excess extracellular Cu^{II} enables it to be helpful in improving the conditions of the patients with left ventricular hypertrophy in diabetes.[61] Many clinical studies of TETA´s treatment has proved that it can act to increase the strength of cardiomyocyte structure, alleviation of heart failure with diabetic patients and normalization of the cardiac diastolic function. The oral treatment of TETA can cause significant improvement in decreasing the elevated levels of LV collagen and β1-integrin in the presence of constantly high blood circulating sugar. However, treatment with TETA can considered as a novel potential therapeutic channel in patients suffering from heart failure in diabetes but the possibility of causing some severe side effects can not be excluded. As the mechanism of action of TETA in diabetes is not much clear so, it can cause a depletion of copper in patients suffering from diabetes.[62]

TETA is not only a therapeutic orphan drug for treating Wilson´s disease and patients suffering from heart failure in diabetes but can also be considered as a potential therapeutic agent for the treatment of cancer. There are different mechanisms that have been proposed that how TETA can act on different tumour cells but the confirmation of any mechanism by which TETA proceeds in the cancerous cells is yet to be known. One possible mechanism of action of TETA to show its cytotoxicity on tumour cells is to inhibit telomerase activity. Telomerase plays a key role in cellular immortalization and tumourigenesis, which are appeared in more than 80% of all human cancers. The reports suggested that TETA has the ability to stabilize both inter and intra-molecular G-quadruplex because of its tendency to act as a ligand for G-quadruplex.[63]

TETA´s other mechanism of action against cancer can be anti-angiogenesis. Chelation of copper by TETA can play a very vital role to suppress several angiogenic mediators. These mediators include vascular endothelial growth factor-1(VEGF-1), fibroblast growth factor-1(FGF-1), interleukin-1 (IL-1), IL-6, IL-8 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). By suppressing the activities of these modulators, it is thought to exhibit its properties as anti-angiogenic effects in cancerous cells.[64] Treatment of TETA could overcome cisplatin resistance in human ovarian cell culture via inhibition of superoxide dismutase 1/ Cu / Zn superoxide dismutase.

TETA can also induce apoptosis in murine fibrosarcoma cells by activating p38 mitogen-activated protein kinase (MAPK) pathway. One other way of introducing apoptosis could be chronic depletion of copper by chelation with TETA, which might induce the expression of antioxidants and triggers apoptosis in neuroblastoma cells.[65]

All these above mentioned mechanisms of action against tumour cells are only suggested routes by which polyamines interact within a biological system of an organism. One clear mechanism of action in cancer pharmacology of TETA is yet to be determined. Although, non of the mechanisms can be strictly applied to cancer pharmacology but all these different mechanism might have their role to play in different cancer tissues or combination of different mechanism might act to inhibit the growth of cancer cells. Anyhow, complete knowledge different mechanism of TETA´s pharmacology in different cancerous cells can helpful in developing different drugs for the treatment of different diseases especially cancer.

9. Analysis of TETA, MAT and DAT

TETA is mostly used in clinics in the form of dihydrochloride salt as it dissolves in aqueous solutions and appears as free-based TETA. Most recently its disuccinate form has also been developed.[66] The analytical investigation and detection of TETA and its two major metabolites, which are actually it´s terminally mono and diacetylated forms, known as MAT and DAT is very difficult. Due to very polar structure of TETA it elutes very inefficiently from conventional high performance liquid chromatography (HPLC) columns. It also absorbs poorly at accessible ultra violet (UV) detection wavelength. There are several methods which have been applied recently. One of them is fluorescent-labelling reagents method which is very helpful to derivatize TETA and its derivatives.[67] The fluorescent-labelling method is very challenging and highly demanding as it requires a high level of accuracy while labelling analytes and also it presents difficulty in the detection of peaks weather they are separated from their respective metabolites or from other metabolites or not. This problem of inconsistent labelling of targeted analytes can give rise a difficulty of complex chromatogram. An HPLC conductivity method has also been developed but it renders poor sensitivity due to high detection limit.[68] In recent days, a method combining liquid chromatography (LC) and mass spectrometry (MS) has been developed.[69] It can be used to detect TETA and its two major metabolites MAT and DAT simultaneously in the aqueous solution, providing better sensitivity for detection and analytical power. Now in the presence of the liquid chromatography (LC) combined with tandem mass spectrometry (MS-MS), LC-MS-MS technology, a method with higher resolution, sensitivity and accuracy can be developed that will be used to investigate TETA and its major metabolites in the human samples, which will ultimately help us to predict its future pharmacology and clinical usage of TETA.

B. Experimental Part

10. Materials and Methods

All of the materials were purchased from the commercial suppliers and used without further purification. The solvents that were dried and distilled are acetic anhydride, methanol, ACN, TFA, THF, DMF and DCM. Thin layer chromatography was performed on silica gel, Kiesel gel. Chromatography column was carried out on silica gel. The NMR analysis was done on Bruker AVANCE DRX spectrometer operating at 500.13 MHz. The mass of the Deuterated product was measured on QSTAR XL MS/MS spectrometer using electrospray ionization.

11. Synthesis

11.1. Synthesis of 2,2'-(ethane-1,2-diylbis(benzylazanediyl))diacetonitrile (**1**)

N, N´- dibenzylethylenediamine (2.0 g, 8.3 mmol), chloroacetonitrile (1.885 g, 24.97 mmol), K_2CO_3 (6.9 g, 49.93 mmol), KI (1.38 g, 8.31 mmol) were measured in a round bottomed 100 ml flask and dried DMF (40 mL,) was added. The mixture was refluxed for 4 hours in nitrogen atmosphere dried *in vacuo*. The product was dissolved in DCM (15 mL) and washed with water (5x20 mL). Organic layer was separated and dried with $MgSO_4$. After filtering the product, it was dried again *in vacuo* to give 2,2'-(ethane-1,2-diylbis(benzylazanediyl))diacetonitrile (**1**) as brown solid (1.3562 g, 68%) which melted at 89 °C.

^{1}H NMR (500.0 MHz, $CDCl_3$) δ: 3.665(4H,s),3.516(4H,s),2.787(4H,s),7.234-7.332(10H,m)
^{13}C NMR (125.8 MHz, $CDCl_3$) δ: 136.67, 114.74, 126.97-129.01(5 C), 58.91, 51.26, 41.37

11.2. Synthesis of (3,6-dibenzyl)-1,8-diamino-3,6-diazaoctane (**2**)

$LiAlH_4$ (0.0954 g, 2.51 mmol) in finely grounded form was dissolved in diethyl ether (20 mL) refluxed in nitrogen atmosphere for one hour. After one hour, the reaction mixture was cooled down for 30 minutes at 0-4 °C in an ice bath. **1** was dissolved (0.2 g, 0.62 mmol) in diethyl ether (20 mL) and added slowly to the reaction mixture drop wise in approximately 20 minutes with gentle mixing. The mixture was warmed up to room temperature and refluxed again for four hours. After four hours, reaction mixture was cooled to 0-4 °C in ice bath. The reaction was stopped by adding

0.2 mL of distilled water and 0.6 mL of 5M NaOH in the reaction mixture. The mixture was warmed up to room temperature and mixed gently for 30 minutes. The organic layer was filtered and the residue was washed with DCM (3x10 mL). $MgSO_4$ was added in organic part and the product was dried *in vacuo* after filtration. The product was purified on TLC using MeOH as a solvent giving (3,6-dibenzyl)-1,8-diamino-3,6-diazaoctane (**2**, 0.119g, 60%)as white crystals.

^{1}H NMR (500.0 MHz, MeOD) δ: 3.678(4H,s),2.599(4H,m),2.616(4H,m),2.800(4H,s),7.228-7.274(10H,m)
^{13}C NMR (125.8 MHz, MeOD) δ: 137.69, 127.97-128.01(5 C), 64.91, 57.37, 52.28, 38.50

11.3. Synthesis of N, N´-((ethane-1, 2- diylbis(benzylazanediyl))bis(ethane-2,1-diyl))diacetamide (**3**)

2 (0.1800 g, 0.55 mmol) was dissolved in toluene (20 mL) and pre-distilled pure and dried acetic anhydride (0.125 g) was added slowly. The reaction mixture stirred for one hour at room temperature and the solvents were dried away *in vacuo*. Product was purified on silica gel column chromatography using MeOH as eluent giving *N, N´*-((ethane-1, 2-diylbis (benzylazanediyl))bis(ethane-2,1-diyl))diacetamide (**3**, 0.12g, 67%) as yellowish crystals.

^{1}H NMR (500.0 MHz, MeOD) δ: 1.863(6H,m),3.279(4H,s),2.592(4H,m),2.513(4H,m), 3.547(4H,s),7.244-7.252(10H,m)
^{13}C NMR (125.8 MHz, MeOD) δ: 171.1, 138.20, 127.85-129.77(5 C), 65.10, 58.78, 53.63, 36.97, 21.63

11.4. Synthesis of N^1,N^{10}-diacetyltriethylenetetramine (DAT)

In first method of deprotection step, Pd/C 5% (0.015 g, 0.14 mmol) was measured in two necked flask. **3.** (0.1000 g, 0.24 mmol) was dissolved in MeOH (5 mL) and added in the flask containing the catalyst. The reaction vial was put to the catalytic hydrogenation chamber at 1 atm standard pressure in room temperature for 4-5 hours. The product was filtered through celite plug and dried *in vacuo*.

In second method of deprotection step, $PdCl_2$ 100% (0.010 g, 0.05 mmol) was measured in two necked flask. **3** (0.1000 g, 0.24 mmol) was dissolved in MeOH (20 mL) and added in the flask

containing the catalyst. The reaction vial was put to the hydrogenation at normal pressure in room temperature for 4-5 hours. The product was filtered through celite plug and dried *in vacuo*.

In third method of deprotection step, $PdCl_2$ 100% (0.010 g, 0.05 mmol) was measured in two necked flask. **3** (0.1000 g, 0.24 mmol) was dissolved in MeOH (20 mL) and added in the flask containing the catalyst. The reaction mixture was hydrogenated for overnight at 4 bar pressure. The product was filtered through a celite plug with MeOH (20 mL) and dried *in vacuo*.

In fourth method of deprotection step, Pd/C 5% (0.015 g, 0.14 mmol) was measured in high pressure hydrogenation flask. **3** (0.1000 g, 0.24 mmol) was dissolved in MeOH (19 mL) and added acetic acid (1 mL) was added and put it to the reaction vial. The reaction mixture was hydrogenated for overnight under 4 bar pressure. The product was filtered through a celite plug with MeOH (20 mL) and dried *in vacuo*.

11.5. Synthesis of 2,2'-(ethane-1,2-diylbis(azanediyl))diacetonitrile(**4**)

Ethylenediamine (0.5 g, 8.3 mmol), chloroacetonitrile (1.319 g, 1.65 mmol), K_2CO_3 (2.3 g, 1.66 mmol) were measured in a round bottomed 100 ml flask and dried ACN (20 mL) was added. The mixture was refluxed for 4 hours with vigorous stirring in nitrogen atmosphere. The reaction mixture filtered and dried *in vacuo*. The product was dissolved in DCM (10 mL) to remove the impurities and dried *in vacuo* again to give 2,2'-(ethane-1,2-diylbis(azanediyl))diacetonitrile (**4**) as white colour powder (0.4064 g, 81%).

^{1}H NMR (500.0 MHz, $CDCl_3$) δ: 3.599-3.701(4H,m),2.87(4H,s)
^{13}C NMR (125.8 MHz, $CDCl_3$) δ: 118.01, 47.87, 37.58

11.6. Synthesis of di-tert-butyl ethane-1,2-diylbis((cyanomethyl)carbamate)(**5**)

4 (0.2410g, 1.74 mmol) was dissloved in DCM (20 mL) in a 100 ml round bottomed flask. A solution of di-*t*-butyl dicarbonate (Boc_2O) (0.7804g, 3.57 mmol) was made in DCM (10 mL). This solution was added slowly drop wise to the first flask keeping it in an ice chamber. The contents of the flask were stirred for 1 hour at room temperature. The solvent was evaporated and dried *in vacuo* to give di-tert-butyl ethane-1,2-diylbis((cyanomethyl)carbamate) (**5**) white long shaped crystals (0.5021g, 64%)

^{1}H NMR (500.0 MHz, $CDCl_3$) δ: 4.202(4H,s),3.427(4H,s),1.333-1.587(18H,m)
^{13}C NMR (125.8 MHz, $CDCl_3$) δ: 154.78, 116.24, 80.27, 47.98, 40.29, 28.43(3 C)

11.7. Synthesis of di-tert-butyl ethane-1,2-diylbis((2-aminoethyl)carbamate)(**6a**)

$LiAlH_4$ (0.337 g, 8.55 mmol) in finely grounded form was dissolved in diethyl ether (20 mL) and refluxed in nitrogen atmosphere for one hour. After one hour, the reaction mixture was cooled down for 30 minutes at 0-4 °C in an ice bath. **5** was dissolved (0.5 g, 1.47 mmol) in diethyl ether (20 mL) and added slowly to the reaction mixture drop wise in approximately 20 minutes with gentle mixing. The mixture was warmed up to room temperature and refluxed again for four hours. After four hours, reaction mixture was cooled to 0-4 °C in ice bath. The reaction was stopped by adding 0.6 mL of distilled water and 0.3 mL of 5M NaOH in the reaction mixture. The mixture was warmed up to room temperature and mixed gently for 30 minutes. The organic layer was filtered and the residue was washed with hot DCM (3x10 mL). $MgSO_4$ was added in organic part and the product was dried *in vacuo* after filtration. To this product ethyl acetate (10 mL) and acetic acid (0.02 mL) were added to get the desired crystals of the product. Solvents were evaporated again in *vacuo* giving di-tert-butyl ethane-1,2-diylbis((2-aminoethyl)carbamate)(**6a**) (0.275g, 55%) as foamy white crystals.

^{1}H NMR (500.0 MHz, MeOD) δ: 3.025(4H,s),3.279(4H,s),3.384(4H,d),1.45(18H,s)
^{13}C NMR (125.8 MHz, $CDCl_3$) δ: 155.78, 80.20, 50.98, 46.35, 38.59, 28.81(3 C)

11.8. Synthesis of deuterated di-tert-butyl ethane-1,2-diylbis((2-aminoethyl)carbamate)(**6b**)

$LiAlD_4$ (0.297 g, 7.08 mmol) in finely grounded form was dissolved in diethyl ether (20 mL) refluxed in nitrogen atmosphere for one hour. After one hour, the reaction mixture was cooled down for 30 minutes at 0-4 °C in an ice bath. **5** was dissolved (0.4 g, 1.18 mmol) in diethyl ether (20 mL) and added slowly to the reaction mixture drop wise in approximately 20 minutes with gentle mixing. The mixture was warmed up to room temperature and refluxed again for four hours. After four hours, reaction mixture was cooled to 0-4 °C in ice bath. The reaction was stopped by adding 0.6 mL of distilled water and 0.3 mL of 5M NaOH in the reaction mixture. The mixture was warmed up to room temperature and mixed gently for 30 minutes. The organic layer was filtered and the residue was washed with hot DCM (3x10 mL). $MgSO_4$ was added in organic part and the product was dried *in vacuo* after filtration. To this product ethyl acetate (10 mL) and acetic acid

(0.02 mL) were added to precipitate the desired crystals of the product. Solvents were evaporated again *in vacuo* giving di-tert-butyl ethane-1,2-diylbis((2-aminoethyl)carbamate)(**6a**) (0.22g, 55%) as foamy white crystals.

MS Data: m/z 389.2738$[M+K]^+$, 373.2985$[M+Na]^+$,351.3150$[M+H]^+$,251.2559$[M+H\text{-}Boc]^+$

11.9. Synthesis of di-tert-butyl ethane-1,2-diylbis((2-acetamidoethyl)carbamate)(**7**)

6a (0.200 g, 0.42 mmol) was dissolved in dry ACN (20 mL) and pre-distilled pure and dried acetic anhydride (0.0438 g) was added slowly. The reaction mixture stirred for one hour at room temperature and the solvents were dried away *in vacuo*. Product was purified on silica gel column chromatography using MeOH as eluent giving di-tert-butyl ethane-1,2-diylbis((2-acetamidoethyl)carbamate)(**7**)(0.080g, 40%) as yellowish crystalline powder.

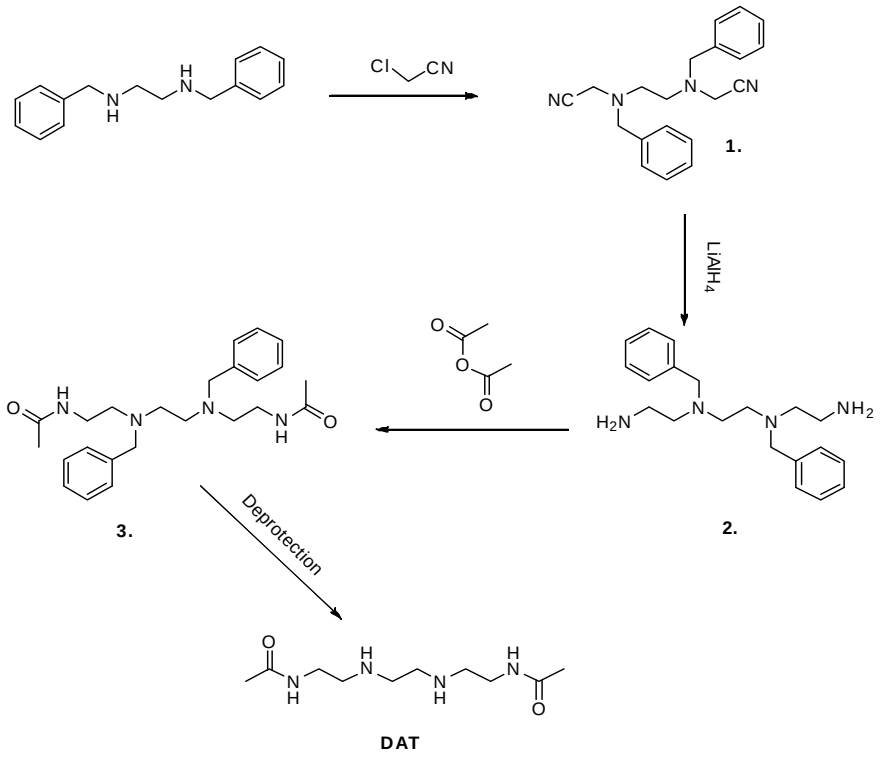
^{1}H NMR (500.0 MHz, MeOD) δ: 1.820-1.872(6H,m), 3.661-3.729(4H,m), 3.304(4H,s), 3.358(4H,d), 1.556(18H,bs)
^{13}C NMR (125.8 MHz, $CDCl_3$) δ: 170.9, 154.87, 81.02, 51.24, 48.35, 36.51, 28.91(3 C), 27.671

11.10. Second synthesis method of N^1,N^{10}-diacetyltriethylenetetramine (DAT)

7. (0.080g, 0.18 mmol) was dissolved in 60% TFA in DCM (3 mL) for the removal of protective groups. The reaction mixture was allowed to stir at room temperature for 30 minutes in order to ensure thorough mixing. The solvent evaporated and product was dried *in vacuo* to give N^1,N^{10}-diacetyltriethylenetetramine (DAT) as di trifluoroacetate salt (0.050g, 63%) as off white powder.

^{1}H NMR (500.0 MHz, D_2O) δ: 3.42-3.37(4H, m),3.34 (4H,bs), 3.15-3.11(4H,m), 1.872 (6H,s)
^{13}C NMR (125.8 MHz, D_2O) δ: 175.88, 48.41, 43.58, 36.20, 22.22

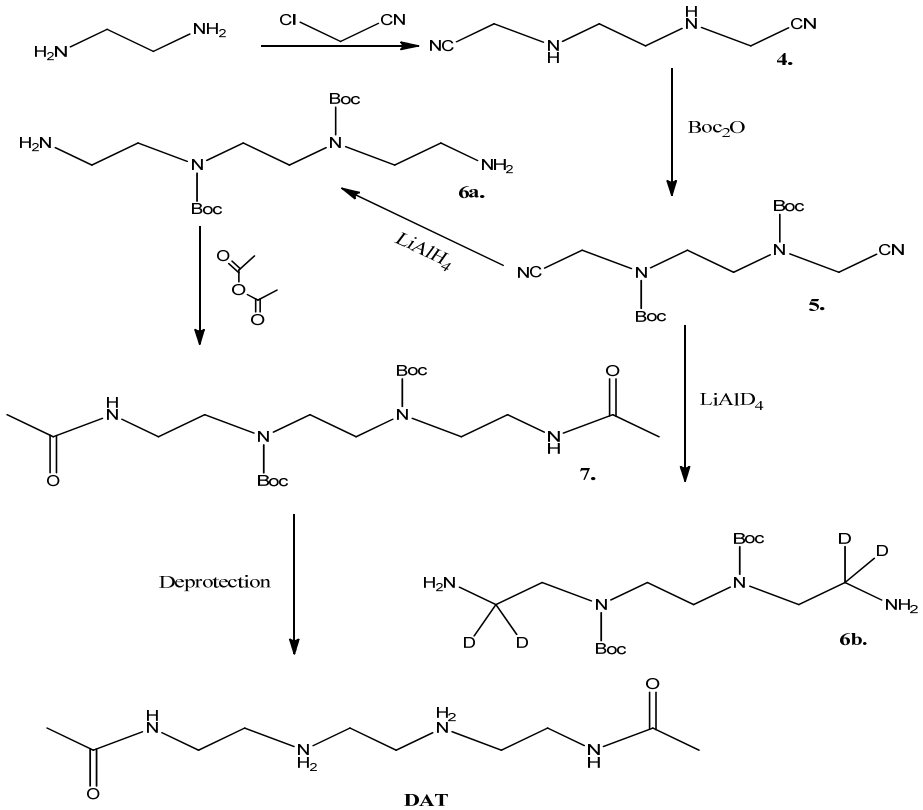
12. Results and Discussion



Synthesis scheme1 The synthesis of DAT

In the beginning of research project shown in scheme 1, the starting material N, N´-dibenzylethylenediamine was made to react with chloroacetonitrile in the presence of different solvents like DMF and ethanol including K_2CO_3 and KI. The first step of the synthesis in which ethanol was used as a solvent produced a mixture of product and by-product ending up having very low yield of the original product. When the solvent was changed to DMF a big increase in the purity and the yield of the product was observed. This particular route was upgraded to higher molar concentrations to get a bigger amount of product **1** for the next step. In second step, the reduction of the product **1** obtained from the first step was carried out using $LiAlH_4$ in diethyl ether and in THF. The reaction with THF did not proceed well and reaction ended in the partial reduction of the product **1**. The reaction with diethyl ether produced the product with higher % yield and higher purity. Then the molar concentrations of the step with diethyl ether were upgraded to get higher amount of the product **2**. In third step of the synthesis, the product **2** was acetylated with distilled acetic anhydride to get the mono and diacetylated products. Acetic acid and toluene were used as solvents during these reaction routes that helped in obtaining the mixture of mono and diacetylated products in these reactions. Column chromatography was used in order to get them

separated. Only the diacetylated form has been obtained after column chromatography because of the presence of very minute quantities of mono acetylated from which were almost impossible to separate. After obtaining diacetylated product **3** in the third step, the main aim of the project was to remove aromatic protective groups in order to get the desired products. The fourth step of the synthesis was catalytic hydrogenation, which was performed at low pressure using (Pd/C) 5% catalyst and methanol and toluene as solvents, and also at high pressure using the same catalyst and acetic acid along with the methanol to get the DAT as a final product. The deprotection did not take place even under high pressure catalytic hydrogenation using acetic acid along with the solvents. All these reagents remained unable to cleave the aromatic protective group although they have been kept under high pressure catalytic hydrogenation for over night. Hence the deprotection did not proceed as planned and has to be optimized. After analysing NMR spectra by both of these methods, it is concluded that deprotection or removal of aromatic rings did not work according to the synthesis planned.



Synthesis scheme2 The synthesis of DAT

In second synthetic route shown in scheme 2, the starting material ethylenediamine was made to react with chloroacetonitrile in the presence of dry ACN as a solvent, Cs_2CO_3 and K_2CO_3 were also

tested as base. The reaction with Cs_2CO_3 produced lots of impurities and lower yield was obtained at the end of the reaction using this base but the reaction route with K_2CO_3 gave the higher yield of product **4** so it was upgraded to get more amount of the product. In the second step, the protection of the product **4** was done with the help of Boc protective group. Different solvents were tested in this step, like dry triethyleneamine, dry DCM and after words this step was upgraded using the same protective group and dry DCM as solvent as they gave the better purity and yield of product **5** as compared to other solvents used. In the next step, reduction of the product **5** was carried out by two parallel reducing agents $LiAlH_4$ and $LiAlD_4$ using diethyl ether as solvent. Both of these reduction steps went along quite well in terms of purity of the product **6a** and **6b** respectively so these steps were upgraded using higher molar concentrations of the reagents. In both of these step re crystallization of the product was done using ethyl acetate and acetic acid and a better crop of crystals is obtained in terms of purity and yield. The reduction with $LiAlD_4$ is performed in order to get deuterated form of the 6a mentioned as **6b** in the scheme 2. The next step of acetylation was carried out using product 6a as starting material. It was acetylated using dried and distilled acetic anhydride and dry ACN as solvent to get mono and diacetylated product in this step. Column chromatography was used in order to get them separated and diacetylated product **7** was obtained. The deprotection of this product **7** was done using 60% TFA in DCM solution. After analysing the NMR spectra of our product **7**, we came to know that deprotection went successfully as compared to first scheme in order to give us DAT trifuoloroacetate salt as our final product which was one of our desired products of the project work.

13. Conclusion

The first synthetic route did not work because of the problems arising from the difficult deprotection of the diacetylated product (**3**). Hence it was concluded that if the problem in deprotection can be solved, then this synthesis route can be applied for the synthesis of deuterated TETA, MAT and DAT. Anyhow the second synthesis strategy was successfully applied for the synthesis of DAT, thus providing the methodology for the synthesis of Deuterated compounds. These deuterated polyamines might be used as internal standards in mass spectrometry for their quantitative and analytical investigation.

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