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Investigation of New Actinium Complexation Systems for Therapeutic Applications

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Abstract

This thesis is devoted to the synthesis of new chelating agents for actinium and actnoids (An), as part of a research effort to the production of new radio immune drugs.

The first part is dedicated to the synthesis of the first member of a brand new class of An chelating agents based on dendrimers. These compounds are particular interesting because they can give drugs with an higher dose load (i.e. with more than one An atom bound by the same chelating agent), extremely difficult to be realized with traditional methodology. The identified target molecule is benzoyl-(Glu)G3-OEt^{i} a dendrimer of glutamic acid. When this thesis started there was no article or hypothesis at all on the complexing properties of dendrimers towards An, but very recently the chelating properties of dendrimers towards uranium have been assessed. Although this takes away the primogeniture of the idea, it supports the validity and interest of this research field.

The second part is dedicated to the synthesis of new nitrogenated chelating agents. New synthetic ways for the synthesis of N-nitroso compounds are inspected. These species have been chosen by using one of the synergic strategies nowadays so widely recommended in industrial management manuals. In fact their complexing properties toward transition metals and lanthanoids are well known and it is of scientific and applicative interest to synthesize new suitable chelating agents to make possible the study of their chelating affinity towards An. At the same time, they can be used as both starting material for the synthesis of hydrazinic chelating agents and synthetic scaffolds for the synthesis of a wide range of pendant-type-macrocyclic bifunctionalised chelating agents .

The third part is dedicated to the identification of new synthetic pathway towards classical pendant-type macrocyclic bifunctionalised chelating agents. A lot of syntheses have been published on this argument, both cumbersome and with low yields. The basic idea of this work was to avoid the total ring synthesis by exploiting

the well-known acidity of proton in α position with respect to a nitroso group. Unluckily, this kind of approach did not give any result, probably because of conformational problems. Also other attempts of direct introduction of a pendant on a functionalized ring performed with several different basis and activating agents, did not bring to the desired products.

Since all previous attempts were unsuccessful, an optimization of the classical total ring synthesis focused on the cyclisation step (that all authors performed under extreme diluted conditions with very low yields) has been enquired.

In conclusion, during the work of this thesis the first member of a brand new class of possible chelating agents has been synthesized along with new potential macrocyclic chelating agents containing hydrazine and N-nitroso substituted nitrogen atoms. Literature does not report any suitable gram scale synthesis of pendant-type macrocyclic chelating agents, either by using N-nitroso macrocyclic chelating agents as starting materials or by performing the total ring synthesis. Evaluation of the actual An chelating properties of the synthesized molecules is well beyond the scope of this thesis and can only be investigated in a facility suited to work with highly radioactive isotopes.

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Table of abbreviations

APCI-SIM	Atmospheric Pressure Chemical Ionization-Single Ionization Molecule
CA	Chelating Agents
CI	Chemical Impact
BCA	Bifunctionalised Chelating Agent
BOC	Di-tert-butyl dicarbonate
Bipy	2,2'-bipyridine
CAMM	Computer Assisted Molecular Modeling
CDR	Complementarity Determining Region
D-BCA	Dendrimeric Bi-functionalized Chelating Agent
D-PT-BCA	Dendrimeric Pendant Type Bifunctionalized Chelating Agent
DTPA	DiethyleneTriaminePentaAcetic acid
EDC	N-Ethyl-N'-(3-dimethylaminopropyl)-carbodimide
EDTA	Ethylendiaminotetraacetic acid
EDTMP	Ethylenediaminetetramethylenephosphonic acid
Fab	Fragment antigen binding
Fc	Crystallisable Fragment
FGI	Functional Group Interconvention
FR	antibody Framework Region
HAMA	Human Anti-Mouse Antibodies
HEHA	1,4,7,10,13,16-exaazacyclooctadecane-1,4,7,10,13,16-hexaacetate
HEHA-NCS	1,4,7,10,13,16-exaazacyclooctadecane-1,4,7,10,13,16-hexaacetate-2-*p*-benzylisotiocianate

HOBt	Hydroxybenzotriazole
HOMO	Higher Occupied Molecular Orbital
HV	antibody HyperVariable region
Ig	Immunoglobulin
LDA	lithium diisopropylamide
LET	Linear Energy Transfer
MAb	Monoclonal Antibody
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization-Time Of Flight
MCA	Macrocyclic Chelating Agent
MRI	Magnetic Resonance Imaging
NBS	N-bromosuccinimide
Ophen	1, 10-ortophenantroline
PT-MBCA	Pendant-Type Macrocyclic Bifunctionalised Chelating Agent
Rad	radiation absorbed dose
RID	Radio Immune Diagnostic
RIT	Radio Immune Therapy
TAT	Targeted α-immunotherapy
TFA	TriFluoroacetic Acid
THF	Tetrahydrofurane
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
TPTZ	2,4,6-tris(2'-pyridine)-1,3,5-triazine
TTHA	Triethylenetetramine-N,N,N',N'',N''',N'''-hexaacetic Acid

Part I

State of the Art

Chapter 1

Radioimmunotherapic Drugs

1.1 General concepts

Aim of this work is the synthesis of BCA, the molecular moiety in RIT and RID drugs that bind together the antibody (that seeks for cells that express the related antigen) and the radioactive isotope (whose decay will kill the malignant cells).

Immunotherapeutic and immunodiagnostic drugs, whose characteristic is their ability to selectively recognize and bind to specific targets, have been proposed at the beginning of last century by Paul Herlich[ii]. He suggested the side chain theory for antibody and antigen interaction just seven years after the discovery of antibodies in 1890, when Emil von Behring and his student Shibasaburo Kitasato described their activity against diphtheria and tetanus toxins. In this theory he hypothesized that receptors (described as “side chains”) on the surface of cells could bind specifically to toxins – in a "lock-and-key" interaction – and that this binding reaction was the trigger for the production of antibodies, Fig. 1.1.

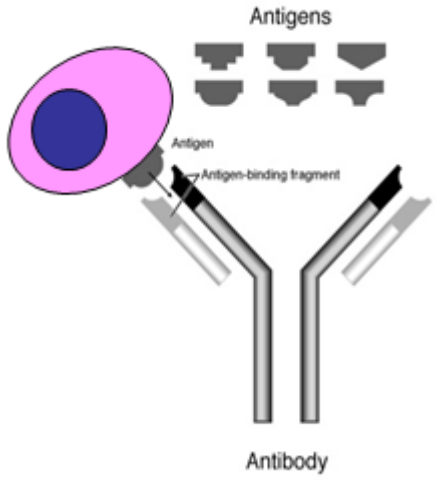


Fig. 1.1: Antibody and lock-and-key interaction with its antigen

The next logical step was to imagine, like he made in 1904, to use this natural pointing system to address substances that can induce cellular apoptosis into cancer cells and other pathogens, i.e. immunotherapeutic drugs.

Since the global performance of these drugs and their viability are determined by each of its moiety and each part interacts with the other two, it is important -before to begin any research in this field- to understand their basic functioning and the restraining condition they pose each other.

1.2 Monoclonal antibodies[iii]

An immune system is a collection of mechanisms within an organism that protects it against disease by identifying and killing pathogens and tumor cells. It detects a wide variety of agents, and needs to distinguish between organism's own healthy cells and tissues (self) and foreign substances (non-self) in order to function properly. Detection is complicated as pathogens adapt and evolve new ways to disguise themselves and successfully infect the host organism.

To survive this challenge, more and more sophisticated mechanisms have evolved that recognize and neutralize pathogens. Even simple unicellular organisms such as bacteria possess enzyme systems that protect against viral infections. More sophisticated mechanisms developed with the evolution of vertebrates, even if the primitive mechanisms are still operating. In the most evolved of these mechanism DNA of B germline lymphocytes undergoes to random mutations, generating millions of “clones”, each one producing a different kind of antibody specific for its own antigen. All this antibodies, also

called immunoglobulin (Ig), have the same base structure, consisting of four polypeptides –two heavy chains and two light chains- joined by disulphide bonds to form a “Y” shaped molecule.

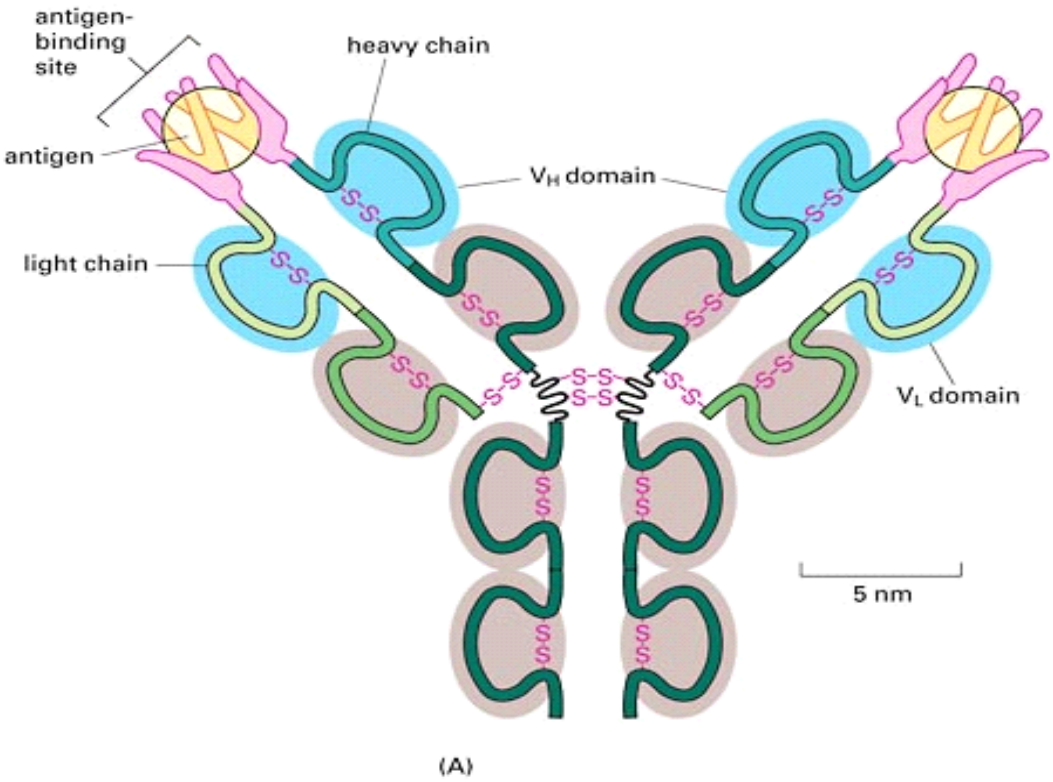


Fig. 1.2: Antibody structure

It is the amino acid sequence on "Y" tips, composed of 110-130 amino acids, that varies greatly among different antibodies and gives to each of them its specificity toward one antigen. The variable region includes the ends of the light and heavy chains. Treating the antibody with a protease can cleave this region, producing the so called Fragment Antigen Binding (Fab). The constant region, named crystallisable fragment (Fc), determines the mechanism used by the body to destroy the detected antigen.

The variable region is further subdivided into hypervariable (HV) and framework (FR) regions. Hypervariable regions have a high ratio of different amino acids in a given position, relative to the most common amino acid in that position. They directly contact a portion of the antigen's surface. For this reason are also sometimes referred to as complementarity determining regions (CDR). Within light and heavy chains, three hypervariable regions exist, separated by four FR regions which have more stable amino acids sequences.

1.2.1 Murine Mab

To exploit antibody for drugs an industrial scale procedure for their selection and production was needed. In the 70's of last century the set up of cellular hybridization technique by Köhler and Milstein[iv] allowed big scale production of Monoclonal antibody (Mab), generating a wave of scientific and economical enthusiasm equivalent of the unbridled optimism that nowadays surrounds stem cells.

This basic production process involves injecting a specific antigen into a mouse, thereby inducing the mouse's B lymphocytes antibodies production. To harvest such antibodies, scientists would ideally pluck only the B cells that make them. But finding those cells and getting them to make large quantities of the antibodies takes some doing.

Part of the complex procedure involves fusing B cells from the mice to immortalized (endlessly replicating) cells in culture to create cells called hybridoma. Each cell ("clone") is bread, non immortal hybridoma dies after a while and (among the immortal surviving cells) those producing the required antibody are selected. The drawback of these particular hybridoma is that they produce murine antibodies, which the human immune system can perceive as interlopers.

Patients who have received infusions of murine monoclonals have experienced a so-called Human Anti-Mouse Antibodies (HAMA) response. This syndrome includes joint swelling, rashes and kidney failure and can be life-threatening. It also destroys the antibodies. As consequence rodent antibodies cannot be used for prolonged human therapy: Clinical trials failed, stocks plunged and millions of dollars were lost.

1.2.2 Chimerical and humanized Mab

Several different strategies have been set up by scientist to solve this problem: Some point to make murine antibody more human. Since antibody specificity is due to interactions between its Fab region and the antigen, replacing the murine Fc region with a human one should not affect its specificity and reduce the likelihood that it is recognize as non-self by other antibodies. Some of the monoclonals now on sale are such chimerical –part mouse, part human- antibodies. Since this approach gave positive results, it was extended and more and more parts of the murine antibodies have been humanized. First the FR

moieties of the Fab and subsequently even parts of the "CDR grafting" have been replaced by human counterparts.

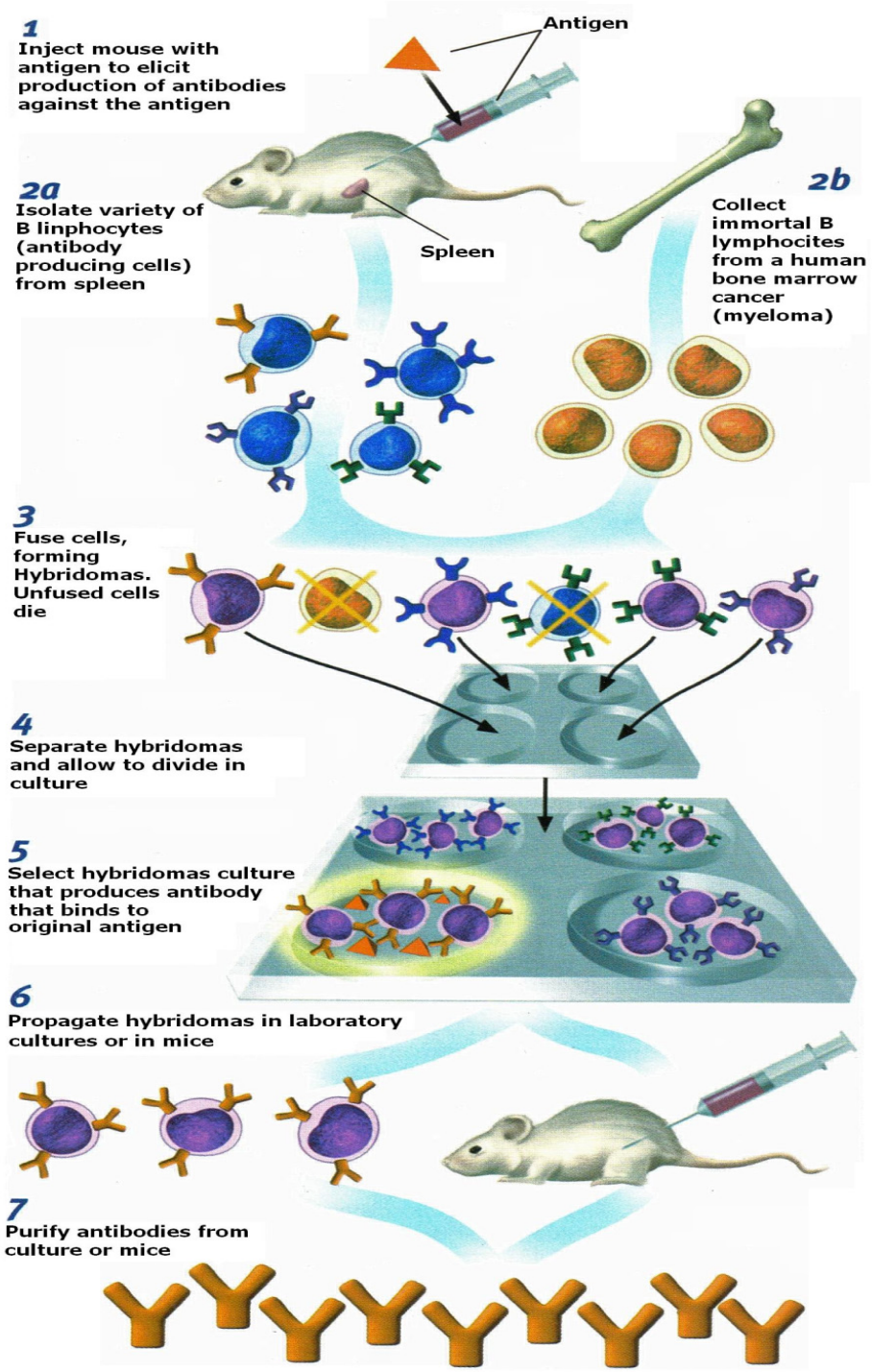


Fig. 1.3: Production of monoclonal antibodies.

1.2.3 Fully human Mab

To achieve this goal investigators are attempting to mass-produce monoclonals without the aid of mice. Abraham Karpas of the University of Cambridge finally fused human B cells to human immortalized cells to create hybridoma that generate fully human antibodies[v]. It is important to underline that this does not mean that no immune response from the immune system of the patient can be elicited, since somatic differences between antibodies of different people still exist.

Another strategy entails using genetic engineering to induce mice to produce fully human antibodies. Mice are genetically altered to contain human antibody genes; injecting the mice with antigens, the animals produce antibodies that are human in every way.

1.2.4 Mab by phage display

The most promising technique called phage display not only avoids the use of rodents, but at the same time helps to find the most specific monoclonals against a particular antigen. It takes advantage of a long, stringy virus called filamentous phage that infects bacteria. Researchers can isolate DNA from human B lymphocytes, insert this DNA into bacteria such as Escherichia coli and then allow filamentous phages to infect the bacteria. As the phages produce new copies of themselves, they automatically make the proteins encoded by the antibody genes of the various B lymphocytes and add them to the surfaces of newly forming phage particles. Scientists can then use the antigen they intend to target, such as a receptor on cancer cells, to fish out the phages containing the gene for the most specific antibody to that antigen. To produce a lot of that antibody, they can either have one phage infect more bacteria or insert the antibody gene into cultured cells.

1.3 Radionuclide

Radioactive decay is a spontaneous nuclear transition of unstable nuclei from a definite quantum state of the original nuclide towards more stable nuclear quantum states of the daughter nuclide(s). It has been shown to be unaffected by pressure, temperature, chemical form, etc. This insensitivity to extranuclear conditions allows the characterization of radioactive nuclei (without regard to their physical or chemical condition) by their decay period and their mode and

energy of decay. All this properties have been considered for the choice of the most appropriate radionuclides in radioimmune drugs.

1.3.1: Half-life

Radioactive decay is a random process: among the atoms in an hot sample is not possible to identify which specific atom will be the next to decay. The number of disintegration per unit time A, called the *decay rate*, is proportional to N, the number of radioactive atoms present: $A = -\frac{dN}{dt} = \lambda N$, where λ is the proportional constant known as *decay constant*. If the number of radioactive nuclei and the number of decays per unit time are sufficiently great to permit a statistical treatment, calling A_0 the activity present at some original time t=0, an easy mathematical treatment shows the exponential nature of radioactive decay: $A = A_0 e^{-\lambda t}$. Instead of λ is commonly used the *half-live* ($t_{½}$), defined as the time required for one-half of the radioactive atoms in a sample to undergo decay (in practice is the time for the measured radioactivity of a sample to decrease to one-half of its previous value).

In the choice of this parameter a trade-off between two opposite exigencies must be set. On one side drug feasible manufacturing and application require a long half-life. On the other side a short half-life would minimize the quantity of radioisotope to be given to the patient. It must be keep in mind that a big amount of the element would potentially deliver unnecessary doses into the environment –with the connected potential health damages- and increased waste managements procedures.

For biological application it must also be considered that not all the radioactive nuclei given to a patient decay in the body, but each kind of radioimmune drug (such any other substance) has its own metabolism. This important pharmacokinetic parameter is expressed by *biological half-life* (t_b), defined as the time required for half of a given substance to be removed from an organism by either a physical or a chemical process. With radiopharmaceutical drugs both nuclear and biological half-lives must be considered, and the representative parameter is called effective half-life (t_{eff}): $\frac{1}{t_{eff}} = \frac{1}{t_n} + \frac{1}{t_b}$. As in the case of half-life a trade-off must be set in the choice of this parameter between the time required to the drug to reach the target and data accumulation on one side and kinetic of elimination on the other. A too short t_{eff} will lead to a

high number of disintegration before that the vector could reach the target, which means a bad dose ratio between the target and the health tissues. On the other side with a too long t_{eff} a big aliquot of the given dose would be eliminated from the body (usually by the kidneys) before to reach the target tissue, impairing the therapeutic effect. To overcome this problem a higher dose could be given, on the cost of a higher toxicity of health organs, kidneys *in primis*.

In radiodiagnostic application the half-life is between six and a couple of hundreds of hours, in radiotherapeutic spans between some days and a couple of weeks.

1.3.2 The dose

Travelling trough the matter all radiations transfer their energy to the environment. This energy is measured by the *dose,* the amount of radiation energy absorbed per unit mass: $D = \frac{dE_{abs}}{dm}$. In the SI system it equals to 1 Joule per Kg and is called *Gray* (Gy), while in cgs system the unit was 100 time littler and was called rad (radiation absorbed dose).

For biological porpoises it is important to know not only the whole quantity of energy released in the living, but also its density. There is in fact a big difference (Fig and Table) in the consequence on the living if energy is released in tenths of meters (as in the case of gamma rays) or in some µm (as in the case of alpha radiations). This phenomenon is quantified by the *linear energy transfer* (LET), defined as the energy absorbed in matter per unit path length traveled by a radiation: $LET = \frac{dE_{abs}}{dx} \left[\frac{J}{m}\right]$.

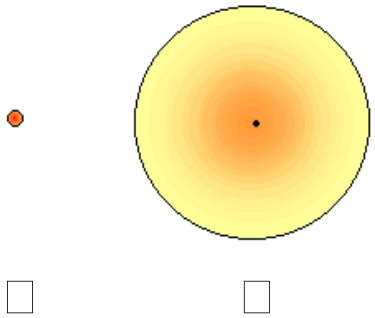


Fig. 1.4: Area involved in a decay event and LET

Tab 1: Comparison between α/β decay

Decay mode	α	β
Energy [MeV]	6	1
Avarage path [mm]	0.1	5
LET $\left[\frac{J}{m}\right]$	60	0.2

An even more accurate parameter, which considers also the dimension of the radioactive particles and the different nature of the living tissues that are travelled by radiations, is the *equivalent dose*, measured in Sievert (sv).

1.3.3 Decay modes

The mode of radioactive decay is dependent upon the particular nuclides involved. The energy difference between the two quantum levels involved in the transition corresponds to the *decay energy*. This decay energy appears in form of electromagnetic radiation and as products kinetic energy. Depending on the radioactive products of the process three main different decay mode have been identified and must be considered: α, β and γ:

- **Alpha decay** is the emission of an helium nucleus.

  It is observed for elements heavier than lead and for a few nuclei as light as Ln. It can be symbolically written as: ${}^{A}_{Z}X \rightarrow {}_{Z-2}{}^{A-4}Y + {}^{4}_{2}He$. As usual in nuclear equation X and Y indicates any element defined by its nuclear charge (Z and Z-2 in this case) and A is the atomic mass.

  Alpha radiations are characterized by their high energy (6-7 MeV), with a high ionizing power and a very short average free path -in a biologic medium no more than 40-80 μm. This two characteristic make α rays the radiation of election for immunotherapy uses on blood tumors and metastases, because of their high LET that kills more then 70% of the targeted cells with low damages on the surrounding health cells. For big tumor masses the short average free path results in high micro dosimetric effects (i.e. non homogeneous irradiation) and reduced *crossfire effect* (the irradiation of non targeted cells by radiation arising from radioimmunotherapic drug on neighborhood cells) the most effective death cause of stem cancer cells.

- **Beta decay** is the emission of an electron or of its antiparticle.

  These processes include electron emission (β^- or ${}^{0}_{-1}e$), positron emission (β^+ or ${}^{0}_{+1}e$) and electron capture (EC).

  β^- process can be written symbolically as follow: ${}^{A}_{Z}X \rightarrow {}_{Z+1}{}^{A}Y + {}^{0}_{-1}\beta + \bar{\nu}$, where $\bar{\nu}$ is the antineutrino, an atomic particle that plays no role in this work. Like alpha particles beta ones loose their

energy ionizing the environment, but since they are lighter, faster and bear a limited electric charge they interact less with the matter. As consequence they have a longer average free path, from some hundreds of μm to some mm. It is not enough to allow these radiations to escape out of the body making them useful in radio diagnostic applications, but this longer path means less micro dosimetric effects (i.e. bigger irradiation homogeneity) and is enough to damage a relevant amount of cells surrounding the targeted one with crossfire. This is of particular relevance in dealing with tumors with bulky necrotic tissues, because inner malignant cells must not be directly targeted to cure the cancer. One of the limits of the Targeted Alpha Immunotherapy is in fact the need to hit directly all tumor cells. To try to reach this objective a quantity of drug exceeding the number of the specific receptors for drug Ig must be given to the patient. This excess cause unwonted collateral damages to other organs in general and to kidneys in particular. Even worse is that in the biggest number of case not all the tumor cells present the Ig antigens (specially the so called stem tumor cell have a different cell surface) and can be reached by alpha immunotherapy. To overcome this problem integrated alpha and beta immunotherapy protocols are under investigation.

β^+ processes are schemetized by the formula: ${}^{A}_{Z}X \rightarrow ({}_{Z+1}{}^{A}Y^{-} + {}^{0}_{+1}\beta + \nu$, with basically the same interaction with matter of the former decay mode. Nonetheless, once the positron has been thermalised, it undergoes to an annihilation process with an electron giving rise to two photons of equal energy, 511 KeV, emitted at 180° one from the other. These photons can be detected in coincidence allowing separating their signal from the background noise allowing a better tridimensional resolution of the organs. PET [vi] (Positron Emission Tomography) is based on this effect.

- **Gamma decay** is the emission of electromagnetic radiation.

  Alpha and beta decays can leave the daughter nucleus in an excited state. This energy is usually removed by a process that is the nuclear equivalent of the Frank-Cordon effect, called gamma emission.

  Since they are not particles but photons (even if very hard ones, with an energy that ranges from some KeV to a couple of MeV), they can travel trough matter until they not interact with some nucleus or electron that adsorb them. For this reason they are never completely halted but just

exponentially attenuated by interaction with matter. This characteristic makes them useful for radiodiagnostic applications. For detection sensibility, radioprotection and tissue self-absorption reasons the energy range of this radiation must be comprised between 100 and 600 KeV.

1.3.4 Selection of the Radionuclide

From the consideration of the previous paragraphs come out that the ideal radionuclide to be used in immunotherapy drug aimed at therapeutic purposes must be an alpha or a beta emitter with half-life in the range between some days and a couple of weeks. In the following tables we can see the candidate β and α emitters.

Tab. 1.2: β *emitter radionuclide of medical interest half-life ordered.*

Nuclide	Half-life [days]	Energy of the β emission [KeV]	Energy of the strongest γ emission [KeV]
^{127}Tl	0.39	700	
^{188}Rn	0.70	2130	155
^{142}Pr	0.80	2160	1600
^{105}Rh	1.48	740	320
^{77}As	1.62	680	239
^{153}Sm	1.95	800	1030
^{149}Pm	2.21	1070	289
^{67}Cu	2.58	540	185
^{90}Y	2.67	2280	
^{198}Au	2.70	470	911
^{186}Rn	3.77	1080	131
^{111}Ag	7.47	1050	340
^{131}J	8.01	600	364

The first (and until some years ago the only) radioisotope to be used for therapeutic aims was ^{131}J. It has a good specific activity, appropriate half-life and is relatively cheap. As an halogen it can be easily inserted into organic compounds. Its Achilles' heel are de-halogenation reactions, that causes dispersion of radioactive iodine trough the body with consequent irradiation of heal tissues. For this reason it would be sensible to replace iodine with other radionuclide (^{90}Y, which present no γ emission and has a shorter half-life would be the best choice) and suitable BCA for this aim are under investigation. Since some Targeted Beta Immunotherapeutic drugs relying on BCA are in phase III

clinical investigation or already commercialized (Zevalin, developed by San Diego-based IDEC Pharmaceuticals and Schering AG, uses ^{90}Y bounded to an Ig specific for an antigen called CD20 on the surfaces of B lymphocytes, cells that grow uncontrollably in non-Hodgkin's lymphoma) it was scientifically more interesting to focus onto alphaimmunotherapy.

Of the potential candidates in the following table only the possible use of ^{211}At and ^{212}Bi has been already enquired by other groups. This thesis deal with the possible use of ^{225}Ac: the absence of collateral emissions in its decay process and the half-live of 10 days make it in fact an ideal candidate for radio immunotherapic applications.

Tab. 1.3: α emitter radionuclide of medical interest half-life ordered.

Nuclide	Half-life [hours]	Energy of the α emission [MeV]	Mode of the strongest cascade emission	Energy of the strongest cascade emission [KeV]
^{213}Bi	0.77	6	β	444
^{212}Bi	1.01	6	β	492
^{149}Tb	4.00	4	γ	43
^{211}At	7.20	6	β	80
^{225}At	240	6		
^{223}Ra	272	6	γ	80

1.4 The Chelating Agent

The core of this thesis, as organic chemist, is the identification and synthesis of suitable Chelating Agents.

First trials of TAT (Targeted α-immunotherapy) drugs[vii] used commercially available CA, with the sacrifice of one CA donor functional group to form the covalent bond with the antibody. Nowadays two different approaches are worldwide followed on the choice of CA for Targeted Immunotherapy: the first can be ascribed to the Australian group of B. J. Allen at the St. Jorge Hospital in Sydney[viii], the second to the American company Actinium Pharmaceuticals of M. W. Brechbiel[ix].

- **The Australian group** deems that supplies problems of molecules synthesized at university laboratories overcome the advantages of higher stability constants of the Actinium-Chelating Agent complex and of lesser

interference with the Ig quaternary structure. This row of priorities leads them to optimize the use of commercially available CA, mainly Open-chain CA, in clinical trials.

- **The American group** on the contrary judge that a higher distortion of the antibody structure and mainly the effects of released Ac in the haematic flux can not be ignored. As consequence they have started a row of collaborations with universities and research center for the syntheses of tailored molecules that lead *inter alia* to the syntheses of Macrocyclic CA.

In both approaches first step is donor atom identification, and then the structural frame that allows an optimal spatial arrangement of these donor atoms around the metallic ion is enquired.

It must always be clear that since the final drug must be used in haematic environment the absolute thermodynamic stability is not the parameter that must be maximized: more important is the whole *in vivo* stability constant. In fact, even if as rule of thumb the higher the thermodynamic constant (at pH = 4.7 and around 37 °C) the higher the *in vivo* stability, the *in vivo* constant considers also transmetallation[1]† and transchelation[2]† phenomena, of paramount importance in an environment with an high concentration of metallic ions (consider for instance Ca^{++} -present in haematic environment with a concentration of around 2.5 mmol/dm^3) and of several different species that show chelating properties (like serines and albumins).

1.4.1 The Donor Atom

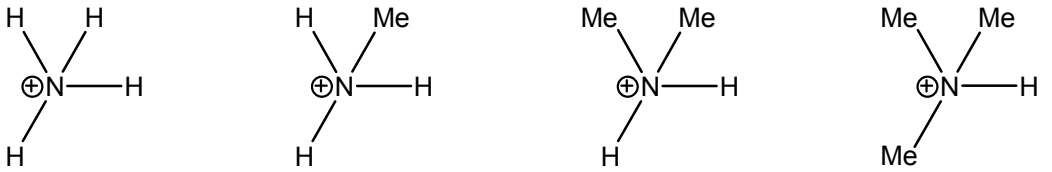
Two different parameters have been taken in account to identified the best suited candidate atom: basicity and electronic density.

- **Basicity**: An electronic lone pair must be provided by the donor atom to the metal ion to form a complex. As first approximation it can be tough that the more the basicity of the donor atom the higher the stability of the complex. From this point of view aminic groups should be better chelating agents then ethers or carboxylic groups.

  Since the substitution of one proton with an alkyl chain in amino groups increase its basicity (because of the electron-donating character of the alkyl group), it should be expected that the more the substitution around the N

[1]† When an atomic ion in the solution displace the metal in the complex.
[2]† When a chelating agent snatches the metal ion from another one.

atom the higher its basicity and the stronger the complexation affinity toward cations. However the pK_a values do not increase in a regular way with increasing alkyl substitution. In fact, tertiary amines are typically less basic than secondary systems. Solvation is responsible for this observation: increasing the number of alkyl groups on the amine nitrogen increase the unfavorable steric disruption of the solvent stabilizing shell of the conjugate acid.



pKa = 9.24 pKa = 10.62 pKa = 10.73 pKa = 9.79

Scheme 1.1: pK_a values of a Series of Simple Ammonium Ions.

It can be ascribed to this effect the increase in stability of amino complexes in the series ammonia < tertiary < primary < secondary. So far a secondary aminic functionality seems to be the best candidate to design Chelating Agents.

- **Soft-Hard interactions:** The basicity of the donor atom and consequently its attitude to share its lone pair with the metal ion influences only at first approximation the stability constant of the complex. Even more important are a good superimposition between the ligand orbital containing the lone pair and the empty orbital of the metal cation and an equal charge density of the two orbital. These considerations are the base of Pearson's theory of hard and soft interactions, that can explain, *inter alia*, why oxygen (an harder atom with Pauli electro negativity of 3.5) builds better complexes with alkaline metals, Ln and An while nitrogen (a softer atom with Pauli electronegativity of just 3.0) builds better complexes with transition and post-transition metals.

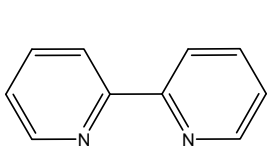
In this optic the hard nature of Ac^{+++} ions must be taken in account: it is the first member of the post transition series where 5f atomic orbital are progressively filled up and that is named actinides after him. The valence electrons of the elements of this series and of the corresponding ions are, like the electron of the Ln series, quite insensitive toward their chemical environment, it

is to say they are hard and form ionic bonds. For this reason negatively charged anions and oxygen are more suited to build stable complexes with it than nitrogen. The ideal candidates turn out to be oxygen containing ones, such as carboxylic group, phosphonic acids or hydroxylamine.

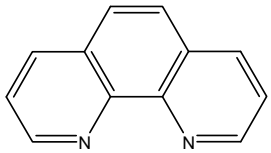
1.4.2: The Chelating Structure

All An are big atoms that form bulky ions with a radio, determined on the basis of the distance ligand-atom/metal in a series of isostructural compounds[x], in the range 9-12 nm. Ac in particular, as the first member of its series and for the well known horizontal properties of the periodic table, is the biggest one. For this reason the most favorable coordination indexes are in 6-9 range and stable complexes formation requires the presence on the Chelating Agent of at least 6 donor atoms.

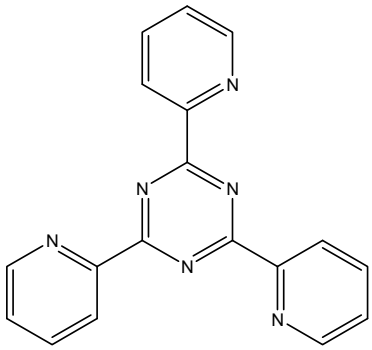
- **Open-chain Chelating Agents**: Historically first attempts to identify compounds with good chelating properties for Ac have been performed with commercially available nitrogen heteroaromatic compounds, such as 1, 10-ortophenantroline (Ophen); 2,2'-bipyridine (Bipy) and 2,4,6-tris(2'-pyridine)-1,3,5-triazine (TPTZ), scheme 1.2.



bipyridine



1,10-phenanthroline



2,4,6-tri(pyridin-2-yl)-1,3,5-triazine

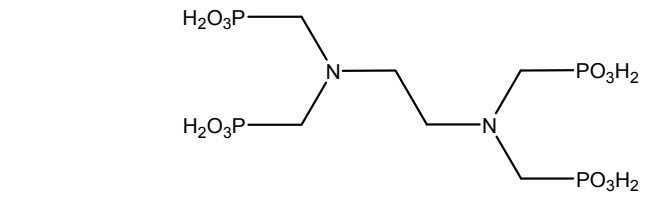
Scheme 1.2: Nitrogen Chelating Agents studied for radio immunotherapeutic applications.

These molecules are good chelating agents for An and the soft qualities of nitrogen lone pairs are used in nuclear facilities for separation operation of the

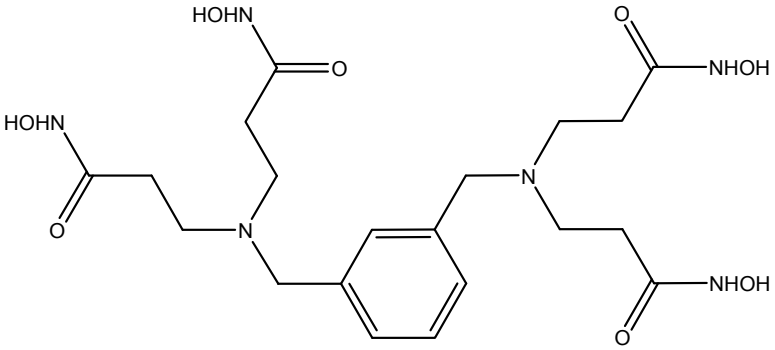
hard An ions from the harder Ln ions. Nonetheless their stability constants are not big enough to foresee their use as chelating agents in drugs. To enhance their stability constant values harder donor atoms, i.e. Oxygen, must be present in the molecule.

Experiments have then been performed with commercially available chelating agents containing carboxylic group [xi] (EDTA, perhaps the most ubiquitous used chelating agent, and its superior homologues, DTPA and TTHA), phosphonic acids (EDTMP, used in spent nuclear fuel reprocessing plants [xii]) or hydroxylamine (THA), Fig. 1.5 and 1.6.

Fig. 1.5: Oxygenated Chelating Agents



EDTMP



THA

- **Macrocyclic CA**: As already said, some research groups were not satisfied of the stability constant of the former Open-chain CA (TTHA, the most stable, has a $k_f \approx 10^{18}$) and prompted the synthesis of tailored CA, using the Macrocyclic Effect that was identified in the fifty years of last century. When e.g. in an ammonia complex two ammonia ligands are replaced by two methylamine molecules, its stability increases as expected as consequence of the higher basicity of amino groups. When two ammonia molecules are replaced by one molecule of 1,2-diaminoethane the stability increases even more. Two effects are responsible:
  - A geometric conformation that keep the second nitrogen atom near the metallic cation once that the first nitrogen has coordinated the metal
  - A geometric rigidity that keeps the two nitrogen atoms apart, minimizing their interactions and mutual repulsion.

The influence of these factors does not stop at the first substitution of ammonia atoms with more organized structures, but becomes more relevant with more complexes structures.

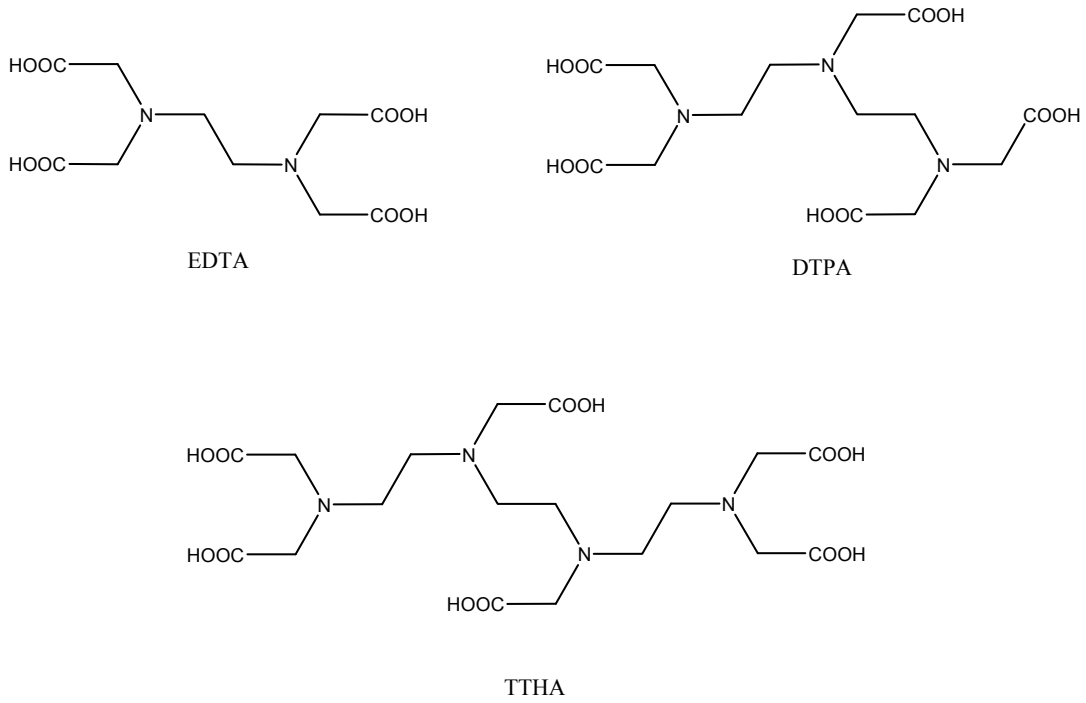


Fig. 1.6: Carboxyl Chelating Agents

To exploit this effect innumerable CA for charged species have been synthesized, from the two-dimensional crown ethers, to scorpionates (that use a tail that springs from the ion-molecule plane and form a bond with the metal ion from a direction incident to that plane) and to the cage molecules that surround and kidnap the ion like a prisoner in a jail. Among these possible structures Brechbiel[xiii] and Ouady[xiv] decided to synthesize a cyclic polyaza structure with carboxylic tentacles, the 1,4,7,10,13,16-exaazacyclooctadecane-1,4,7,10,13,16-hexaacetate (HEHA, Fig. 1.7).

They claim that this structure have the right mix between soft (nitrogen) and hard (oxygen) atoms needed to bind firmly Ac^{+++} ions without prompting transmetallation effect with the haematic Ca^{++}. As explain in chapter 3 this work do not agree with this point of view: probably nitrogen atoms do not play a

central role in directly chelating the Ac^{+++} ion but are necessary to give to the tentacles the required configurational flexibility by the inversion process.

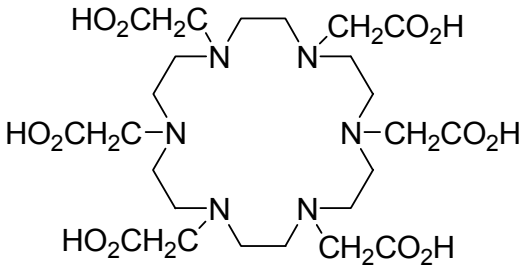


Fig. 1.7: HEHA, Macrocyclic CA

Of the several geometric parameters that can be tuned in polyheterocyclic molecules, the dimension of the prong between two heteroatoms has been particularly studied.

- **Dendrimeric CA**: One of the problems left open from the two previous class of molecules is the dose load. Each BCA can bear just one Ac^{+++} ion. Unfortunately, as can be seen in tab. 1.4, this leads to the death of the tumor cell just in the 70% of the sample population. To overcome this problem two ways, both unsatisfactory, are covered. The first one consists of increasing the number of BCA for antibody, but this often interfere with its quaternary structure and impairs its ability in recognizing the marker on the tumor cell. The second method is to increase the quantity of Ig-BCA given to the patient, but in this way the number of drug molecules that do not target the malignance increases and a higher dangerous dose harms health organs, mainly kidneys.

  - Tab. 01.4: Compared alpha and beta emitter efficiency.

% Tumor Cells Killed	alphas per Cell	betas per Cell
99.9	6	720000
99	4	480000
90	2	240000
70	1	120000
60	0.8	100000
50	0.6	72000
40	0.45	54000

The solution proposed in the chapter 4 of this thesis is the synthesis of dendrimeric CA, whose number of chelating tentacles change in accordance with the generation of the dendrimer and can be easily modulated with the exigencies of the drug to bind one or more Ac^{+++} ion.

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[i] For the peculiar nomenclature of these compounds see paragraph 4.3.5.

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Part II

Results

Chapter 2

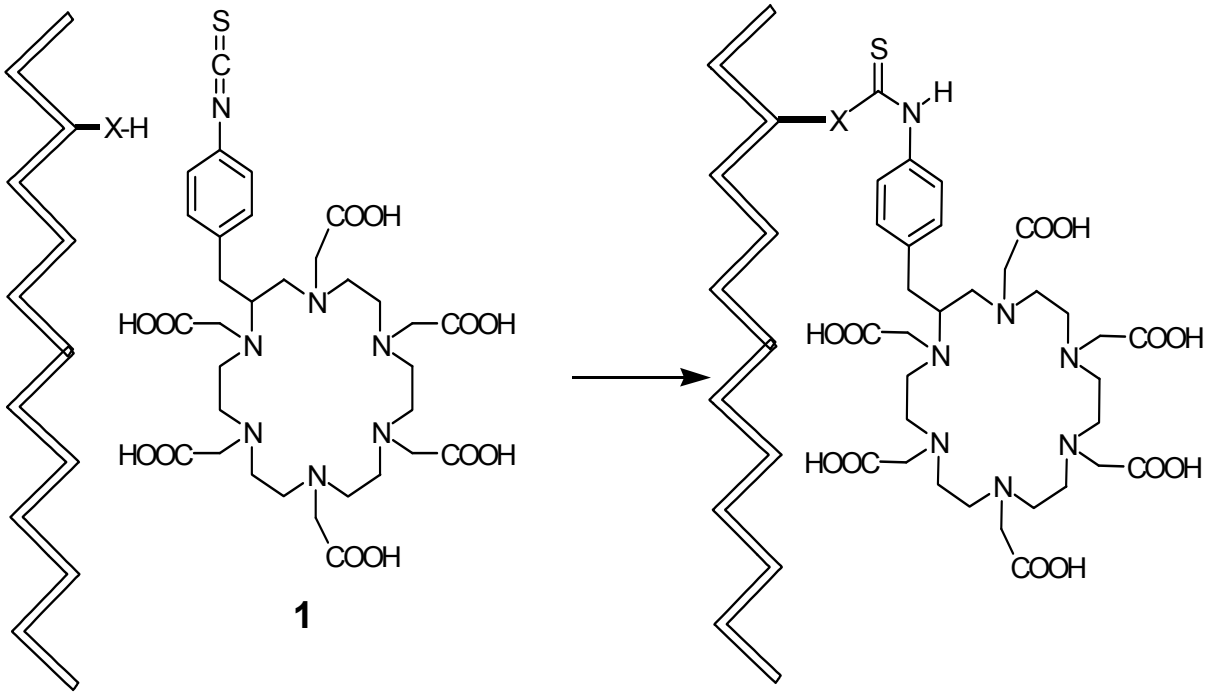
PT-HEHA: Macrocyclic Pendant-Type BCA

2.1 Structure

As stated in the previous chapter one of the most promising molecule that encompasses the characteristic required to act as good BCA toward Ac^{+++} is the 1,4,7,10,13,16-exaazacyclooctadecane-1,4,7,10,13,16-hexaacetate-2-*p*-benzylisotiocianate (HEHA-NCS, **1**). The PhD work of Dr. Ouadi[1] demonstrated that the non bifunctionalized 1,4,7,10,13,16-exaazacyclooctadecane-1,4,7,10,13,16-hexaacetate (HEHA, **2**) has indeed the foreseen good chelating properties toward Ac^{+++}, this encourages to make the hypothesis that also HEHA-NCS has akin chelating properties.

These cage molecules are composed by a polyaza ring moiety containing 6 nitrogen atoms, each with his lone pair available for interaction with the Ac^{+++} ion, and 6 acetic tentacles, each with its own carboxylic group, able to give their contribute in binding the metallic ion. The aromatic group in the pendant moiety gives both rigidity (a rigid pendant can keep the chelating moiety apart from the antibody minimizing the distortion of its quaternary structure) and it can provide an useful chromophore for analytical purposes. The covalent bond

with the Ig is provided by the good electrophilic isothiocyanate group, whose C=N double bond, with help of the thiocarboxylic moiety of the molecule, gives easily addition reaction with polarized molecules X-H, such as alcohols, acids and primary amines (Scheme 2.1). This group can also be used to bind the BCA to other supports, like polymers, to produce for instance chromatographic columns to explore a possible use of HEHA and of M-PT-BCA in general in An separations procedures.



Scheme 2.1: Linkage of a BCA to a substrate.

Alike to other chelating agents, also for HEHA-NCS its conformational equilibrium plays a crucial role: its lone pair and respective molecular orbital must be in the right spatial arrangement to overlap with the empty acidic acceptor orbitals of metal cations. Also the interaction of Ig and pendant moiety on the CA must be enquired. It is in fact possible that as BCA generates interference with Ig quaternary structure that can weaken its selectivity toward the specific antigen, so the effects of pendant and Ig on the CA could prejudice its chelating ability.

Conformational analyses performed with CAMM (Computer Assisted Molecular Modeling) techniques, based on energy minimization with MM+ model, show a different dislocation of electron lone pairs in HEHA, HEHA-NCS and in HEHA-NCS-Polymer:

- **HEHA** has three carboxylic groups, available to bind the metallic ion, on each side of its ring, Fig. 2.1, and its cage has adequate size to allocate a Ln atom

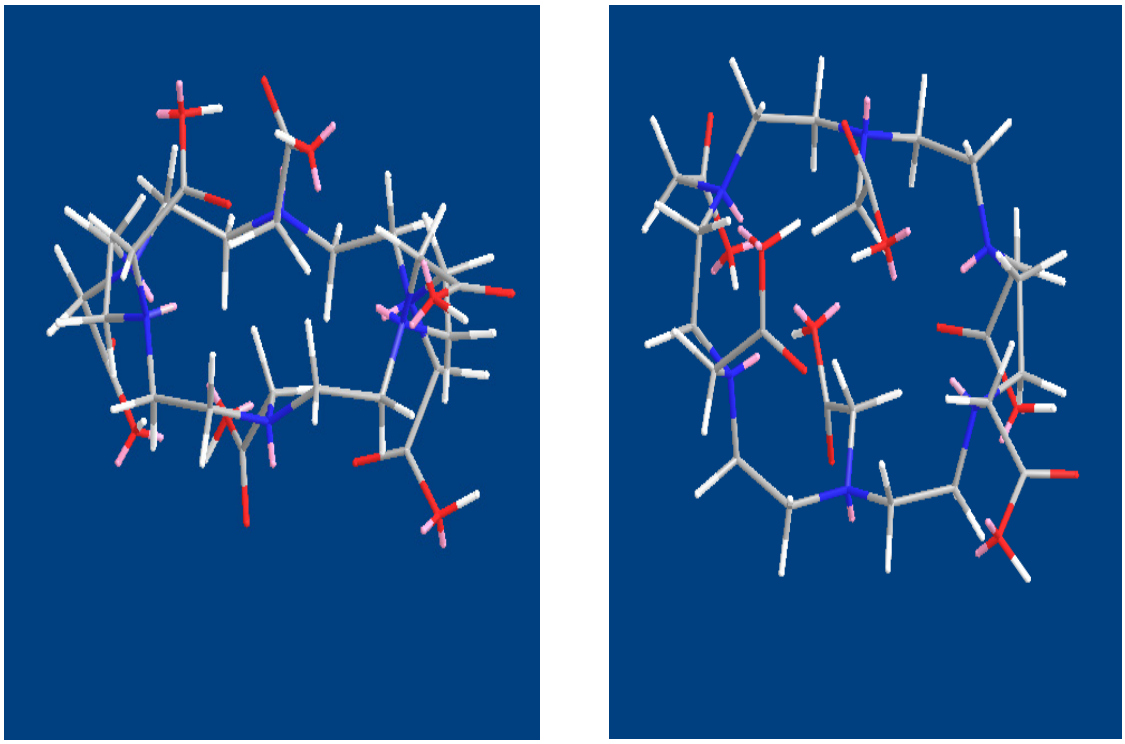


Fig. 2.1: Spatial disposition of carboxylic acid groups around the polyaza ring (left) and molecular cage (right). Color attribution: Blue nitrogen, Red oxygen, Gray carbon, White hydrogen, Pink lone pairs.

- **HEHA-NCS** has the pendant benzyl-isothiocyanate that introduces an element of dissymmetry in the molecule. The pendant completely alters the conformational structure and the 6 carboxylic tentacles are asymmetrically divided between the 2 faces of the molecular ring: 4 of them are on one face and just 2 remain on the opposite one, Fig. 2.2. While the face with 4 tentacles can easily catch an Ac^{+++} ion, the 2 groups on the other one could not exert enough strong interaction to firmly bind the metal ion.

- **HEHA-NCS-Polymer**, of interest for separation applications in column and that act as a model for BCA-Ig interactions, shows the same spatial distribution of carboxylic tentacles of the non functionalized HEHA, Fig. 2.3.

Even if some group[2] deems that the conformational structure of free HEHA-NCS impairs its employ in radioimmunotherapy applications, it must be kept in mind the dynamic nature of the binding process: the interaction between the charged ion and the BCA alters the conformational structure of the latter during the chelating process.

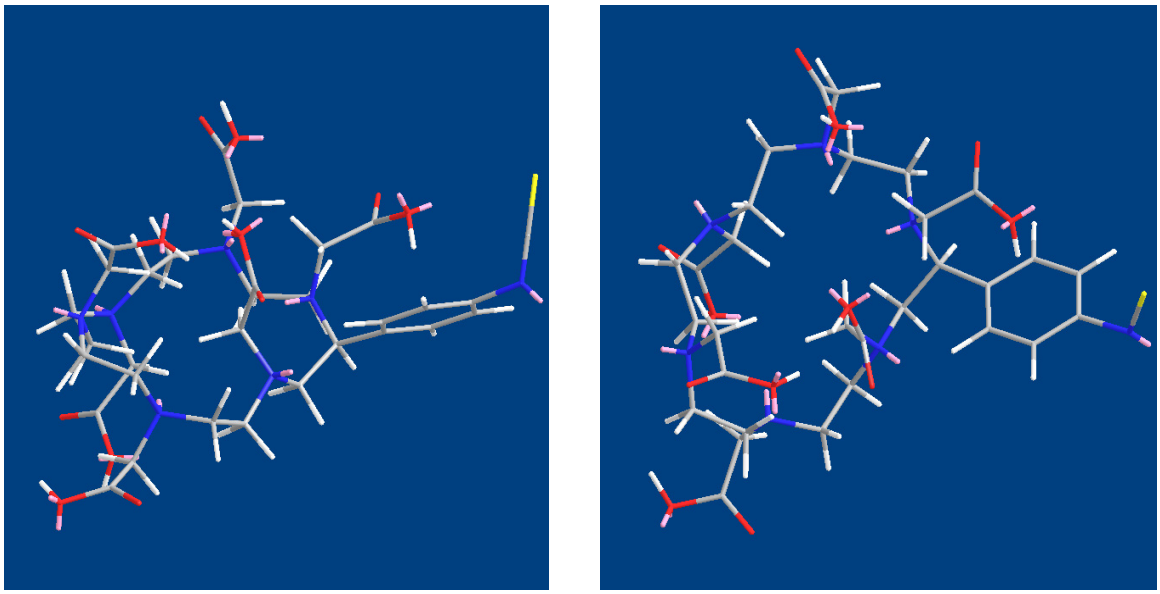


Fig. 2.2: Spatial disposition of carboxylic acid groups around the polyaza ring in HEHA-NCS. Color attribution: Blu nitrogen, Red oxygen, Gray carbon, White hydrogen, Pink lone pairs.

Each carboxylic tentacle of the CA flips rapidly about the nitrogen atom it is bond to, going trough a planar sp^2 intermediate, with the well known inversion phenomenon of amines. If this phenomenon were hindered enantiomers were present and it could also be possible to identify and separate them. The experimental consideration that it does not happen supports our assumption. In a dynamic process, once that a couple of tentacles begins to interact with the cation the whole conformational structure of the CA changes, the flipping tentacles spend the biggest part of their time on the side of the molecule where the cation is, attracted by its positive charge, and all 6 carboxylic groups contribute to form the complex. It must not be forgotten that when all the carboxylic groups are on the same side of the molecule, all the nitrogen lone pairs point on the opposite side, straight away from the metallic ion. From this point of view the role of nitrogen atoms into the polyaza ring is not, as Ouadi and Brechbiel assert, to participate directly in binding the An cation and to give it the right mix of hard and soft ligands required to bind An, but just to give to the structure the necessary conformational flexibility.

These analyses and considerations support the choice of HEHA-NCS as useful BCA in radioimmunotherapy studies.

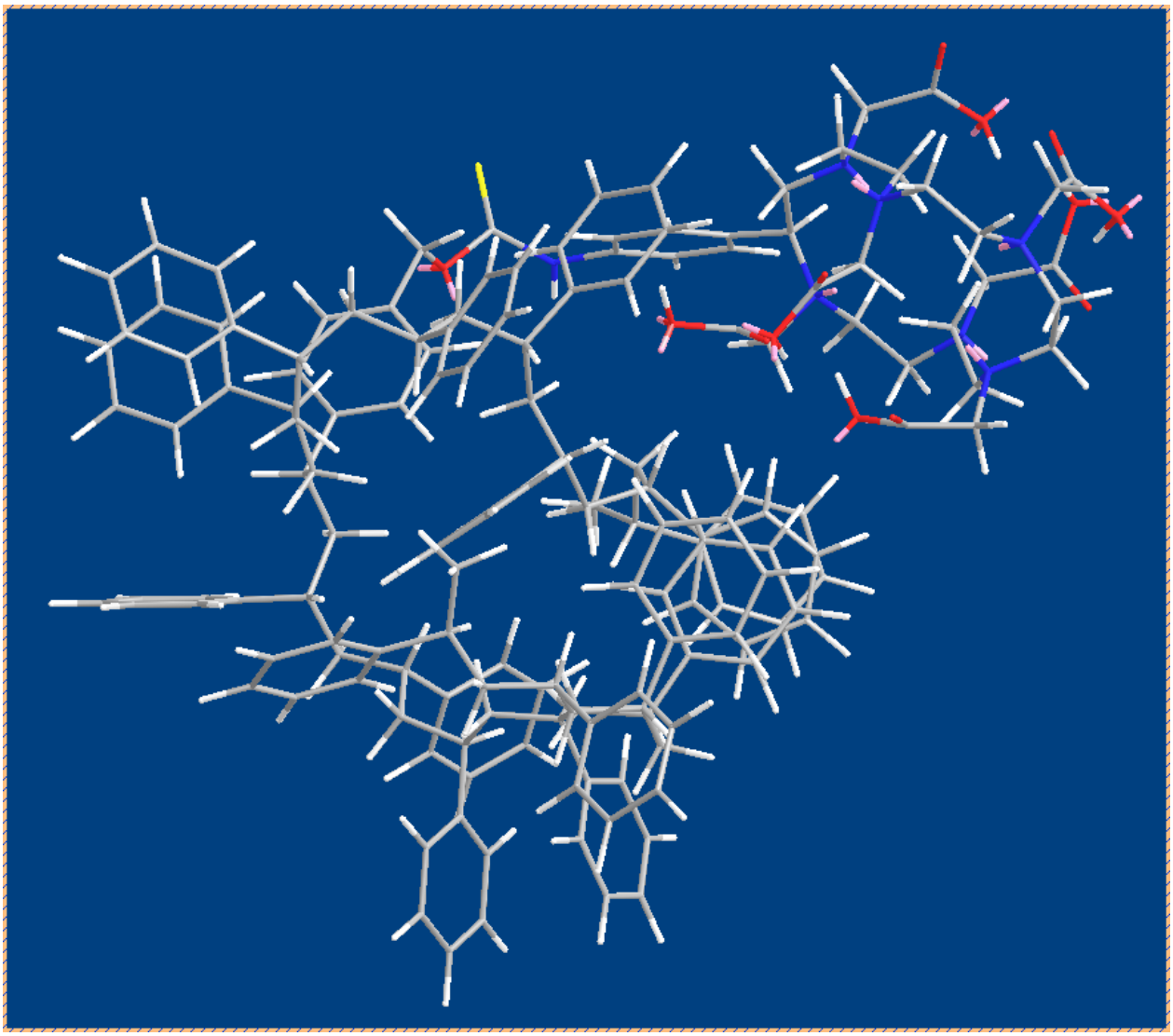
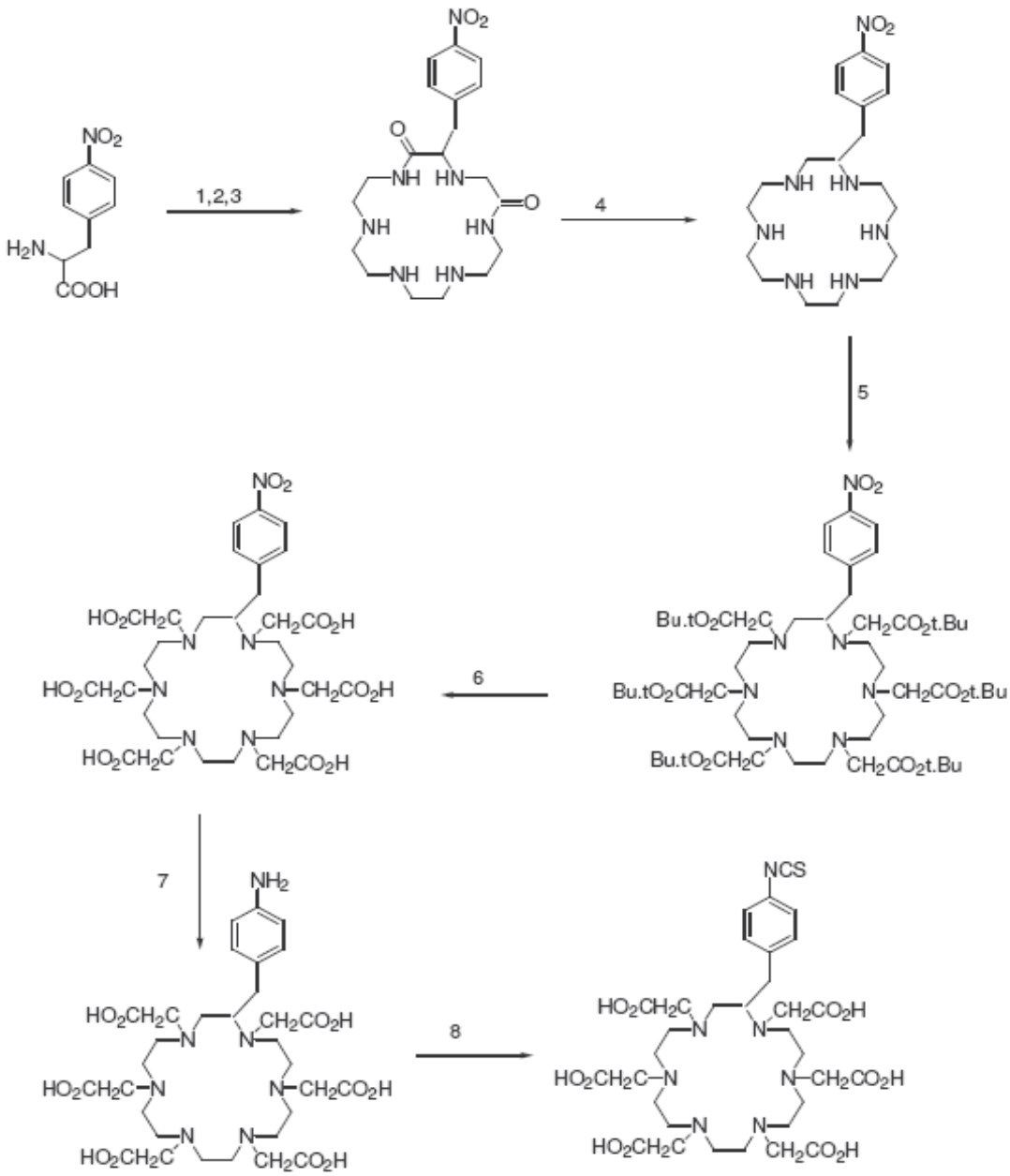


Fig. 2.3: Spatial disposition of carboxylic acids around the polyaza ring in HEHA-NCS-Polymer. Color attribution: Blu Nitrogen, Red Oxygen, Gray Carbon, White Hydrogen, Pink lone pairs.

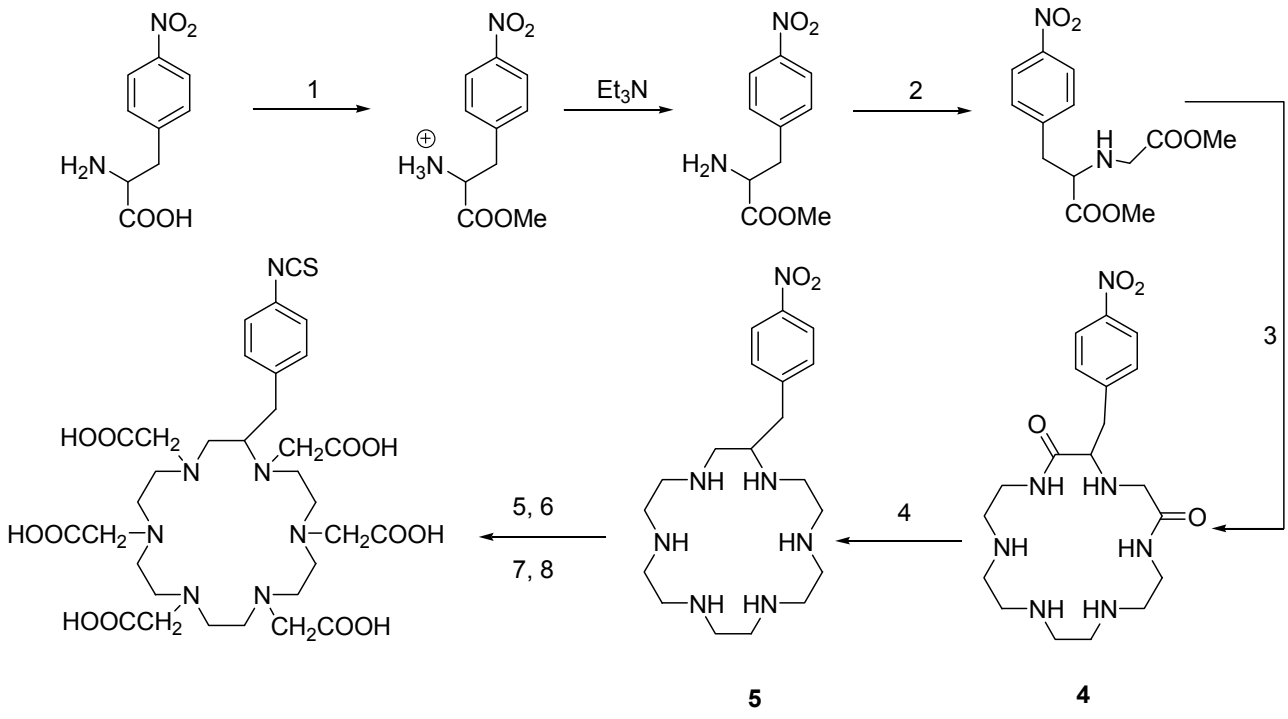
2.2 Total Ring Syntheses: 5+1 Approach

Even if some syntheses of functionalized HEHA are known, a gram scale preparation procedure of the product, indispensable to proceed to a better characterization of the drug and to the subsequent clinical trials phases, has not still been set up.



Scheme 2.2: 5+1 synthesis of HEHA-NCS, steps 4-8. 1: MeOH/HClg, 100%; 2 :Net_3/$BrCH_2CO_2Me$/THF, 90%; 3: NaOMe/TMPA/MeOH/reflux, 50%; 4: BH_3/THF, 55%; 5: Na_2CO_3/$BrCH_2CO_2But$/DMF, 82%; 6: $SnCl_2$/EtOH, 62%; 7: TFA, 90%; 8: $CSCl_2$, 90%

The procedure with the highest yield, proposed by Ouadi, can be seen in Scheme 2.2. As usual in macrocycles syntheses the critical step is the ring formation. In this case to close the polyazaaminic ring a double condensation between a fragment containing 5 nitrogen atoms and a fragment with just one nitrogen atom is proposed (Step 3 in Scheme 2.2). Because of the size of the two fragments involved in this cyclization reaction the whole approach is named 5+1. Even if some of its steps have an acceptable yield, the alleged yield of the whole sequence starting from the precursor is just of 7% and the characterization of the final product is not complete. In fact in the reported paper[3] only 100 mg of the final product are obtained and its only characterization is represented by a mass spectrometry analysis where the sole molecular peak is reported.



Scheme 2.3: 5+1 synthesis of HEHA-NCS, steps 1-4. 1: MeOH/HClg, 100%; 2: $NEt_3/BrCH_2CO_2Me$/THF, 90%; 3: NaOMe/TMPA/MeOH/riflusso, 50%; 4: BH_3/THF, 55%; 5: $Na_2CO_3/BrCH_2CO_2t$-Bu/DMF, 82%; 6: $SnCl_2$/EtOH, 62%; 7: TFA, 90%; 8: $CSCl_2$, 90%.

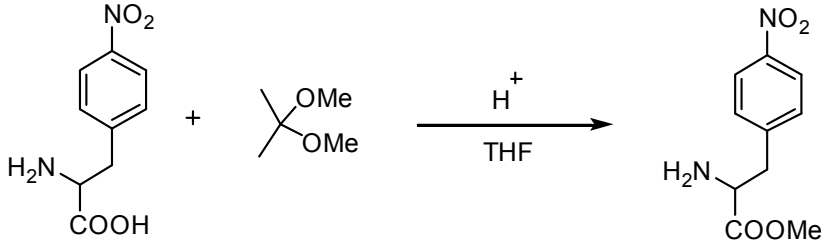
first three steps are crucial and could be modified. In detail starting from (R,S) 2-amino-3-(4'-nitrophenyl) propanoic acid (commercially available, albeit quite expensive) the synthetic strategy goes through the protection of the carboxylic acid groups as ester, employs the amino group as basis for the insertion of a carbomethoxymethyl substituent and allows the obtained methyl 2-

((methoxycarbonyl)methylamino)-3-(4'-nitrophenyl)propanoate (**3**) to react with 1,11-diamino-3,6,9-triazaundecane to obtain the cyclic diamide 3-(4'-nitrobenzyl)-2,6-dioxo-1,4,7,10,13,16-hexaazacyclooctadecane **4**. This amide, after reduction with $NaBH_4$•THF, should give the key intermediate 2-*p*-nitrobenzyl-1,4,7,10,13,16-hexaazacyclooctadecane (**5**), which with subsequent transformation (Scheme 2.3) would have given the target molecule **1**.

To achieve a gram scale synthesis it was necessary to improve the experimental procedures described in the previous Scheme to enhance the overall yield.

2.2.1 Synthesis of methyl 2-amino-3-(4'-nitrophenyl)propanoate

The first step, that in literature was performed with gaseous hydrogen chloride and anhydrous Methanol was drastically modified. The production of gaseous hydrogen chloride adding oleum to a solution of hydrochloric acid 37%, and subsequently bubbling the obtained wet gaseous hydrogen chloride through several stripping bottles in the effort to make it anhydrous, is not viable for gram scale syntheses. This reaction is replaced with a transacetalisation (Scheme 2.4) with 2,2-dimethoxypropane in anhydrous THF using *p*-toluenesulfonic acid as catalyst.



Scheme 2.4: Synthesis of methyl 2-amino-3-(4'-nitrophenyl)propanoate

This reaction has been improved after similar reactions described in literature.[4] Reaction driving force is represented by the higher thermodynamic stability of the products: acetone is more stable than its precursor 2,2-dimethoxypropane) and it is not necessary to force the equilibrium by removing one product or using great quantities of expensive reactant.

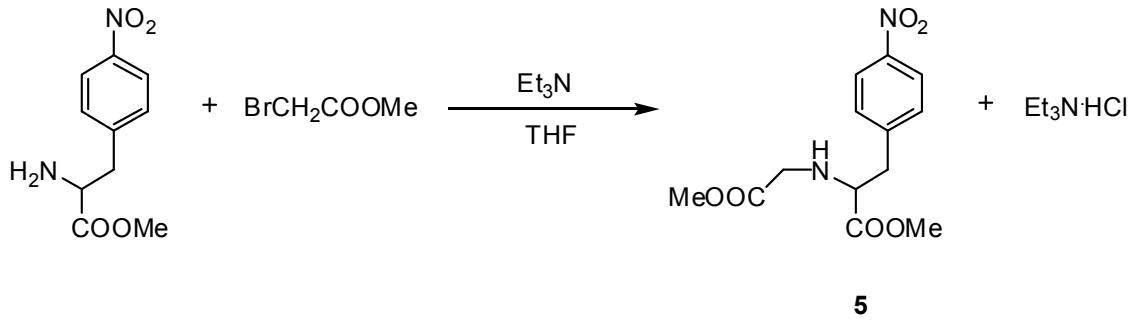
The second strong point of this reaction is to be absolutely anhydrous, being water used neither during the reaction nor during the product work-up. The excellent solubility of amino acids in water, due to their zwitterionic

characteristics, would make extremely difficult the recover of not reacted (R,S) 2-amino-3-(4'-nitrophenyl) propanoic acid from the reaction vessel. The by-products of this new reaction, anhydrous methanol and acetone, can be easily removed at reduced pressure at the end of the reaction, leaving a chromatographically pure product that requires no further work-up.

In the old synthesis two steps were required to achieve the same product, leading to inevitable decrease in yield. It was in fact necessary to neutralize the amino ester salt obtained in the first step during the reaction with gaseous hydrogen chloride with a basic solution of triethylamine. The new reaction gives the product with a chemical purity > 99% in few minutes, to be compared with the long reaction time of the old methodology reported in literature.

2.2.2 Synthesis of methyl 2-((methoxycarbonyl)methylamino)-3-(4'-nitrophenyl)propanoate

Since the previous reaction affords directly the free amine it can react without any preparation or deblocking step with methyl 2-bromoacetate (Scheme 2.5).

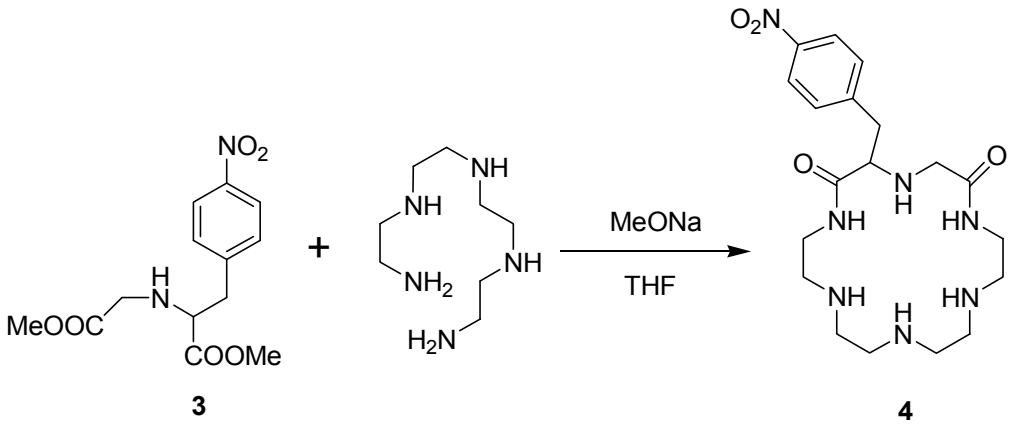


Scheme 2.5: Synthesis of methyl 2-((methoxycarbonyl)methylamino)-3-(4'nitrophenyl)propanoate

This step, performed in anhydrous THF, gives the product, after filtration and evaporation of the solvents under reduced pressure, almost quantitatively, to be compared with the 80% yield of Ouadi's method.

2.2.3 Synthesis of 3-(4'-nitrobenzyl)-2,6-dioxo-1,4,7,10,13,16-hexaazacyclooctadecane

In the third step of the reaction sequence reported in literature, sodium methoxyde was prepared *in situ* in a THF solution. To this solution were added sequentially the compound **3** and 1,11-diamino-3,6,9-triazaundecane dissolved in methanol (Scheme 2.6).



Scheme 2.6: Synthesis of 3-(4'-nitrobenzyl)-2,6-dioxo-1,4,7,10,13,16-hexaazacyclooctadecane

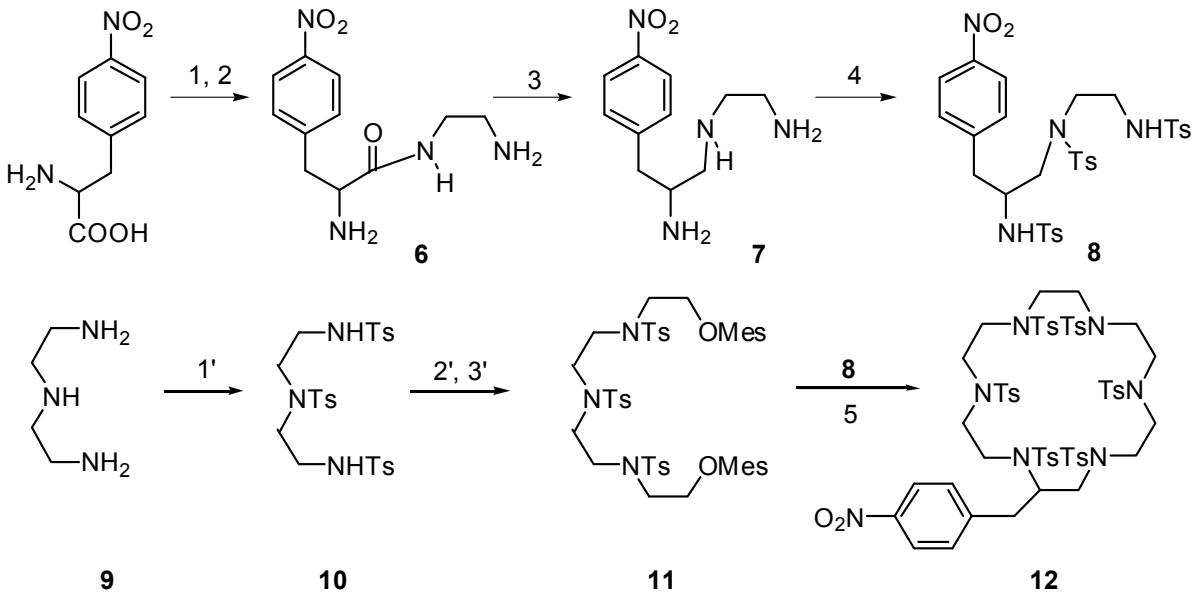
Also in this case the procedure has been modified: commercially available sodium methoxyde is dissolved in THF and the anhydrous tetrahydrofurane solution of 1,11-diamino-3,6,9-triazaundecane is added. After one hour at reflux the product **3** is added and the reaction proceeds for 96 hours. Another experimental improvement has been made in the purification procedures: The crude product is firstly washed with water to eliminate the triethylamine and then purified by flash chromatography (acetone/pentane 20:80 v/v). Unfortunately the reaction yield is just 20%, in accordance with the yield reported by all other groups[5,6] but the French ones, which claims a 50% yield.

2.3 Total Ring Syntheses: 3+3 Approach

This procedure (Scheme 2.7) is also named after the fragments that take part to the cyclization step, both containing 3 nitrogen atoms. The original sequence plans the conversion of the methyl ester of (R,S) 2-amino-3-(4'-nitrophenyl)propanoic acid in the amide **6**, that after reduction to amine **7** is protected[7] and activated through the formation of tosylate **8**. The sodium salt of this specie is finally cyclised with the tri-tosylated amine **11** to give the cyclic compound **12**, an immediate precursor of HEHA. Also in this case the yield of this cyclization step is just around 20%.

The first two steps of this reaction have till now been optimized:

1) The esterification is performed like in Scheme 2.4 (quantitative yield)
2) General conditions of the second step have not been changed, but of the work-up optimization leads also in this case to quantitative yield



Scheme 2.7: 3+3 synthesis of HEHA-NCS. 1: CH_3OH/HCl, 88%: (2,2-dimethoxypropane/p-toluenesulfonic acid, 100%); 2: $CH_3OH/NH_2C_2H_4NH_2$, 90% (100%), 3: THF/BH_3, 80%; 4: $NaOH/pTsCl/H_2O/Et_2O$, 70%; 1') $NaOH/pTsCl/H_2O/Et_2O$, 82%; 2') KOH/Etilen carbonate, No yield reported; 3') MesCl, 80%; 5: NaH/THF, No yield reported. Yields and condition in brackets are referred to this thesis work.

The way to set-up a gram-scale synthesis of HEHA by optimization and tuning of all the steps of the syntheses proposed in literature (Schemes 2.3 and 2.7) is still long, but from the results so far achieved other important upgrades of the processes can be foreseen.

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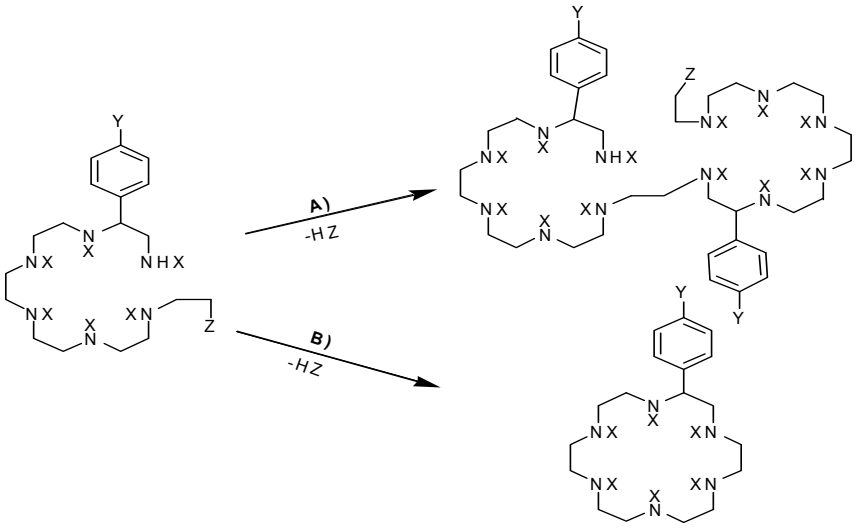
6) Stetter,H.; Frank, W. & Mertens, R. „Darstellung und Komplexbildung von Polyazacycloalkan-N-essigsäuren", *Tetrahedron*, **37**, 767-772 (1981)
7) Greene T.W., *Protective groups in organic synthesis*, Wiley, New York, 1981.

Chapter 3

New Approach to M-PT-BCA Synthesis

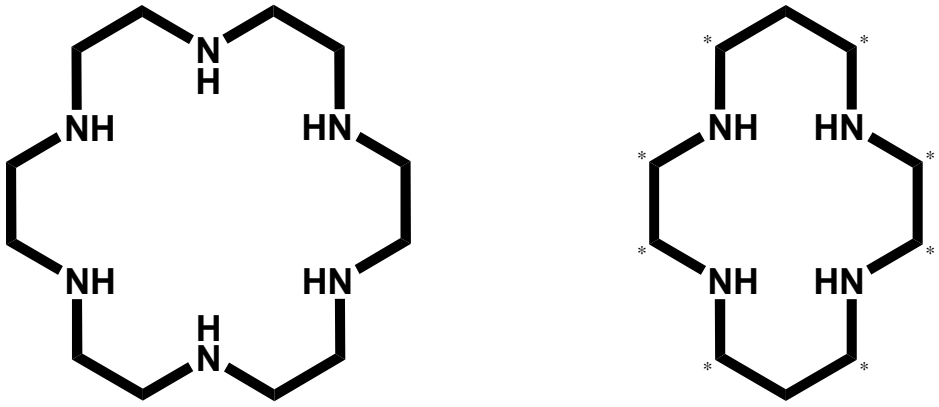
Total cycle syntheses can be easily performed with 5, 6 or 7 member rings but ring closure becomes more and more difficult at ring size increasing. In this step collateral intermolecular polymerization reactions take place, along with the desired intramolecular condensation. Linear dimers and oligomers produced in these side reactions sink dramatically the yield of the cyclization step and of the whole procedure.



Scheme 3.1: Condensation reaction. Path A: dimerization and polymerization byproducts. Path B: target molecule.

These two possible reaction courses are shown in Scheme 3.1 for HEHA-NCS synthesis. To minimize these side reactions it is necessary to work in high-dilution conditions, with extremely precise reaction parameters whose identification is extremely time-consuming and expensive.

To bypass this obstacle a brand new synthetic approach to PT-M-BCA, that could skip the cyclization step, was needed. This implied starting from the polyaza ring structure and converting it in a useful scaffold for the insertion of several kinds of pendant on the cycle, opening the way to the production of a broad range of new M-PT-BCA. It would then be possible, with an "ad hoc" experimental campaign, to chose among these molecules those with the best affinity for the metal ion and the antibody. The considerations expressed in former chapters made natural the choice of 1,4,7,10,13,16-exaazacyclooctadecane (**13**), commercially known as hexacyclene (HEXA, Fig. 3.1), as starting material.



13 **14**

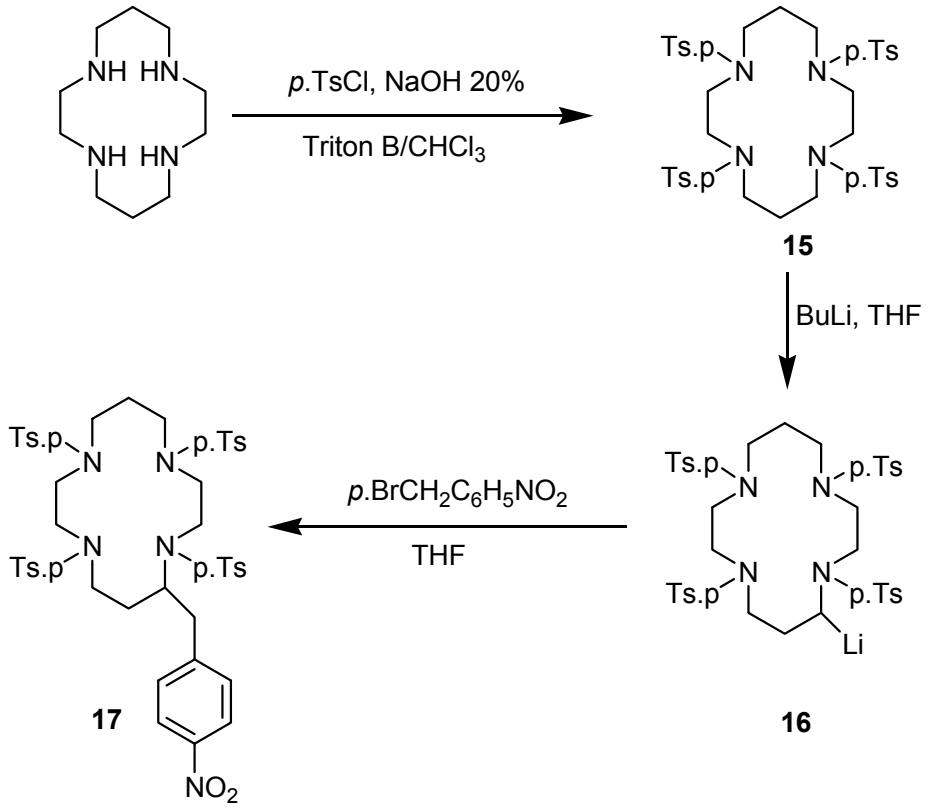
Fig. 3.1: 1,4,7,10,13,16-exaazacyclooctadecane, commercially known as hexacyclene (13) and 1,4,8,11-tetraazacyclotetradecane (14), commercially known as Cyclame

With this approach the task became the introduction of the pendant (for the target molecule the 4-isothiocyanatobenzyl or a suitable precursor) trough the functionalization of only one endocyclic methylene group. Taking into account Hexacyclene high cost, the whole reaction sequence has been implemented employing 1,4,8,11-tetraazacyclotetradecane (**14**), commercially known with the name of Cyclame (Fig. 3.1).

This molecule, available in large amounts in these laboratories, is a smaller homologue of **13** containing the same functional groups. As consequence they should have the same reactivity and procedures set-up on Cyclame should behave similarly to hexacyclene.

3.1 Synthetic Strategy

Possible strategies exploit the endocyclic nitrogens to introduce a functional group, which induces a positive or negative polarity on carbon atoms in α position (marked with a star in Fig. 3.1). The subsequent step is the reaction between the best-suited synthetic equivalent of this synton with a reverse polarity synthetic equivalent of the pendant.



Scheme 3.2: Synthetic route to a cyclame M-PT-BCA using p-tosyl as protective group.

The first synthetic strategy was based on the protection of endocyclic nitrogen atoms, followed by reaction with a suitable strong base, in equimolar amount to the tetra-protected Cyclame, that should lead to the formation of an anionic specie through the removal of one α proton. It is clear that because of the high symmetry of the molecule all atoms in α position to the endocyclic functionalized Nitrogens are equivalent for our synthetic aims. The extraction of a second proton from the same molecule should have been prevented by the negative charge on the formed anion. The reaction of this anion with a positive synthon of the pendant would have given (Scheme 3.2) the functionalized cycle. Kinetic considerations suggest that reaction between the unreacted ion and the PT-MCA to give back the unreacted macrocycle and the $[PT\text{-}MCA]^-$ ion is not competitive.

3.2 Paratoluenesulfonyl as protective group

Paratoluenesulfonyl group was chosen for its good protective features for amino groups[1]: it does not give side reactions with the *n*-butyl lithium, that has an hardness incompatible with the softness of the sulfonyl group,[2] and can be easily cleaved through basic hydrolysis.

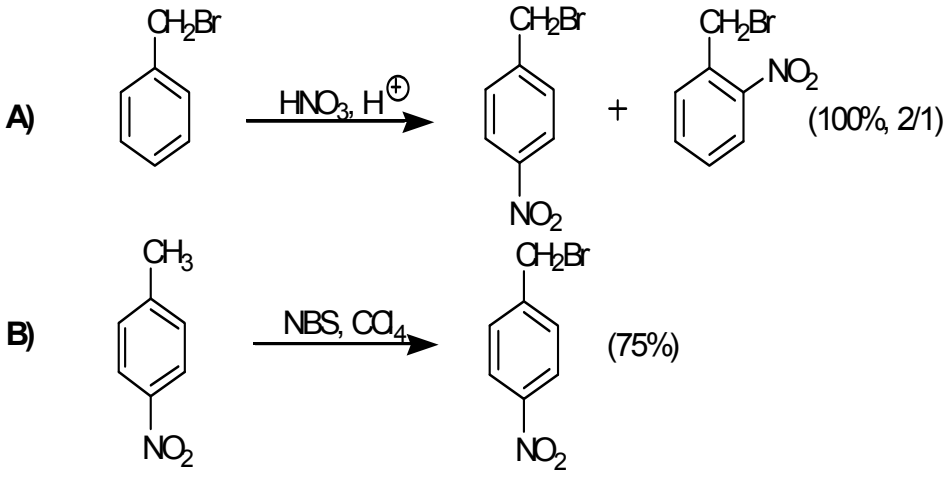
3.2.1 Synthesis of 1,4,8,11-tetraparatoluenesulphonyl-1,4,8,11-tetraaza-cyclotetradecane

In literature is reported[3] an expensive and cumbersome synthesis of 1,4,8,11-tetraparatoluenesulphonyl-1,4,8,11-tetraazacyclotetradecane (**15**). In this synthesis eight equivalents of paratoluenesulfonyl chloride give a mixture (1:1) of tri- and tetratosyl cyclame. The final yield, after purification is only 35%.

In this work a new synthetic procedure has been set-up, based on phase transfer catalysis concept. Cyclame and paratoluenesulfonyl chloride (1:4) react together in a double-phase medium (chloroform/aqueous solution of sodium hydroxide at 20%) using benzyl-trimethylamonium bromide (Triton B) as phase-transfer catalyst. This procedure does not make use of organometallic reagents and can be performed without the employ of anhydrous solvents and inert atmosphere. After 12 hours **15** was recovered almost quantitatively.

3.2.2 Synthesis of 4-nitrobenzyl bromide

The synthesis of 4-nitrobenzyl bromide, immediate precursor of 4-isothiocianate benzyl moiety, was easily carried out according to two different methodologies (Scheme 3.3):



Scheme 3.3: Systhetic strategies used for 1-bromomethyl-4-nitrobenzene preparation.

Nitration in para position of benzyl bromide performed with fuming nitric acid (98%) in presence of Amberlist 15-H, a sulfonic acid resin, as catalyst.[4] Unfortunately this technique produced para and ortho isomers in a 2:1 mixture isolated by flash chromatography[5]

- **Radical bromination** of a benzyl Hydrogen of paranitrotoluene has been developed to avoid the separation step trough the reaction of paranitrotoluene with N-bromosuccinimide (NBS) in carbon tetrachloride. The solution, gauged by GC, was refluxed till no more precursor was present, by means of a procedure described for analogous substrates.[6] The subsequent recrystallization of the reaction crude product gave the chemically pure 1-bromomethyl-4-nitrobenzene in a 75% yield.

3.2.3 Functionalization of the cycle

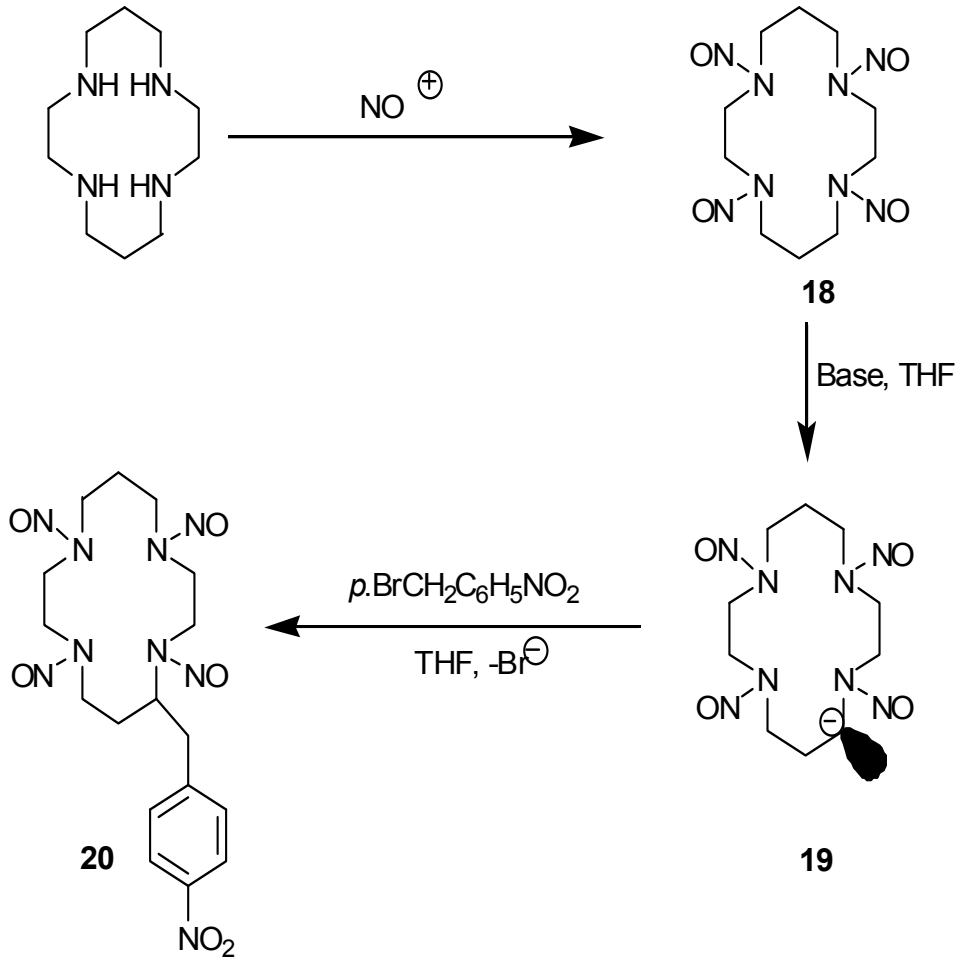
This step went through the reaction of **15** with an equivalent of *n*-Butyl Lithium and, after formation of intermediate **16**, with one equivalent of 1-bromomethyl-4-nitrobenzene. Unfortunately it did not give 2-(4'-nitrobenzyl)-1,4,8,11-tetraparatoluenesulfonyl-1,4,8,11-tetraazacyclotetradecane (**17**), but **15** was quantitatively recovered from the reaction mixture.

3.3 Nitroso as protective/activating group

The lack of reactivity of **15** can be ascribed to an insufficient acidity of the Hydrogen atoms in α position to the endocyclic sulfonamide groups. It was thus necessary to substitute the tosyl group with something that could not just protect the amino functionality, but also enhance the Hydrogen acidity. The ideal candidate was the nitroso group[7] whose reactivity has been widely studied by Seebach[8] and was quite well known. There were two possible approaches:

- **Nitrosation of only one amino group**, trough the protection of all the other endocyclic nitrogen atoms[9], facing the consequent separation of products with none, one or several nitroso groups at this early stage
- **Total nitrosation of the substrate**, that implies separation between mono and poly pendants chelating agents at a later synthetic stage.

The latter strategy (Scheme 3.4) was chosen because it allows a synergic approach, nowadays so widely recommended in industrial management manuals.



Scheme 3.4: Synthetic route to a cyclame M-PT-BCA using NO as protective/activating group.

Cyclic nitrosamines can in fact not only be used as synthetic scaffold for the synthesis of a wide range of PT-MBCA, but their complexing properties toward transition metals and Ln are well known[10]. It is of scientific and applicative interest to study, with new suitable CA, if they form stable complexes also with

It must be kept in mind that even this work deems that nitrogen atoms in HEHA have just a structural function and do not give an appreciable contribution to the Ac^{+++} binding, Ouadi and Brechbiel[11] have the opposite theory: a right balance between hard oxygen atoms and soft nitrogen atom in the same molecule is required to build a good CA for An. Such a balance could be achieved with help of nitroso group. In same N-nitroso complexes in fact both oxygen and nitrogen participate to the complex formation binding the metallic cation.[12]

In addition the cyclic polynitroso compounds produced as intermediates in this synthetic path can be interconverted by reduction in cyclic polyhydrazines, whose chelating properties towards An are also of interest.

3.3.1 N-nitrosamine structure

Nitrosamine are represented by two main resonance structures the second one representing the commonly used 1,3 zwitterionic form with a diazenic double bond, Fig. 3.2.

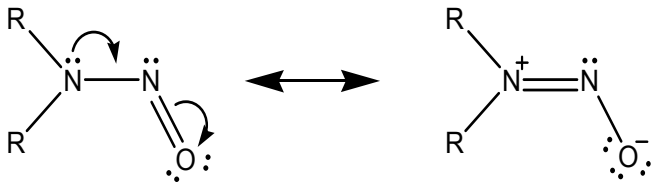


Fig. 3.2: Main N-nitrosamine resonance structures.

As a consequence of the relatively high weight of this limit resonance structure, the free rotation around the N=N bond is somehow hindered.[13] The presence of this partial double-bond characteristics implies the existence of isomerism: E-Z when the simple diazenic bond is considered, syn-anti around the ring structure when a cycle is present. An accurate determination of the relative weigh of this two limit structures, for a better understanding and foreseeing of the reactivity of this class of compounds and of the single nitrosamines, is a field that involved researchers for many years. It must be kept in mind that since the rotational barrier is just around 20 Kcal/mol,[14] compared with the 60 Kcal/mol of a C=C double bond, it is not always correct to speak of stereoisomerism and for compounds with low N-N rotational barrier the denomination conformer is more appropriate. It is also reported[15] a link between the stability of nitroso compounds and their rigidity: products that undergo easy N-NO bond cleavage have little diazenic character and as consequence can rotate more easily around the N-NO bond and exist just as conformers.

Fig. 3.3: E-Z isomerism in a N-nitrosamine. R has a higher Cahn-Ingold-Prelog priority group then CH_3.

NMR is the methodology of election for configurational assignment of nitroso compounds,[16] with this technique it is possible to discriminate for example α-syn protons –whose resonant frequencies are at higher fields- from α-anti protons.[17] Alike to alkenes stereoisomers, “cis” compounds are less stable than “trans” (Fig. 3.3) and the bulker the R group the higher the stability of “trans” stereoisomer.[18]

Tab. 3.1: 1,4,8,11-tetranitroso-1,4,8,11-tetraazacyclotetradecane production methodologies.

Reaction	Reagents	Conditions	Yield
1	$NaNO_2$/HCl in aqueous solution	Controlled pH (5.5) with acetic acid, room temperature	75%
2	$HNO_{2\ gas}$ from $NaNO_2$ and oleum	Bubbling gas at -5°C	70%
3	$SiO_2/H_2O/NaNO_2$/Nafion-H in heterogeneous catalysis	Stirring at room temperature	0
4	TMSi-ONO from $NaNO_2$ and $(CH_3)_3SiCl$	Stirring at room temperature, THF as solvent	90%

Several procedures for the synthesis of these products, summarized in Table 3.1, have been investigated.

Leaving out some exotic reactions like radical[19] ones (that use metallic ions and for this reason are not suited for this work aims)[20] or hydrazines oxidation[21] (where the synthesis of the starting reagent would be more difficult of the synthesis of the product), all syntheses of nitrosamine employ the nucleophilicity of amino nitrogen, Fig. 3.4.

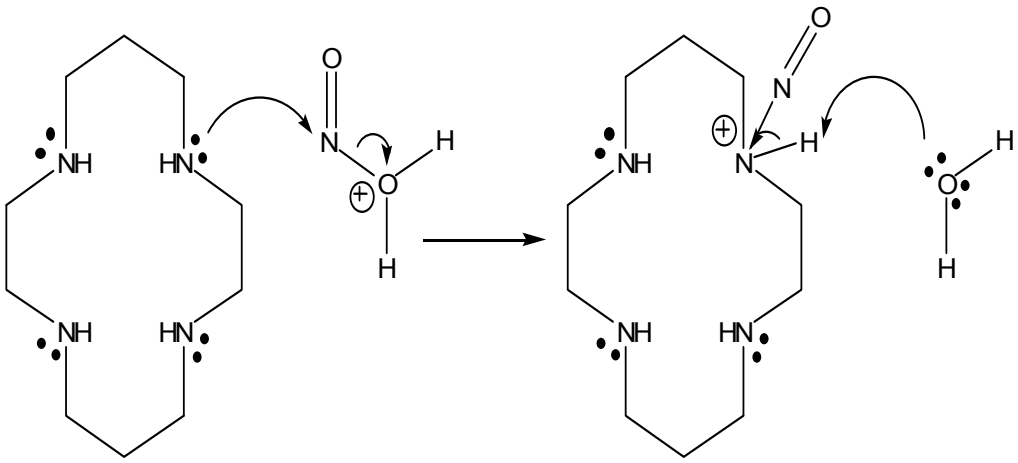


Fig. 3.4: Synthesis of N-nitrosamine using amino nitrogen nucleophilicity.

3.3.2 Synthesis at controlled pH

From Tab. 3.1 arises clearly that Cyclame exhibits a more complex and peculiar reactivity in normal nitrosation conditions than standard aliphatic or alicyclic amines.

It is extremely important, for instance, to keep the reaction medium at a controlled acidity during the reaction of Cyclame with sodium nitrite/hydrochloric acid in aqueous solution. It must be in fact avoided a

complete neutralization of aminic groups with an acid, that would drop nitrogen nucleophilicity, but the solution must be enough acid to allow the existence in appreciable quantities of the nitrosating cation $[H_2NO_2]^+$. For this reason the reaction evolution had to be constantly gauged by a pH-meter and pH value must be kept in 5.0 – 6.0 range with carefully addiction of acetic acid (Table 3.1, reaction 1). The product, 1,4,8,11-tetranitroso-1,4,8,11-tetraazacyclotetradecane (**18**) was recovered after an easy filtration and a careful washing with cold water.

Crystallographic and IR spectra of **18** synthesized with this methodology fit perfectly with literature data. In IR spectra N-H stretching bands (3000 cm^{-1}) are absent, while it is possible to observe the classical double tipped N=O stretching band (1450 and 1428 cm^{-1}), the N-N stretching (1139 cm^{-1}) and the N-N=O bending (555 cm^{-1})[1].

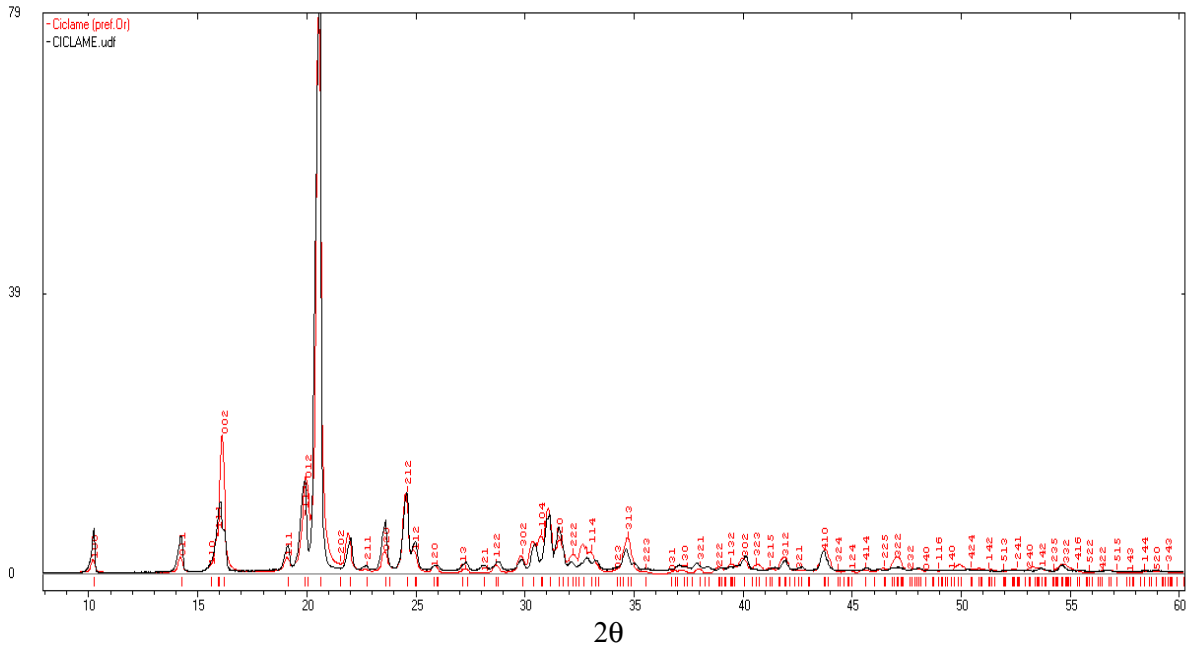


Fig. 3.5: Comparison between 18 measured diffraction spectrum (black) and calculated one (red).

Also the X-ray spectra, registered on a crystal of tiny dimensions, is super imposable to that reported in literature. In Fig. 3.5 it is possible to compare the first 60° of the X diffraction spectra of a polycrystalline sample of **18** prepared during this thesis (black spectrum) with the theoretical spectrum (red line) calculated for a single crystal of the same molecule starting from structural

[1]) These vNO bands are lover than those observed for C-nitroso compounds (1621-1539 cm-1), and this observation is usually used as evidence for the weakened N-O bond due to 1,3 zwitterionic resonance structure in fig. 3 (Rao, C.N.R; Bhaskar, K.R. "The Chemistry of the Nitro and Nitroso groups. Part I. Feuer, H., Ed Interscience, New York, 1969).

data.[22] It is clear that is the same crystal phase of the same substance. Different intensity in same peaks is due to preferential orientation effects.

A relevant discrepancy existed between the solubility data (Table 3.2): the product synthesized in this work is soluble only in boiling water and partially in dimethyl sulfoxide, while Sousa's group claims that the product is also soluble in Acetone, dichloromethane and acetonitrile.

Tab. 3.2: Comparison between solubility data found in this work and data reported in literature.

Solvent	Solubility in this work	Solubility reported
Water	at reflux	soluble
Triethylamine	Insoluble	
Chloroform	"	
THF	"	
DMF	sparingly soluble	
Acetone	Insoluble	soluble
Hexachlorobutadiene	"	
Hexafluoroxylene	"	
Benzene	"	
Nitrobenzene	decomposition at high temperature	
Acetonitrile	"	soluble
Hexametapol	sparingly soluble	
DMSO	"	
Dichloromethane	Insoluble	soluble

To clarify this discrepancy the published procedure has been repeated.

3.3.3 Synthesis with gaseous nitrous acid

The procedure reported in literature for this nitrosation is very awkward and cumbersome: extremely carcinogenic gaseous nitrous acid, produced *in situ* by oleum addition to solid sodium nitrite, is bubbled trough a water solution of Cyclame. No yield is given in the related paper, and the characterization, lacking both ^{1}H and ^{13}C NMR spectra, is incomplete.

Anyway the product obtained in this work had exactly the same IR spectrum and solubility properties of that obtained in the previous paragraph.

3.3.4 Synthesis via Amberlist 15 and wet silica

To be sure that the synthesized product was correctly identified a cross reference, obtained through the synthesis of the same product with other well tested methodologies, was necessary. This necessity was also an opportunity to find reactions with higher yields. The use of several acids, both protic[23] and of Lewis,[24] have been considered, along with the use of halogen ions.[25]

One of these alternative methodologies, applied with mono and diamines, both aliphatic and alicyclic, seemed really promising for its simplicity (Table 3.1, reaction 3). Wet silica,[26] modified by means of nitrous acid, prepared *in situ* using Amberlist 15[27] or Nafion-H®[28] as proton source, is used as heterogeneous catalyst in a reaction where all reagents can be comfortably filtered away at reaction end. Disappointingly, when this procedure was applied to Cyclame no product recovery was possible, probably because of the strong interactions between the solid and the organic phase, that remained adsorbed on the silica. Several attempts have been performed to extract the product, but only with Acetonitrile was possible to recover a compound, namely 2-nitrosoacetonitrile, probably arising from the degradation of the product during the extraction with Soxhlet apparatus.

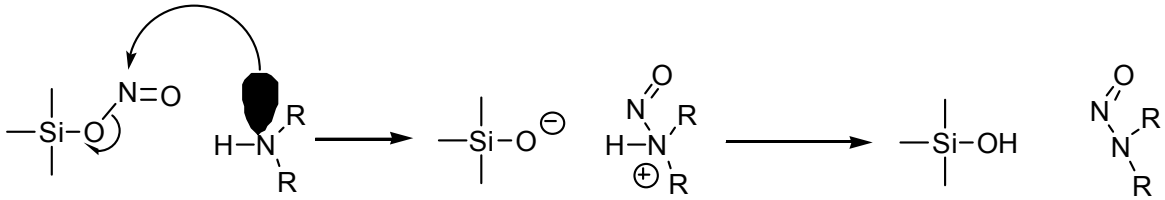
3.3.5 Synthesis with silicon compounds

Still in need of a cross reference **18** has been synthesized with an innovative nitrosation methodology that is currently generalized to other amino substrates in this laboratory.

The starting point of this new strategy is the analysis of the experimental results of the nitrosation reaction carried out with the Zolfigol's[25] strategy in the previous paragraph. This reaction should proceed in two different steps: the formation of a nitroso silyloxyde that subsequently acts (Scheme 3.5) as nitrosating agent toward amino group. The thermodynamic stability of the syliloxyde ion leaving group produced at the reaction end should be the driving force of the whole reaction. Unluckily in the reaction with the Cyclame the interactions between the overwhelming number of Si-OH functionalities and the Nitrogen atoms in the reacting molecules became so strong that a recovery of the product becomes impossible. The problem can be faced employing an 1:1 ratio between the nitrososyliloxyde and the amino group without the interference of Si-OH groups.

To test this idea trimethylnitrososilane has been synthesized for the first time in this laboratory by reaction of Chlorotrimethylsilane with Sodium nitrite in dichloromethane. The product of the subsequent reaction with cyclame, obtained in good yield (Table 3.1, reaction 4), has the same IR spectrum of the molecule produced with the first methodology (Table 3.1, reaction 1) or with the methodology reported in literature (Table 3.1, reaction 2).

At this point it was interesting to verify the nitrosation properties of nitroso trimethylsilyloxyde. This interesting molecule should contain two different electrophilic sites: the hard silicon and the soft nitrogen atoms. Since amines are soft basis, it is sensible to think that the soft nitrogen lone pair can overlap the antibonding N=O orbital, prompting the elimination of the good leaving group trimethylsilyloxyde ion, as sketched in Scheme 3.5.



Scheme 3.5: Synthesis of nitroso compounds using nitroso trimethylsilyloxyde.

Also in this case the obtained product had the same solubility, IR and NMR spectra of the others obtained in the previous syntheses.

3.3.6 Structure determination

As already stated, the synthesis of **18** through so many procedures was necessary because of its insolubility in all more common and exotic solvents, that makes its complete characterization difficult..

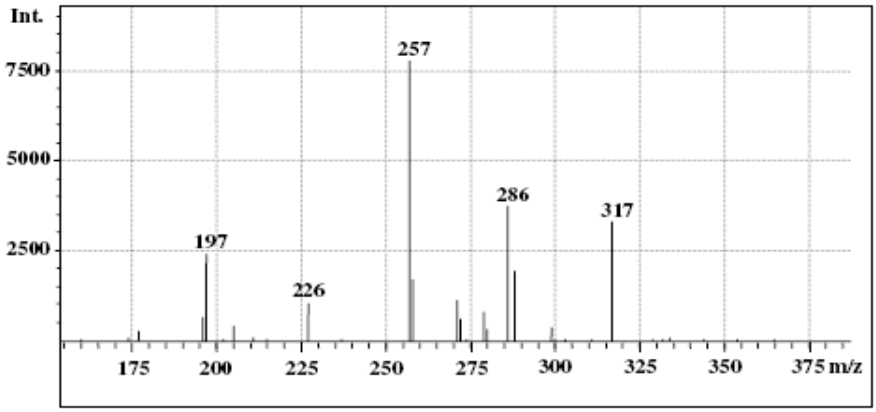


Fig. 3.6: MS of 18 reported in literature performed by APCI-SIM.

It must also be recorded that published mass spectroscopy analysis results does not completely fit with the mass spectroscopy results obtained during this thesis. This discrepancy can be tracked down to the different instruments used to perform the analysis

While Sousa's group utilized a LCMS (Liquid Chromatography - Mass Spectrometry) with a mass spectrometer APCI-SIM (Atmospheric Pressure Chemical Ionization-Single Ionization Molecule) interfaced with a liquid chromatograph (isocratic elution performed with Acetonitrile/water = 80/20 in a Shimadzu column C18 1250 x 20 mm, 4.6 lm with a Shimadzu 2010 equipment), in this work the sample was analyzed with a MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time Of Flight) instrument.

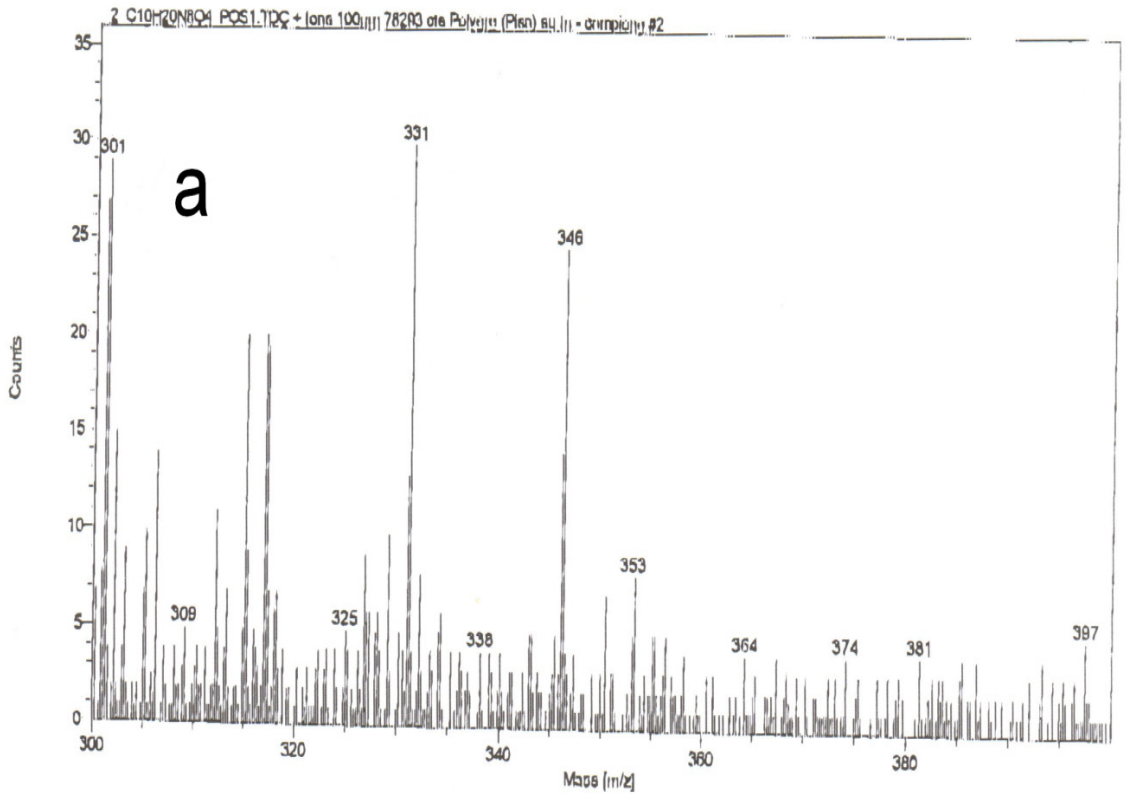


Fig. 3.7 a: MS of 18 obtained in this work with a MALDI-TOF. Mass range300-400.

In reported data (Fig. 3.6) a higher molecular peak M^++1 is detected (m/z = 317), that in our data (Fig. 3.7 and Table 3.3) is present as a smaller peaks flanked by a twin M^+-1 peak (m/z = 315). Along with these two peaks an heavier peak (m/z = 602) corresponding to a more complex dimer structure has been detected. Also peaks corresponding to M^+-NO (m/z=288) and M^+-2(NO) (m/z=257) are identified.

From this data and from the presence of others peaks heavier then the molecular peak of **18** it has been possible to identify a more complex dimer, whose structure and fragmentation path is shown in Table 3.3 and Scheme 3.6.

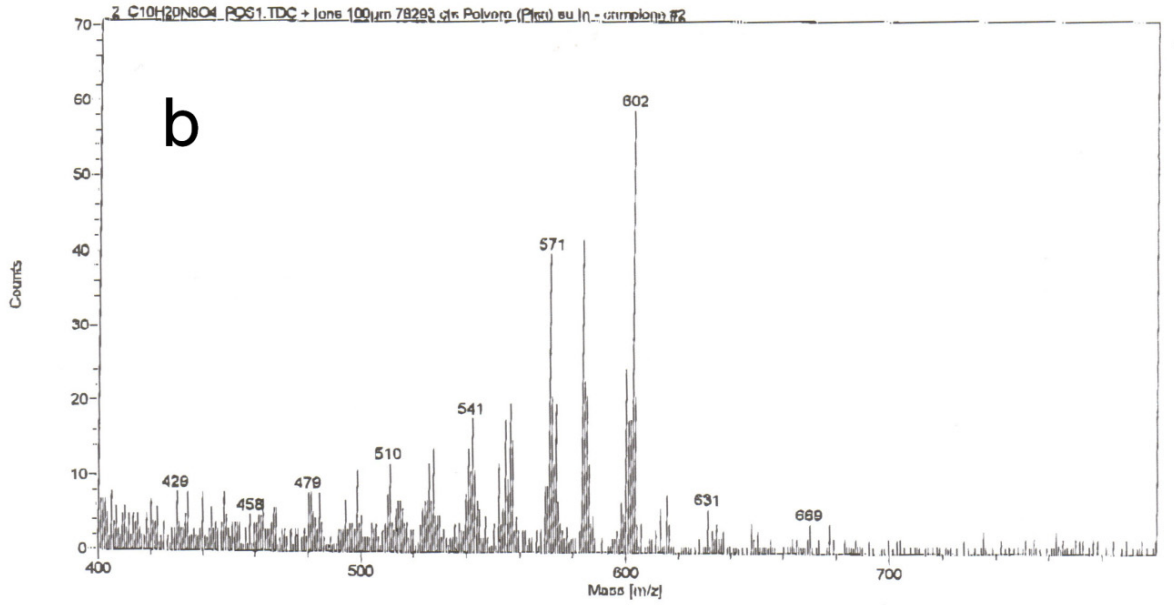
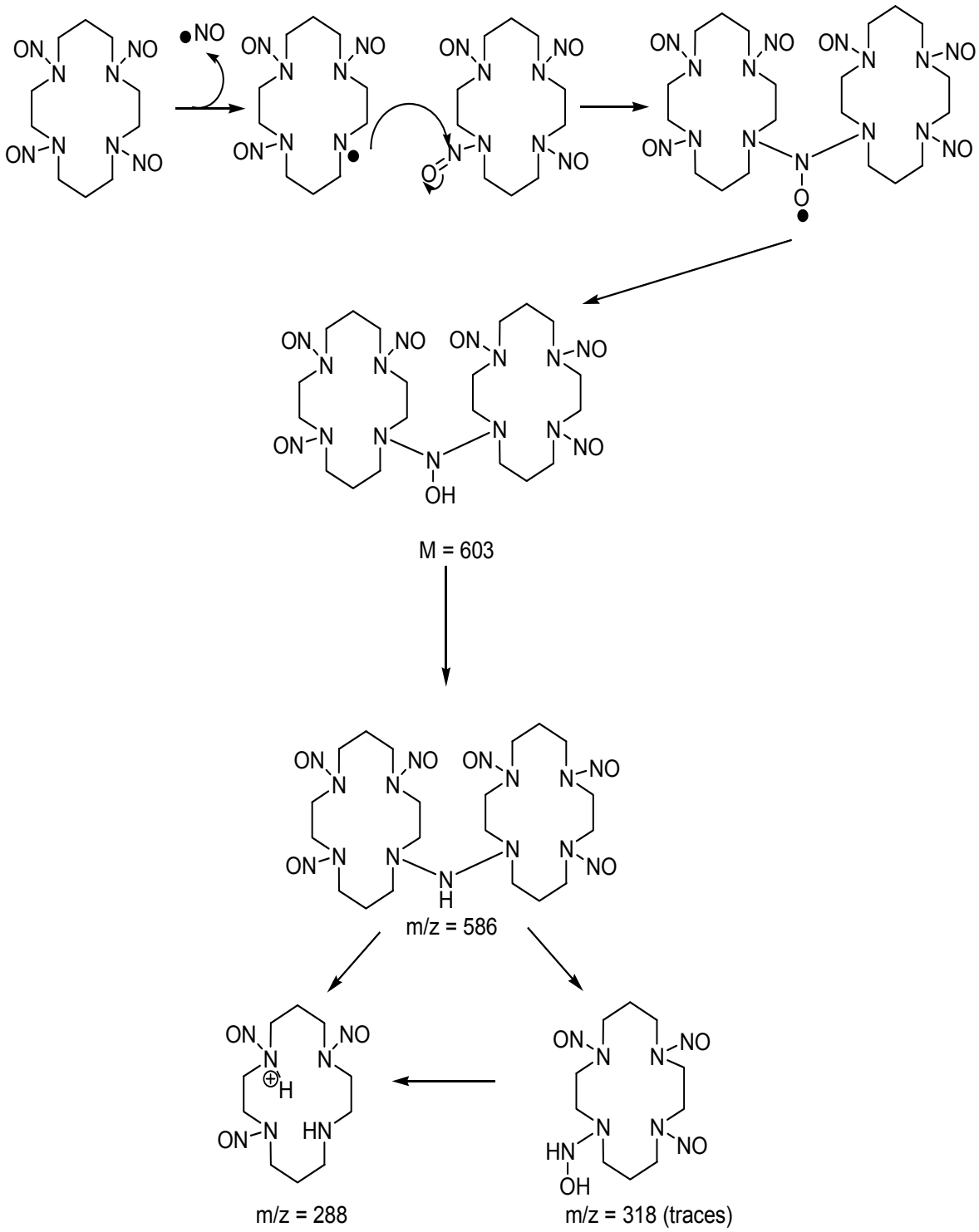


Fig. 3.8 b: MS of 18 obtained in this work with a MALDI-TOF. Mass range 400-800.

Even if some group deems[29] that this dimer is produced during the synthesis, hypothesis that could explicate the different solubility of our product compared with the solubility of the product reported in literature, this work, considering the peaks relative highs is confident that some dimers are generated during the Mass Spectrometric analysis: in MALDI-TOF analysis condition dimers arise from a Chemical Impact (CI) process, between **18** and one of its stable radical produced during the ablation or ionization phase, as shown in Scheme 3.6.

Tab. 3.3: MS fragmentation obtained in this work by MALDI-TOF

m/z	Signal	Fragment	m/z	Signal	Fragment
602	60	$[M\text{-}O+H]^+_2$	315	26	$[M\text{-}H]^+$
571	42	$[M\text{-}NO]^+_2$	301	30	$[M\text{-}O+H]^+$
346	28	$[M+2O\text{-}2H]^+$	288	260	$[M\text{-}NO]^+$
331	31	$[M+O\text{-}H]^+$	257	310	$[M\text{-}(NO)_2+H]^+$
317	26	$[M+H]^+$	196	90	$[C_9H_{18}N_5]^+$



Scheme 3.6: Fragmentation path of 18 by MALDI-TOF MS.

In this work is has also been possible to perform ^{1}H NMR analysis of the sample in DMSO d_6 at 80°C. In this spectrum (Fig. 3.8) it is possible to recognize four different isomers, that can be associated to the four different relative position of the N-NO groups as shown in Fig. 3.9.

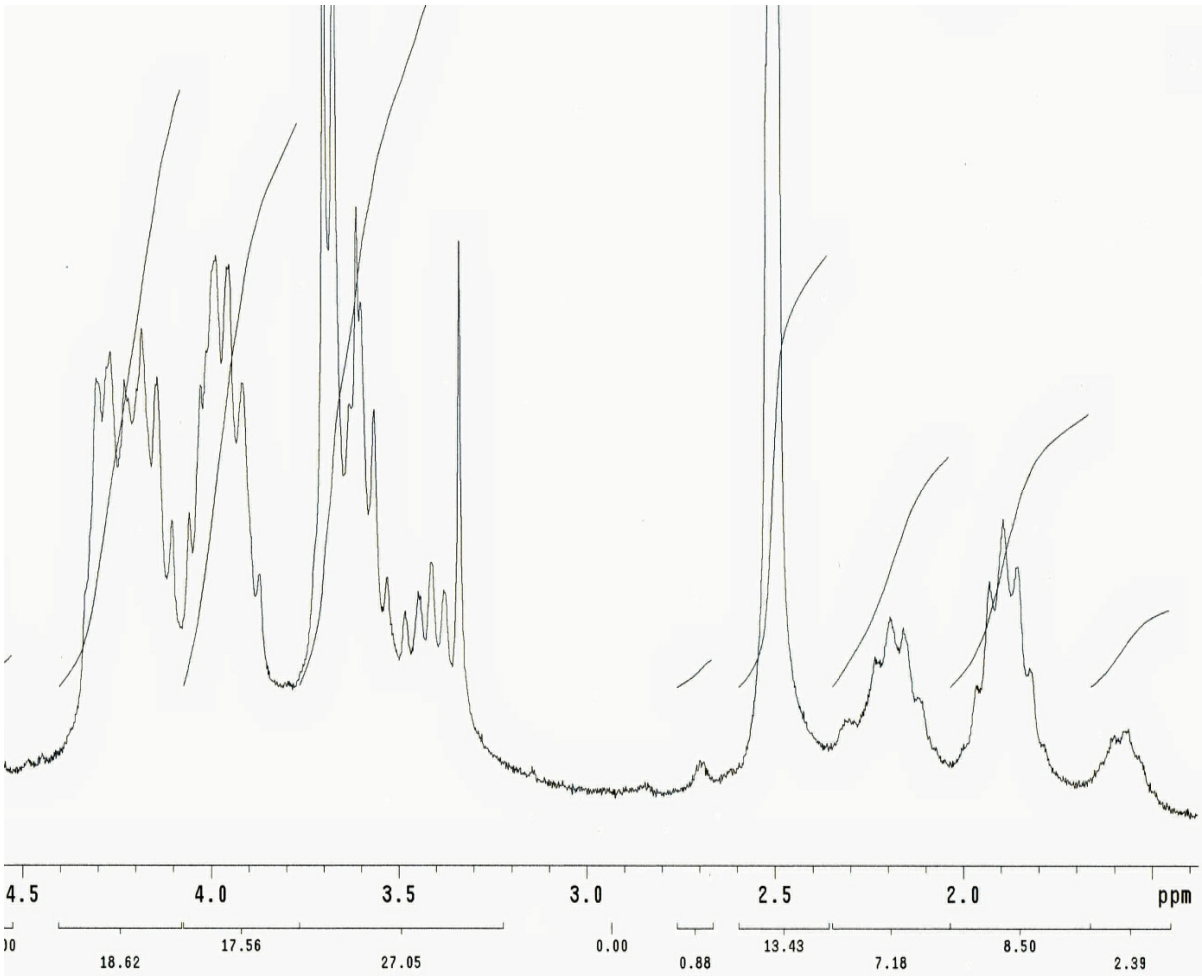


Fig. 3.9: ^{1}H NMR spectrum of 18, acquired in DMSO d_6 at 80°C.

That the product **18** had several isomers and not mere conformational structures was expected as it can be crystallized in boiling water and melts without decomposition, behaviors that attest an extremely high diazenic character and as consequence the existence of stable isomers.

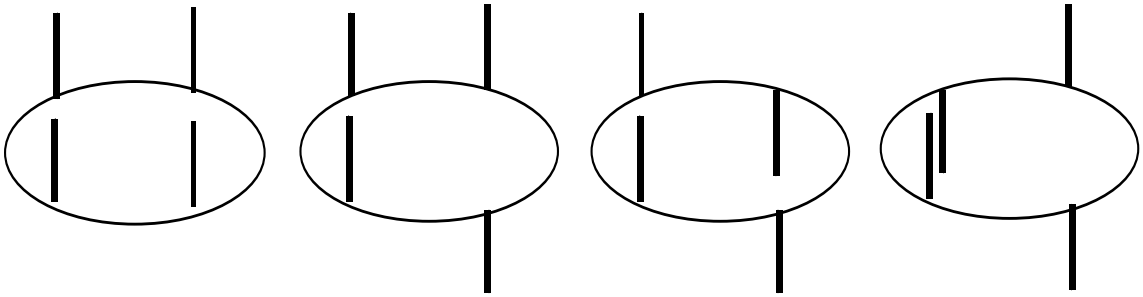


Fig. 3.10: Four possible stereoisomers of 18. Ellipse represent the ring plane and the studs symbolize NO groups.

The third structure of Fig. 3.9 is sketched and simulated as example in Fig. 3.10, with two vicinal nitrosoaminic groups over the ideal molecular plane and the other two under this molecular plane.

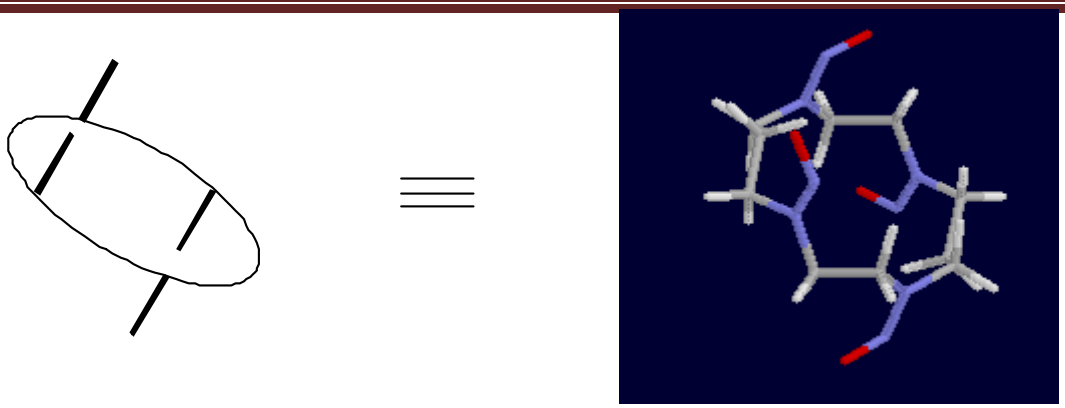


Fig. 3.11: Structure simulation of the third isomer in Fig.9.
Color attribution: Blue nitrogen, Redo, Gray carbon, White hydrogen, Pink lone pairs

^{1}H NMR spectrum for this isomer (Fig. 3.7), simulated by SPINIS software (version 1.94-Impact/m18122/cc558), shows clearly the multiplets for aliphatic methylenes (–CH_2-$\underline{CH_2}$-CH_2-) and a more complex situation for methylene groups in α position to N-nitroso groups. This simulation is in good accordance with the registered spectrum (Fig. 3.8) where the four signal groups correspond to the four identified isomers.

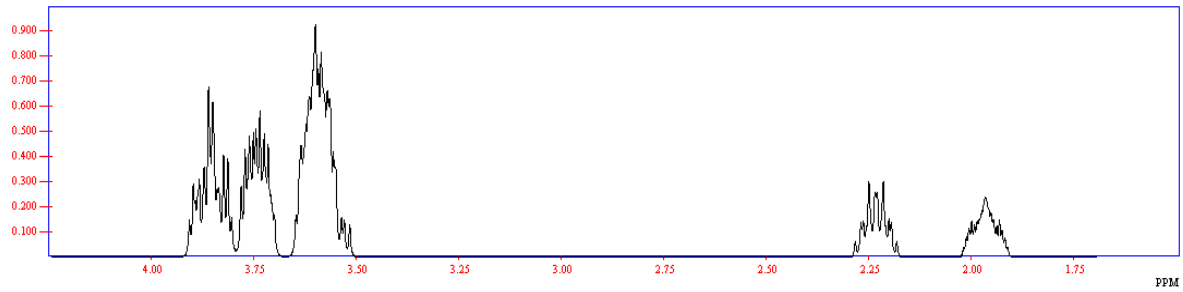


Fig. 3.12: Simulation (SPINUS Software) of ^{1}H NMR spectrum of the isomer of 18 reported in Fig. 3.11.

It was not possible to perform analogues ^{13}C NMR analyses because the product undergoes slow degradation processes and is scarcely soluble even in dimethyl sulfoxide, this means that it is neither possible to acquire data for a long period of time nor to perform analysis on very concentrate solutions.

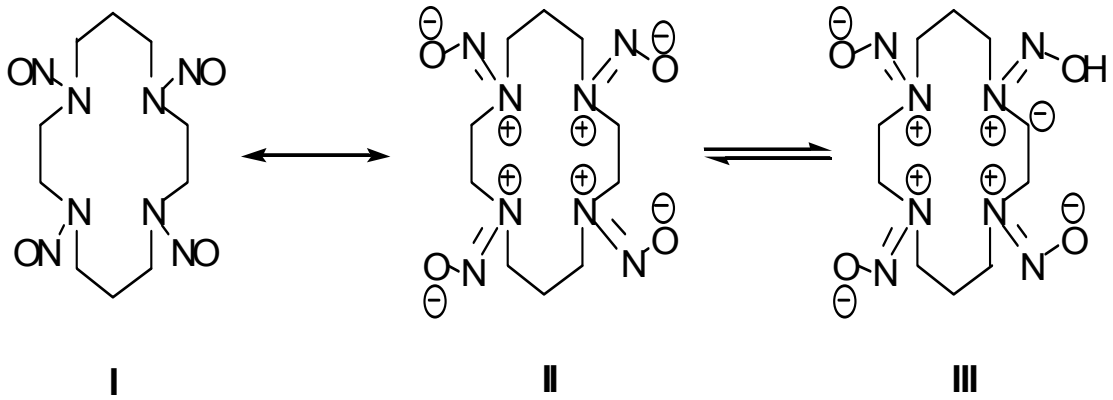
3.3.7 Cycle Functionalization

Once that the structure of compound **18** had been proved, the following step was to exploit the well known acidity of proton in α position to N-NO group for the insertion of a suitable pendant.

The first used base was *n*-butyl lithium. In a equimolecular reaction between **18**, dissolved in anhydrous THF at -20°C, and *n*-BuLi a red-brick solution was obtained. [30]

Unfortunately the addition of 4-nitrobenzyl bromide did not bring to the formation of the foreseen products but, thought a reaction between the strong base and **18,** produced 1-nitrosobutane.

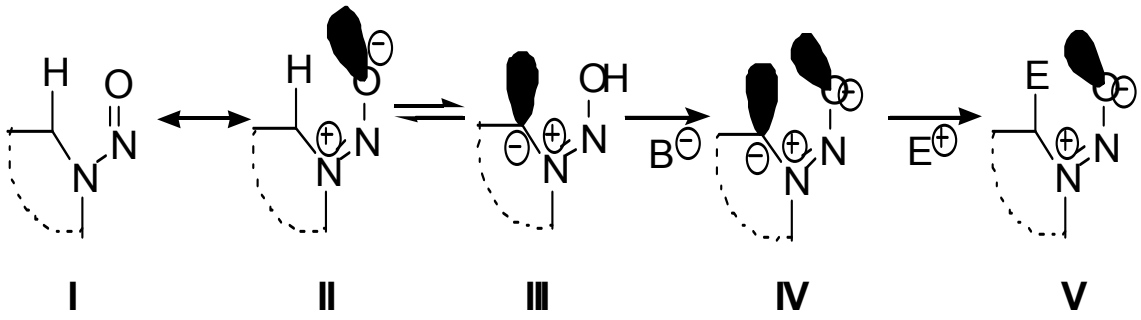
Transnitrosation is a threat that lurks constantly in working with nitroso compounds, [31] that act as nitrosating agents[32] at moderate temperature[33] or with nucleophiles in acid solutions. For this reason we decided to try the use of a milder base as lithium di*iso*propylamide (LDA), the same base used by Seebach in his seminal experiments [34] with aliphatic and alicyclic mono and dinitrosamines. Unfortunately also this attempt did not bring the expected results, in open contrast with what reported in literature for similar compounds. The cause of this unexpected reactivity can be tracked down to the lack of solubility of this compound. Being trapped in a crystal it is not free to bend and rotate reaching the spatial conformation necessary to N-nitrosoaminic group to extract a proton placed on its α Carbon.



Scheme 3.7: Resonance structures of 18.

Acidity of these hydrogens in N-nitrosamines is correlated not only to resonance I ↔ II in Scheme 3.8, but more important is the zwitterionic equilibrium between structure II and III through a cyclic five-ring intermediate, where the proton must be in a syn-periplanar position respect to the negatively charged oxygen atom. This equilibrium is normally shifted on the left side. *Ab initio* studies on the charge densities of the NNO moieties of Nauman and co-workers,[35] based on data obtained via photoelectron and electronic absorption spectra, are compatible with an electronegative oxygen atom, an electropositive nitroso N-atom and a slightly electronegative amine N-atom. They also proposed an involvement and redistribution of molecules' σ electrons. From their calculation the HOMO of R_2NNO is a non-bonding orbital (lone pair) localized mostly on the oxygen atom, with the second HOMO being the nitroso

π orbital. Also UV-visible data,[36] that show absorption in the 340-385 nm (ε~100) and 235 nm (ε~7000) ranges that can be assigned to n → π* and to a π → π* transition respectively, support the assumption of Nauman's group.



Scheme 3.8: Required stereoelectronic conditions for electrophilic functionalization.

The existence of this zwitterionic species has been further demonstrated[37] with a suitable electrophile, that saturated the negative charge on the oxygen atom annihilating the acidity of the hydrogens on its α carbons.

The conclusion is that the formation of the carbanion can occur only if the negative charge on the oxygen atom is in syn-periplanar position respect to the hydrogen that has to be removed. In this contest bases have not the function of directly remove the proton, but to shift the equilibrium within reaction III → IV on the right side (Scheme 3.8).

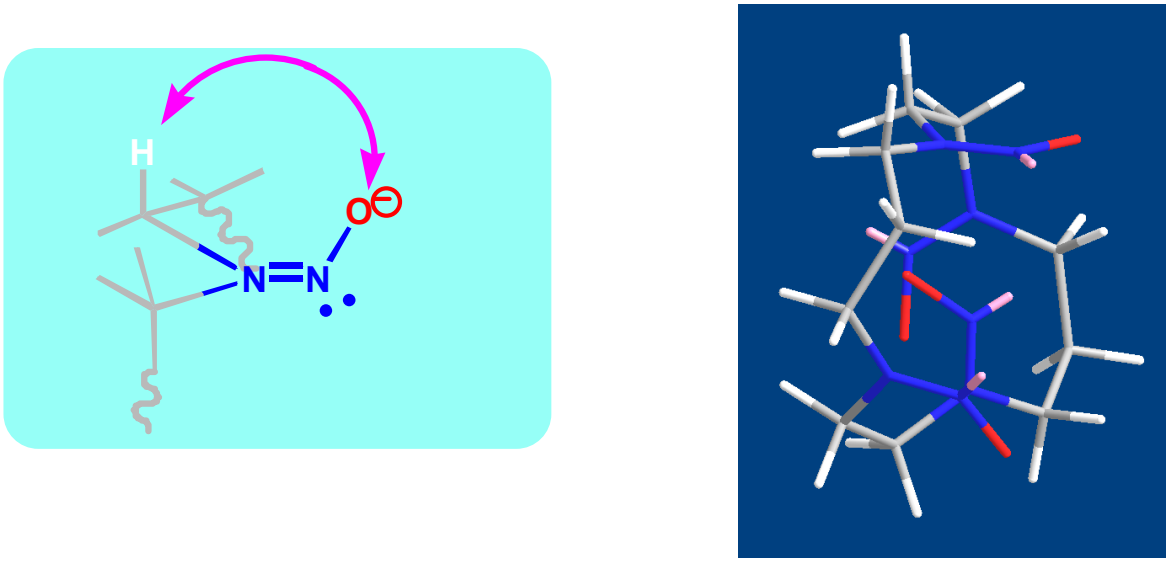


Fig. 3.13: Structure simulation of N-nitroso oxygen atom in syn-periplanar position respect to an hydrogen atom in α position to the N-NO group. Color attribution: Blue nitrogen, Red oxygen, Gray carbon, White hydrogen, Pink lone pairs.

The key step is hardly influenced by the base nature or strength, but principally by relative spatial disposition of the involved groups[38]. Conformational analysis

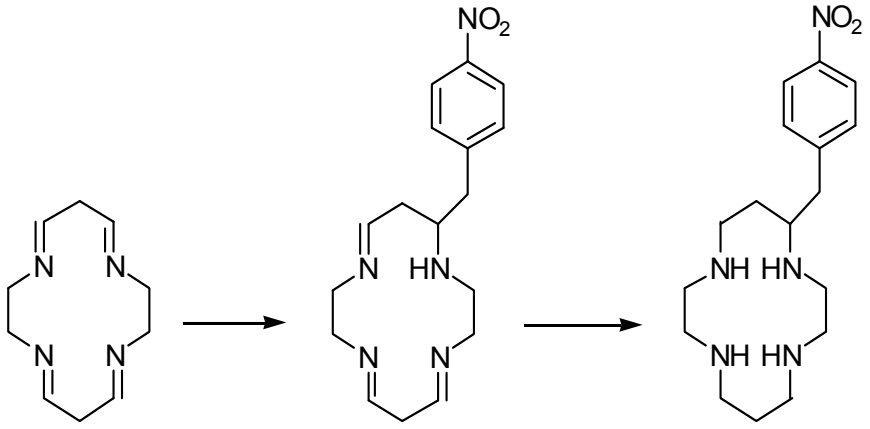
of zwitterion II (Fig. 3.12) shows that the cyclic structure of **18** (Scheme 3.8) hinders a suitable stereoelectronic arrangement of the involved atoms: the conversion of I in V is stereoelectronically forbidden. The reaction between 4-nitro benzyl bromide and the carbanion of **18** does not occur because the latter cannot be synthesized.

It is a remarkable coincidence that the peptidic synthesis of **1** attempted by Moi and Meares[39] did not achieve the target product for analogous intermediates solubility problems.

3.4 Umpolung of the Synthetic strategy

Since the former strategy was not practicable a sensible alternative was the umpolung of the sinthons identified in the retrosynthetic analysis.

It became necessary to identify a suitable Cyclame derivative with a formal or effective positive charge on the α carbon atom to the N-nitroso group that could react with a synthetic equivalent of the anion of 4-nitrotoluene. A good candidate was for instance the 1,5,8,12-tetraazacyclotetradeca-1,4,8,11-tetraene (**21,** Scheme 3.9), along with all the other tetraenes that could be written considering the four double iminic bonds placed on one side or on the opposite side of each nitrogen atom.



21 **22** **23**

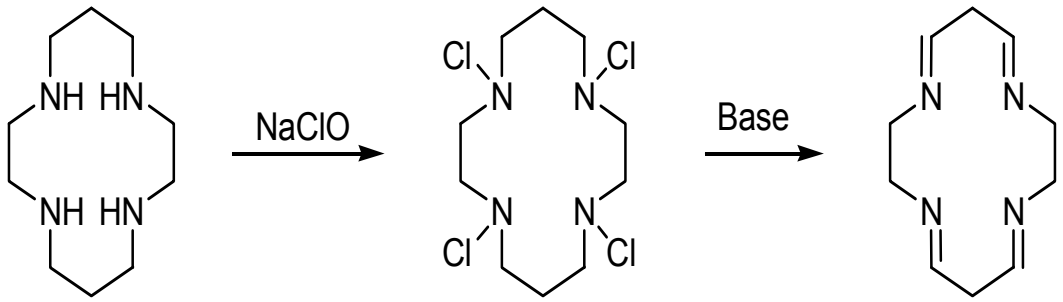
Scheme 3.9: Cycle functionalization via imine formation.

The planned synthetic route went trough a reaction of cyclopolyimine **21** with a suitable nucleophile to give compound **22** (or one of its equivalent with the pendant bounded to any other carbon in α position to one nitrogen atom).

This product after reduction ($NaBH_4$, $H_2/Pd(C)$, $LiAlH_4$) should give the functionalized Cyclame **23**.

3.4.1 Chlorine as protective/activating group

There are not too much synthetic functional group interconversions to transform a secondary amine into an imine. An easy strategy goes trough the halogenation of the secondary amine followed by a formal hydrohalic acid elimination with a strong base.



24 **21**

Scheme 3.10: Synthesis of 1,4,8,11-tetraazacyclo-1,4,8,11-tetrachlorotetradecane (24).

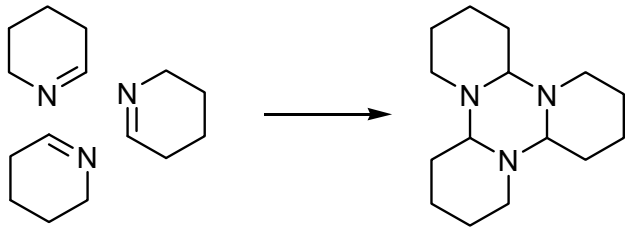
In this thesis the Cyclame was chlorinated in an aqueous solution with an excess of sodium hypochlorite,[40] Scheme 3.10. The powder-pink precipitate, dried under vacuum condition at room temperature, gave with 90% yield 1,4,8,11-tetraazacyclo-1,4,8,11-tetrachlorotetradecane (**24**).

Errore. L'origine riferimento non è stata trovata.

Run	Base	Solvent	Catalyst	Time (h)
1	KO_2	THF	18-Crown-6	4
2	KO_2	THF	18-Crown-6	24
3	K_2CO_3	PhH	18-Crown-6	1
4	K_2CO_3	PhH	18-Crown-6	4
5	$MeCO_2K$	PhH	18-Crown-6	4
6	Et_3N	PhH	-	4
7	Al_2O_3	CH_2Cl_2	-	20
8	KOH/Al_2O_3	CH_2Cl_2	-	4

3.4.2 Synthesis of 1,5,8,12-tetraazacyclotetradeca-1,4,8,11-tetraene.

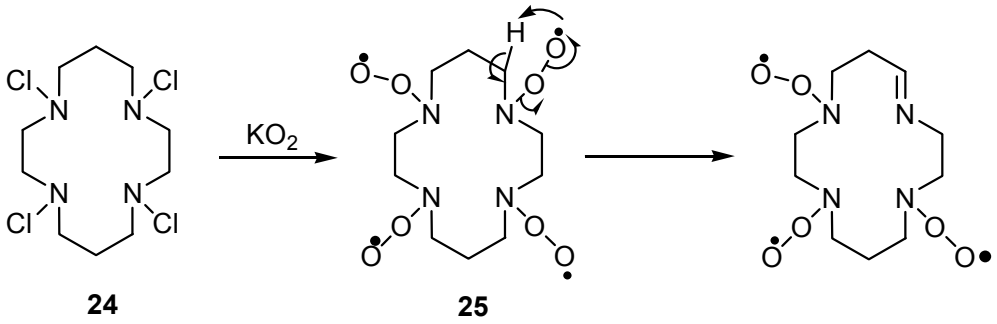
Several attempts to get the compound **21** in different reaction condition (Table 3.4) gave only degradation products. Polyimine **21** (along with its E and Z isomers) is probably formed in reaction 6-8 of Table 3.4, but they are not stable in the used reaction conditions. Imines have in fact a strong tendency to give polymerization reactions. It is for instance known[41] an oligomerization reaction of 2,3,4,5-tetrahydropyridine in similar conditions, Scheme 3.11.



Scheme 3.11: Imine oligomerization.

3.4.3 Potassium Superoxide as protective/activating group

To overcome this problem potassium salts, that can perform the functional group interconversion from chloramines to imines under mild conditions,[42] were used (Table 3.4, entries 1-5). This reaction goes trough the substitution of the Chlorine atom with a basic nucleophile and only in a second step, with an intramolecular mechanism (akin to the pyrolysis of aminic sulfonic esters, acetate or N-oxides) the double bond is produced. For example in Scheme 3.12 is represented the foreseen reaction progress with potassium superoxide.[43]



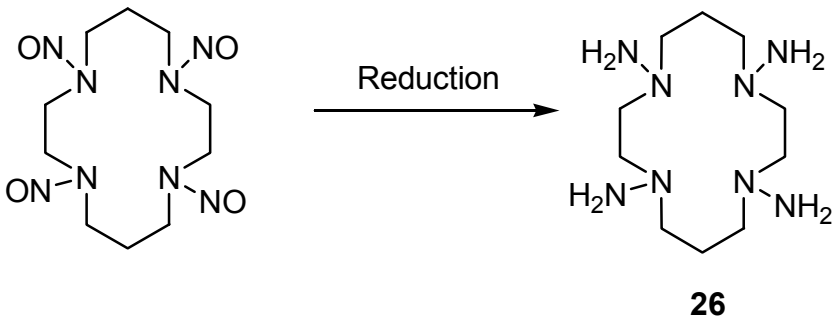
Scheme 3.12: First steps in synthesis of 21 with Potassium Superoxide.

Since also in all these reactions there is an interaction between an N-bonded functional group and an hydrogen on the α carbon to the nitrogen atom, with the formation of an intermolecular cyclic intermediate, also in this case all involved atoms must be placed in one exact spatial position and fulfill stringent stereoelectronic prerequisites. Also in this case the study of molecular models

for the intermediate **25**, as happened with the nitroso group, shows that the required conditions are not accomplished. For this reason, at best of my knowledge, it is not possible to use 1,4,8,11-tetrasubstituted-1,4,8,11-tetraazacyclotetradecane as intermediate for the synthesis of functionalized polyazacycle.

3.5 Cyclic Polyhydrazine CA

As already said at the beginning of this chapter the nitroso group was chosen keeping in mind the possibility of its use as intermediate for the synthesis of cyclic polyhydrazine CA.



26

Scheme 3.13: Synthesis of 1,4,8,11-tetraamino-1,4,8,11-tetraazacyclotetradecane.

The FGI described in Scheme 3.13 can be performed in several ways,[44] but the critical step is not the interconversion itself but the product isolation. Hydrazines have in fact a very strong affinity for water[45] in acid,[46] basic[47] and neutral solutions,[48] and the reaction should be carried on neither in aqueous medium nor with production of water.

For this reason the reduction has been performed in chloroform or benzene at reflux, with cyclohexene in hydrogen transfer conditions and Pd/(C) as catalyst. In this condition the reaction was hardly reproducible and gave different quantities of 1,4,8,11-tetraamino-1,4,8,11-tetraazacyclotetradecane (**26**) along with significant amounts of Cyclame as byproduct. The probable catalytic intermediate palladium hydride is able, due to its softness, to give demolition reaction of the soft N-N bond.

To bypass this problem the reaction has been performed in milder condition (room temperature) using a titanium(III) chloride solution as catalyst. In this condition the reaction gave the expected product, but its extraction and recovery was extremely complicate even using ethyl acetate.

In conclusion, even if this experimental cycle did not open a new synthetic path to the production of PT-MBCA, a new chelating agent candidate, the 1,4,8,11-tetraamino-1,4,8,11-tetraazacyclotetradecane, has been synthesized and new synthetic paths to 1,4,8,11-tetranitroso-1,4,8,11-tetraazacyclotetradecane have been set-up.

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Chapter 4

Synthesis of Dendrimeric Bi-functionalized Chelating Agents

As stated in cap. 1 one of the open problem of Macrocyclic Pendant-Type BCA is their limited dose load. Each complex can bear only one Ac^{+++} ion, that only in 70% case lead to cancer cell death. Both employed solutions (increase of the number of BCA for antibody and of the Ig-BCA given to the patient) have big drawbacks and are unsatisfactory. The number of tentacles in Dendrimeric Chelating Agents depends on the dendrimeric generation and can be easily set to bind more Ac ions.

There was no article or hypothesis on the complexing properties of dendrimers toward An when this thesis began, but last March Mamadu Diallo made an oral report at the 233rd ACS National Meeting in Chicago[1] on the binding properties of poly(amidoamine) and poly(propyleneimine) dendrimers toward U(IV). On one side this take us away the primogeniture of the idea, on the other one support the validity of studying this field.

4.1 Dendrimers: Highly-branched polymers

4.1.1 Structure

Highly-branched polymeric structures can be seen as the chemical equivalent of fractal geometry, where a very simple basic structure or operation is reiterated in a fixed algorithm to obtain complexes, and often esthetically beautiful, structures. Thank to this recursive formulas, that became popular with the works of Benoît Mandelbrot in the 70th of last century, it has been possible to give a mathematical formalization of several natural phenomena, from the growing process of snow crystals to the shape of threes.[2] Some years later Donald Tomalia and co-workers translated the same concepts to chemistry and to synthesized[3] and patented[4] polymers with star or tree shapes, built like onions, where the functional groups on each layer constituted the substrate for the synthesis of a subsequent outer one.

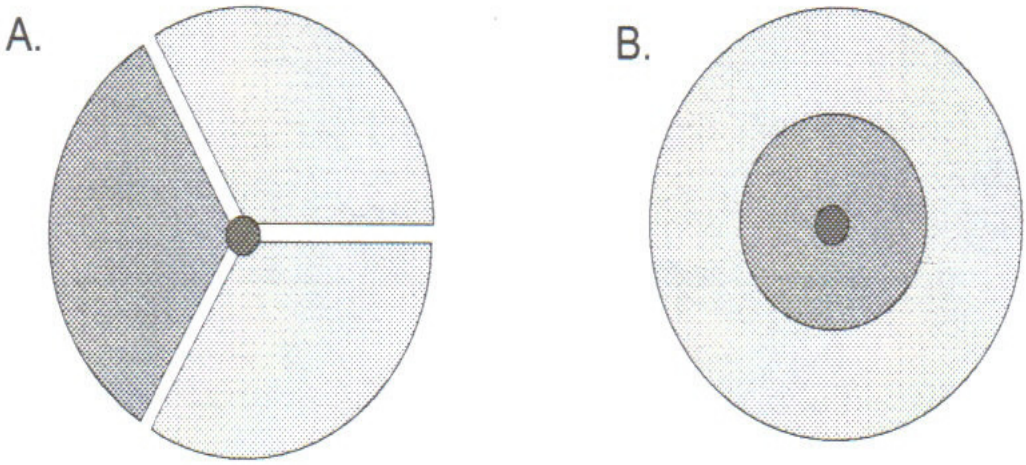
Because their shape, Tomalia named this molecules dendrimers, after δηνδρον meaning a tree in Greek. At the same time, Newkone's group independently reported synthesis of similar macromolecules.[5] They called them arborols from the Latin word 'arbor' also meaning a tree. The term cascade molecule is also used, but 'dendrimer' is the best established one.

As often happens with brand new class of compounds, new symbolism had to be introduced to represent them. As will be seen in more details in the paragraph expressed dedicated to synthesis, two main synthetic ways are used: convergent[6] and divergent.[7]

Alike with linear polymers, dendrimers must not arise from a single kind of monomers, copolymers are often more suitable for specific application.[8] Dendrimeric copolymers are a specific group of dendrimersdivided in two classes:

- **Segment-block dendrimers**[9] are built with dendritic segments of different constitution. They are obtained by attaching different wedges to one polyfunctional core molecule.

- **Layer-block dendrimers**[10] consist of concentric spheres of differing chemistry. They are the result of placing concentric layers around the central core.



Scheme 4.1: Segment (A) and layer (B) block dendrimers

4.1.2 Properties

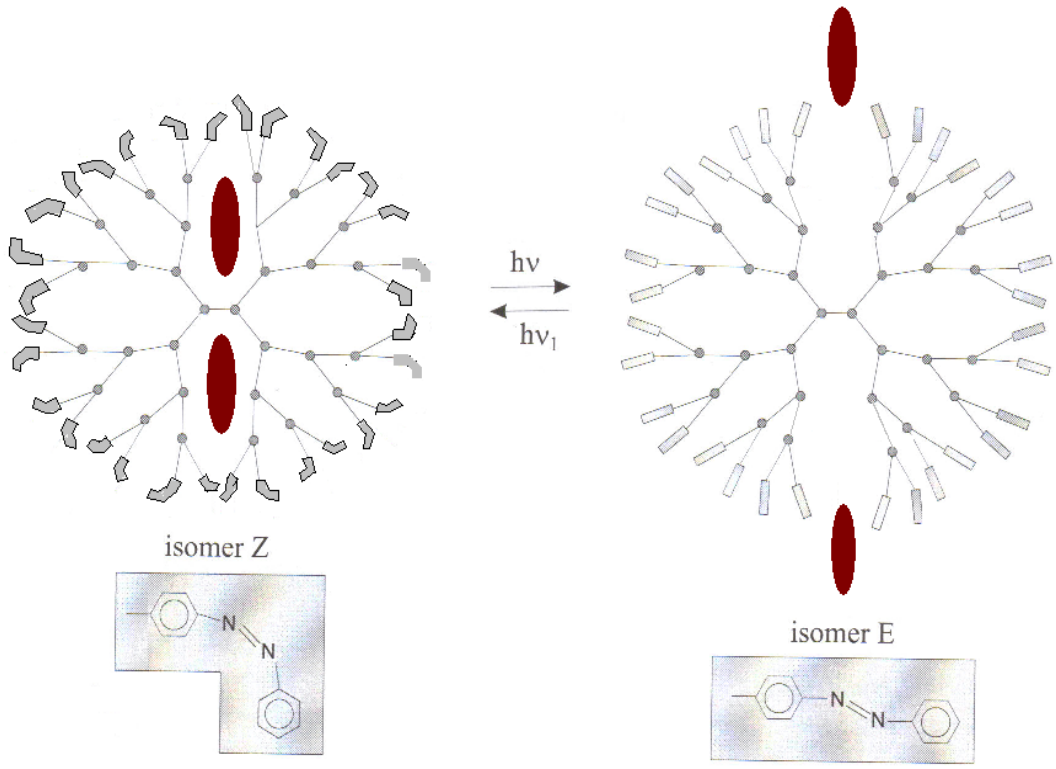
It could be expected, and actually happens, that the properties of highly-branched macromolecules can be very different from linear polymers, which only occasionally contain some smaller or longer branches. The peculiar characteristics of a dendrimer respect to a linear polymer are:

- **Molecular uniformity**: Dendrimers are monodisperse macromolecules, unlike linear polymers. The classical polymerization process which results in linear polymers is usually random in mature and produces molecules of different sizes, whereas size and molecular mass of dendrimers can be specifically controlled during synthesis

- **Multifunctional surface**: Because of their compact tree-like molecular structure, they provide a rich source of surface functionality which makes them useful building blocks and carrier molecules at the nanometer level. Lower generation dendrimers which are large enough to be spherical but do not form a highly packed surface, have enormous surface areas in relation to volume (up to 1000 m^2/g)[11]

- **Spherical shape**: In solution, linear chains exist as flexible coils; in contrast, dendrimers form a tightly packed ball. This has a great impact on their rheological properties. Dendrimer solutions have significantly lower viscosity than linear polymers[12]

- **Internal cavities:** Presence of internal cavities give them the possibility to encapsulate guest molecule in the macromolecule interior. Meijer and co-workers[13] trapped small molecules inside the 'dendritic box', then a shell was formed on the surface of the dendrimer by reacting the terminal functionalities and guest molecules were stably encapsulated inside the box. Cleavage of the outer shell could liberate the guest molecules[14]. Particular interesting is the possibility of a photochemical opening and closing this boxes.[15]



Scheme 4.2: "dendritic box" encapsulating guest molecules & photochemical open-close mechanism

- **Light harvest:**[16] Probably the most recent peculiar characteristic of dendrimers is their ability to act as extremely efficient light-harvesting antennae,[17] extremely useful in laser-dye experiments.[18]

4.1.3 Applications

There are nowadays more than fifty families of dendrimers, each with unique properties, since the surface, interior and core can be tailored to different sorts of applications. The increase of their importance can be easily judged by the number of released patents dealing with this species, that soared from two in the period between 1981-1985 to more than one thousand in last lustrum.[19]

This application can be sorted in three main areas:

- **Commodity** application supplementing and hopefully "pulling" more exotic applications in nanotechnology and biomedicine, as have been the use of dendrimers as additives in commodity plastics and in ink/toner industry

- **Pharmaceutical and biotechnological**, as delivers of drugs and other therapeutic agents that can be locked in dendrimers interiors as well as attached to the surface groups

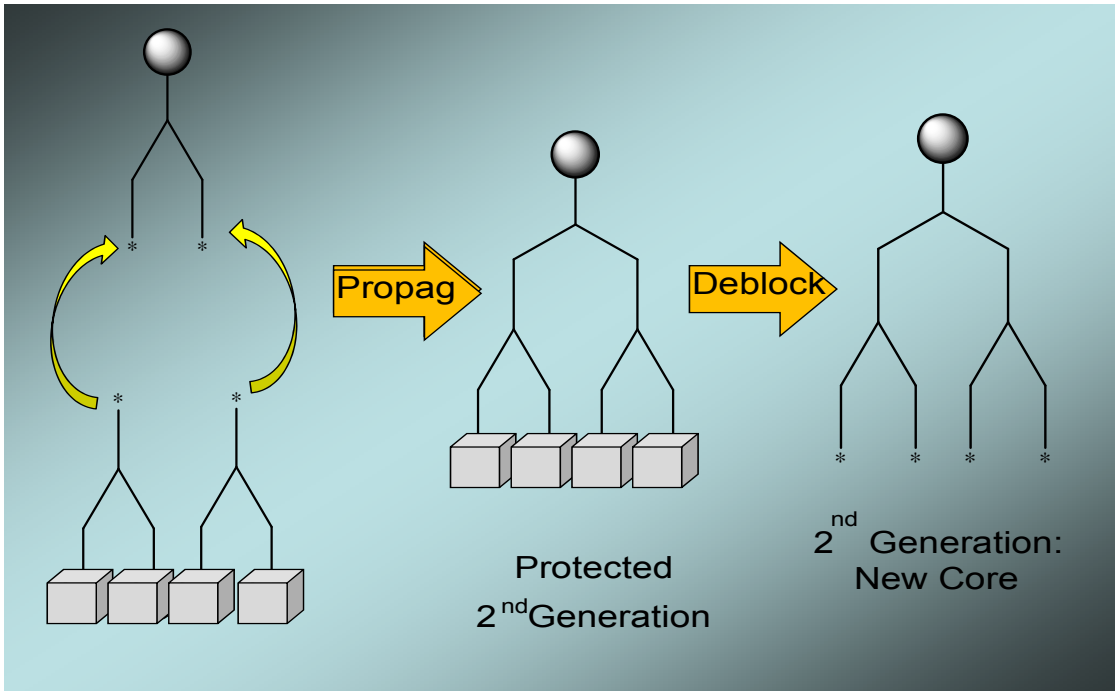
- **Applied research** arising from basic "proof of concept" or mere interesting synthetic chemistry. It is a composed group whose more present application is in industrial processes as nanoscale catalyst,[20] where thanks to dendrimers it is possible to combine the advantages of homogeneous and heterogeneous catalysis.[21]

Of this plethora of uses,[22] the more akin and relevant for my study is the use of copper and rhenium complexes for RIT[23] and of complexes of gadolinium, an heavy lanthanide whose dimension are not too different from An, as contrast agent for Magnetic Resonance Imaging (MRI), the diagnostic equivalent of NMR, that detect the NMR signal of aqueous protons, and as consequence water concentration, in tissues.[24] Addiction of contrast agents (paramagnetic metal cations) improves the sensitivity and specificity of the methodology. Gadolinium salt of diethylenetriaminepentaacetic acid (DTPA) is used clinically but it diffuses into the extravenous area due to its low molecular mass.[25] Dendrimers due to their properties are highly suited for use as image contrast media. Several groups have prepared dendrimers containing gadolinium ions chelated on the surface.[26, 27]

4.1.4 Synthesis

Dendrimers are generally prepared using either a divergent method of a convergent one:[28]

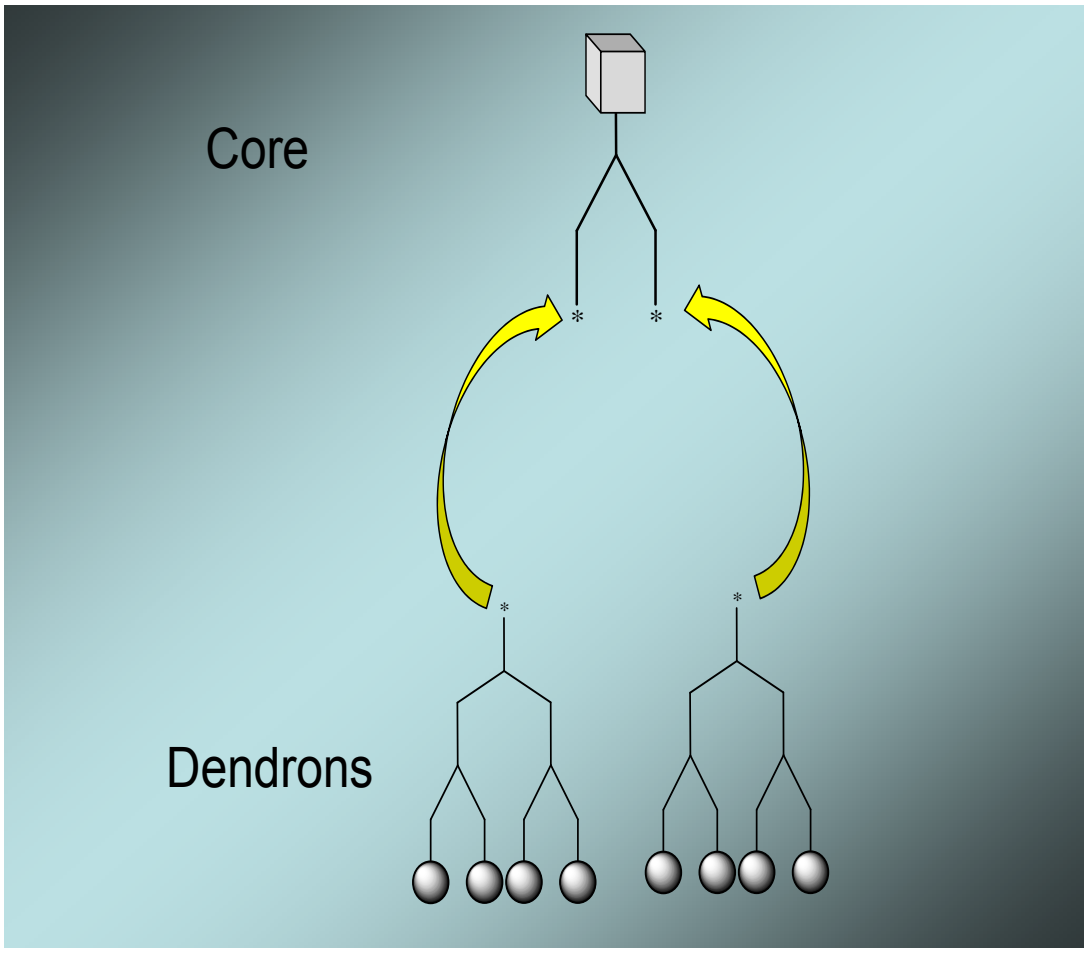
- **Divergent methods**,[29] where dendrimers grow outwards from a multifunctional core molecule.



Scheme 4.3: Dendrimer divergent synthesis

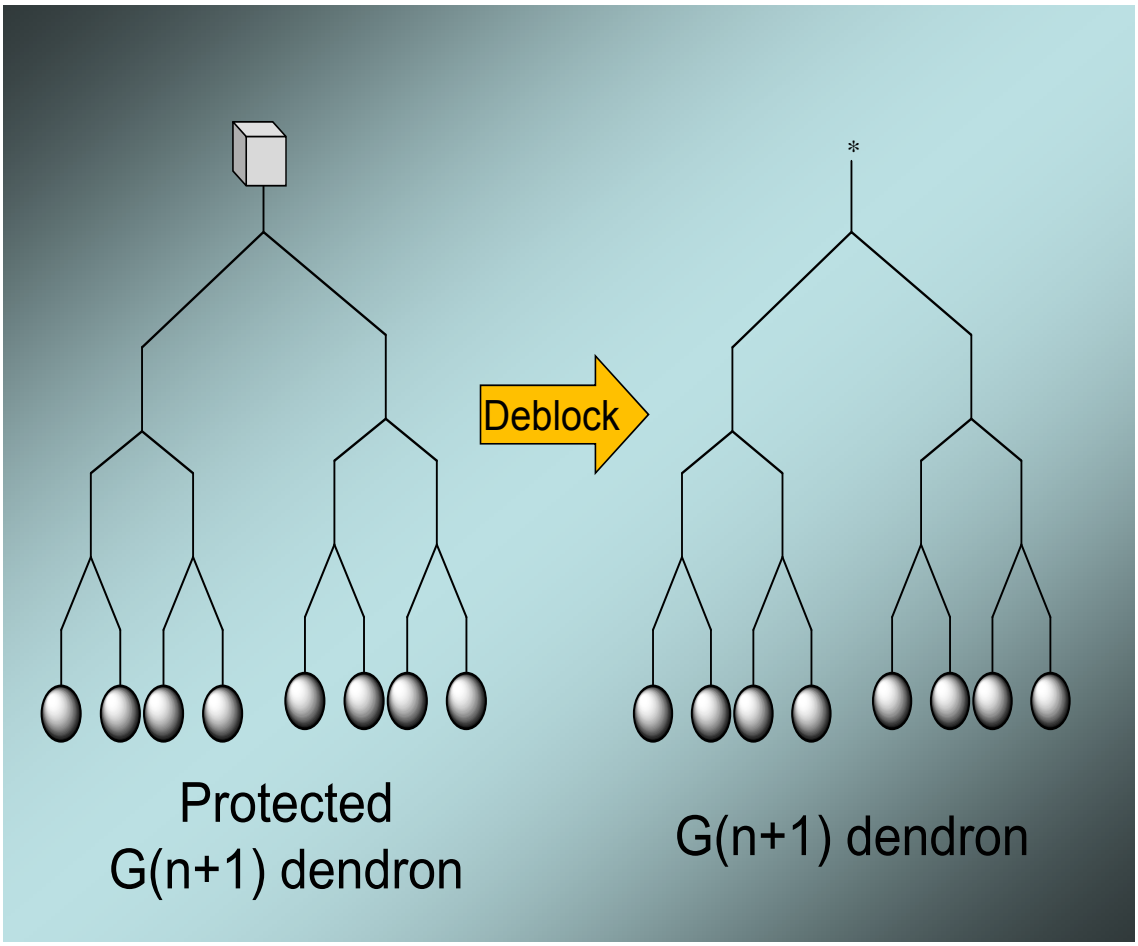
The core molecule reacts with monomer molecules containing one reactive and two dormant groups giving the first generation dendrimer (propagation step). Then the new periphery of the molecules is activated for reactions with more monomers (deblocking step). The product of this reaction becomes the new dendrimer core, the process is repeated for several generations and the dendrimer is built layer after layer. This approach is successful for the production of large quantities of dendrimers. Problems occur from side reactions and incomplete reactions of the end groups that lead to structure defects. To prevent side reactions and to force reactions to completion large excess of reagent is required, that causes some difficulties in the purification of the final products.

- **Convergent methods**,[30] were developed as a response to the weaknesses of the divergent synthesis. Also in the convergent approach the dendrimer is constructed stepwise, but this time starting from the end groups and progressing inwards. When the growing branched polymeric arms, called Dendrons, are large enough, they are attached to a multifunctional core molecule.



Scheme 4.4: Dendrimer convergent synthesis: Propagation step

The convergent growth method has several advantages. Desired products are relatively easy to purify and the occurrence of defects in the final structure is minimized. It becomes possible to introduce subtle engineering into the dendritic structure by precise placement of functional groups at the periphery of the macromolecule. Its limit is that it does not allow the formation of high generations because steric problems occur in the reactions of the dendrons and the core molecule, Scheme 4.6.



- **Self-assembling methods**,[31] were used by Zimmerman's group[32] to synthesize large quantities of high generation dendrimers. They prepared wedge-shaped molecule with adendritic tail in such a manner that subunits could self-assemble in bigger aggregates. Wedges are held together by hydrogen bond and stabilized by van der Waals interactions. This non-covalent structure[33] is responsible for the limit stability of this kind of structures, that disaggregate in diluted solutions, polar solvents and when the temperature is raised.

It is worth to note that two decade since the discovery of dendrimers the quite artisan-like multi-step synthesis still require great effort. Though DSM advocates more cost-friendly, large-scale dendrimers production with the ASTRAMOL™ technology,[34] only few applications, for which the unique dendrimers structure is crucial to pass the cost-benefit test. It is quite trivial to say that most of them are in the pharmaceutical field.

4.2 Identification of the target D-BCAs

As with other classes of polymers, dendrimers are extremely versatile and can be tailored on the custom exigencies to obtain the maximum efficiency from their use. In this paragraph the characteristics of the various dendrimer constituents will be scrutinized to arrive to the identification of suitable target molecules.

4.2.1 The chelating functional group

As said in paragraph 1.3, Ac^{+++} is an hard cation that has stronger interactions with hard anions. Among the electron donating groups stable in water solution (blood, where the complex will find its final application, is a highly complex water solution), the carboxylic one seems to be the more suitable. It is negative charged but does not displace hydrogen atoms from water molecules as alcoholates or amides do, and being harder than thiolates is a better chelating atoms toward An, even if the thiolates have an higher nucleophilicity.

Anionic dendrimers can be used in livings. While “cationic” dendrimers (e.g. amine terminated PAMAM and poly(propylene imine) dendrimers that form cationic groups al low pH) are generally haemolytic and cytotoxic and their toxicity is generation dependent and increases with the number of surface groups[35], anionic dendrimers (like those bearing a carboxylated surface) are not cytotoxic over a broad concentration range.[36]

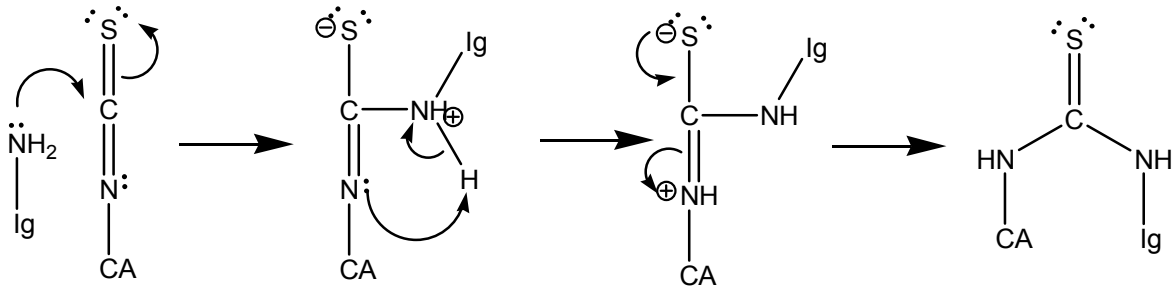
Carboxyl group can be both inserted on the dendrimer surface at the end of the synthesis using dendrimeric superficial functional groups in a specific synthetic step or, because of the high versatility of carboxylic acids and their derivatives, be a functional group of the monomer, allowing more straightforward syntheses.

Since An have a coordination numbers between 6 and 10, a number of complexing groups in the same range must be present on the target molecules to form a complex with one metal atom, and between 12 and 20 functional groups are required to complex two actinides atoms. Since BCAs that can bring a load of just one Ac atom have already been synthesized,[37,38] syntheses of double-load BCAs have more practical interest. Unfortunately double loaded classic Pendant Type Macrocyclic Bifunctionalized Chelating Agents (PT-MBCA)

are really difficult to be synthesized and a new approach toward this problem has to be sought. The target at this initial stage is to prove the feasibility of D-BCA of n-generation (Gn) with around eight chelating functional groups to open the way to D-BCA of n+1 generation (with around 16 chelating functional groups) able to bring a load of two An atoms.

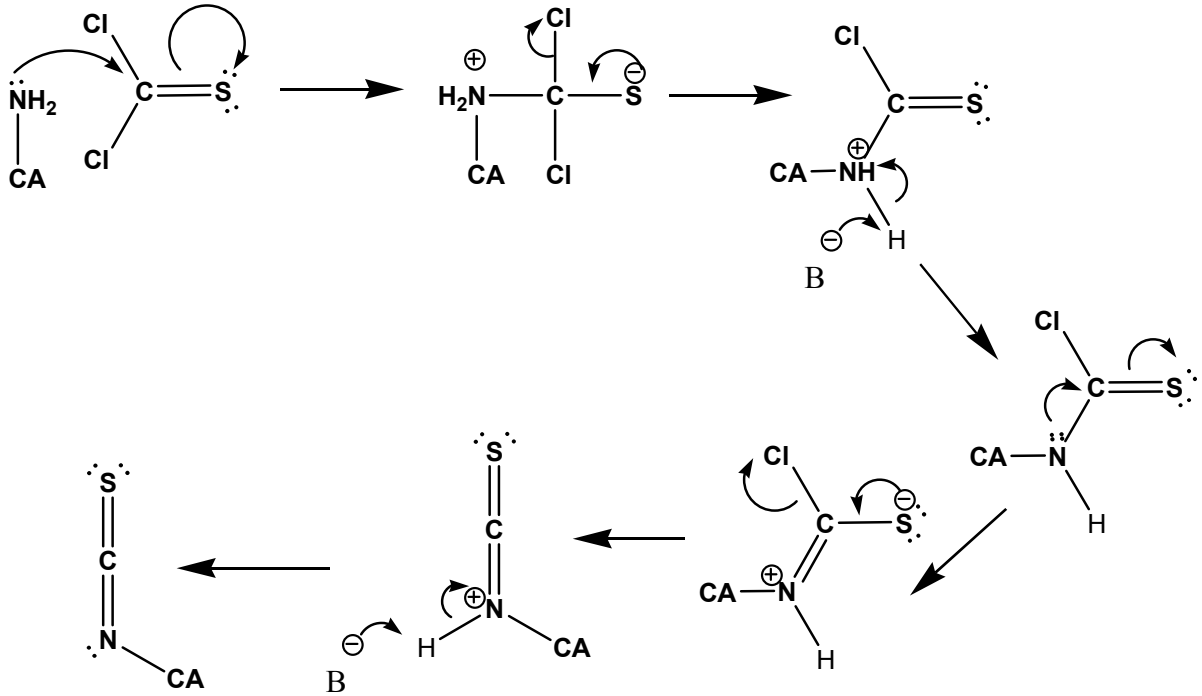
4.2.2 The Ig binding functional group

In § 1.5 we have seen that several functional groups can be use to covalently bind the Ig to the Chelating Agent. Isothiocyanate, that reacts quickly and selectively with an amino group to give substituted thiourea,[39] is the most interesting one.

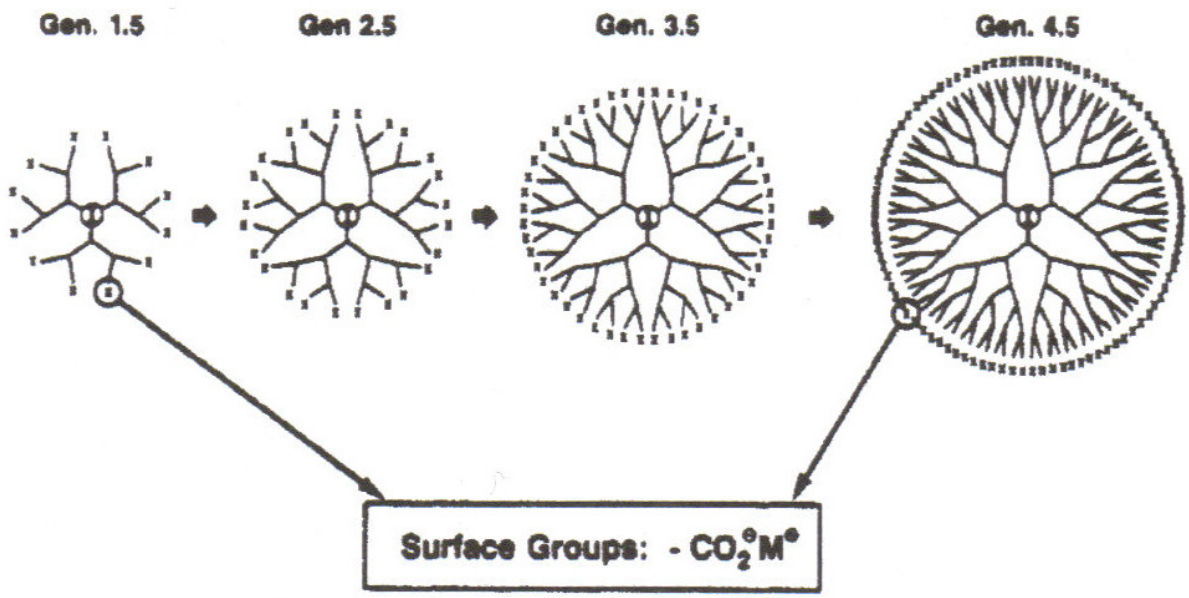


Scheme 4.5: synthesis of Isothiocyanate

It can be easily produced starting from an amine and thiophosgene, along the synthetic path shown in Scheme 4.6.



On a synthetic standpoint both solutions should be possible, since dendrimers of low generations (0^{th}, 1^{st}, 2^{nd} and 3^{rd}) have highly asymmetric shape and possess more open structures as compared to higher generation dendrimers. As the chains growing from the core molecule become longer and more branched (in 5^{th} and higher generation) dendrimers adopt a globular structure[40] and with this dendrimers the use of a pendant, that come out from the globular structure, were unavoidable.



Scheme 4.6: Rapresentation of a typical sturburst dendrimer possessing terminal carcoxylate groups: generation 1.5-5.5. Note the congestion of the surface groups that increases as the generation size increase.

In the already synthesized BCA both solution have been taken, i.e. some use one of the chelating group to create a covalent bond with the antibody, avoiding the use of any pendant, others use a pendant[41] -usually a benzyl group that form a large conjugation with the thiourea giving rise to a long rigid group that keep the chelating agent far from the antibody. Since this group has been proved effective, it makes sense to use it as first trial.

Since the introduction of the isothiocyanate group makes the whole synthesis notably more complex, a wise solution is to prove the feasibility of a D-PT-BCA step by step: First prove that dendrimers forms complexes with An.

Then prove that dendrimeric steric hindrance allows the introduction of a pendant. Third step will be the demonstration that the pendant itself does not interfere with the chelating properties of the dendrimer. Finally the Isothiocyanate group will be introduced. Since the report of Diallo proved the suitability of dendrimers as complexing agents for An, the target of this work became to prove that dendrimeric steric hindrance allows the introduction of a pendant.

4.2.3 The monomer

From the previous paragraphs come out that our target D-BCAs should have around 16 carboxylic functionalities and at list one free amino group, it would make sense to have the same functionalities already present on the monomer, to avoid synthetic steps expressly dedicated to their introduction. In nature there are a lot of molecules that present at the same time a carboxylic and an aminic functionality: the amino acids. Of the 20 more common amino acids used by living beings for protein synthesis, and that are excellent organic synthesis starting materials because of their availability and their cheap costs, only two, aspartic and glutamic acid (Asp and Glu respectively, Fig. 4.1), have two carboxylic functionalities that make them suitable as building block for the synthesis of a dendrimer akin to the target one.

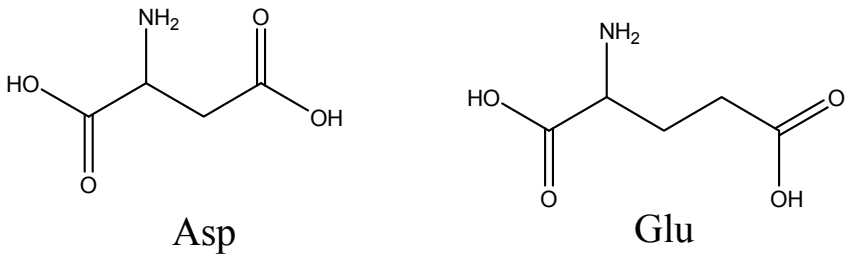


Fig. 4.1: Aspartic (Asp) and glutamic (Glu) amino acids.

The two amino acids differ in a chain carbon atom, so they show the same reactivity and the choice has to be taken on the basis of a shorter or longer alkyl backbone. On one side, having more methylenic groups implies a bulkier D-BCA that can give heavier interference with the quaternary structure of the Ig and compromise its binding ability toward its antigen. This would bent the

scale on the side of aspartic acid. On the other hand longer branches would allow a more comfortable allocation of carboxylic groups around the An ions, bringing higher stability constants and a safer use of the produced drug. Since the latter is the most important parameter, the first attempt is the synthesis of a glutamic acid based D-BCA.

Theoretically it could also be proposed to start with an amino acid with other functional groups, but their introduction would signify the introduction of other reactivity that would complicate the synthesis bringing no advantage. Also the choice of an amino acid with more amino groups would have brought no advantage, since carboxylic functionalities are required on the dendrimeric surface.

4.2.4 The core

There is no need to use as central molecule a different specie than glutamic acid itself. It has an amino group that can be used for the direct introduction of the Isothiocyanate group or of a pendant moiety, and eight carboxylic groups are exactly expressed on the surface of the third generation of a pure Glu dendrimer.

4.2.5 The target molecule

From the previous paragraphs come out that the target molecule is a 3^{th} generation dendrimer of the glutamic acid. Monomers can be built together using peptidic bounds. There are arguments that speak in favor and counterarguments to this choice. On one side this kind of compounds are ubiquitously spread in our organism and they are surely not toxic, on the other one they could be recognized by the immune system as non-self and elicit an immunogenic reaction. Actually some synthetic polypeptides do and other don't, and no universally valid prevision can be made on if, how strong and how much time an immune response would take to appear. Only experiments on the product can give a clear answer.

All the characteristic of the target molecule have been at this point defined: the monomer, the generation number, the core, the pendant and its position. Assembling all the puzzle tesseræ together give the identikit of the target molecule:

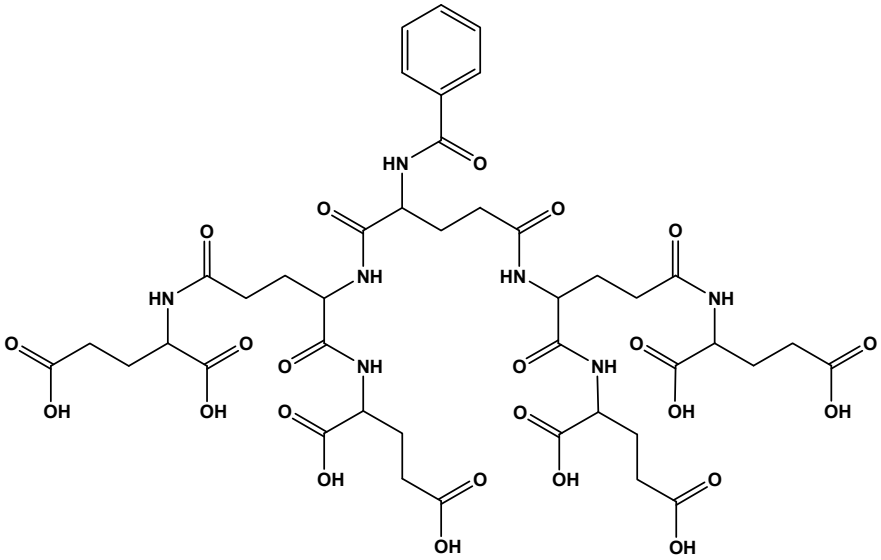
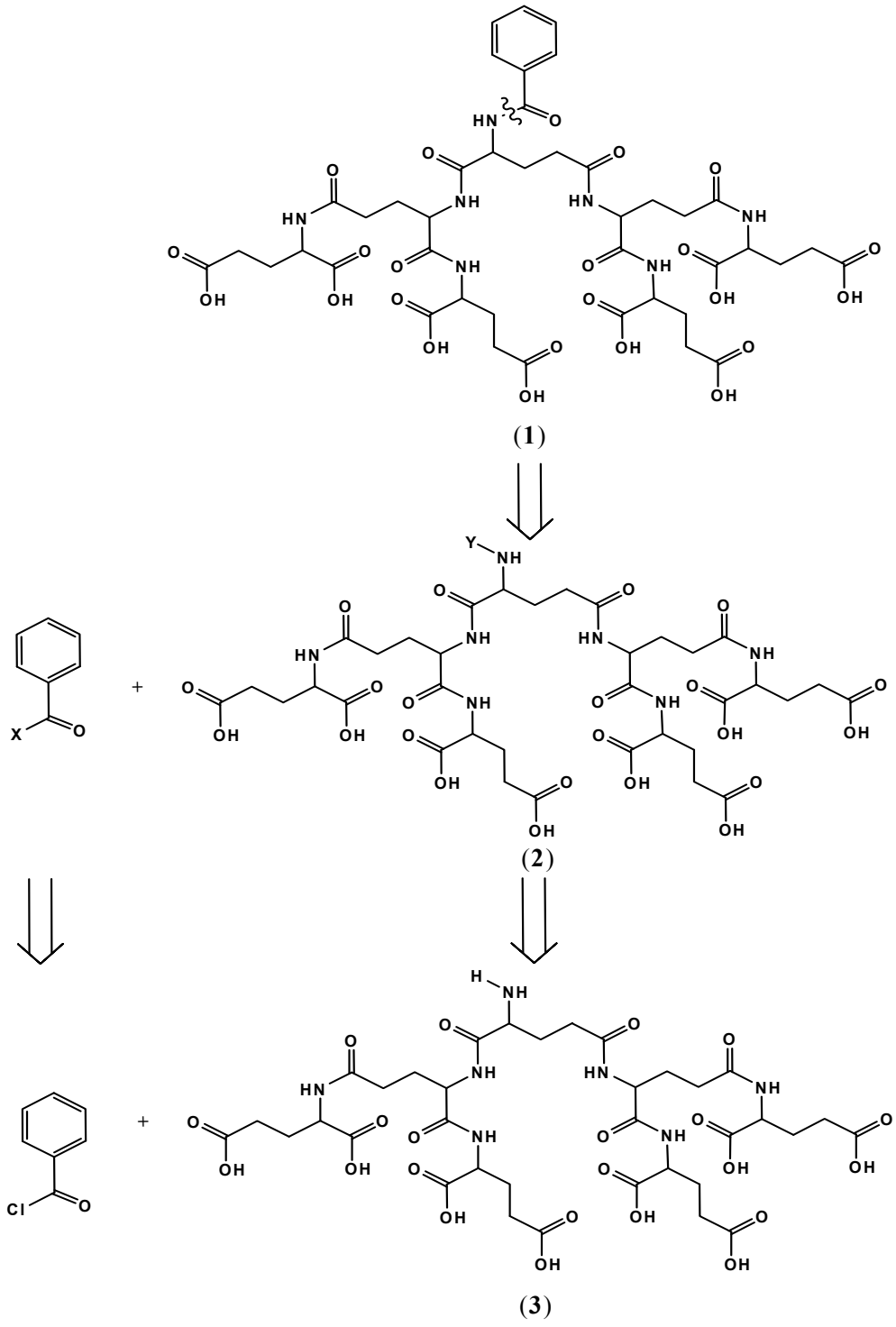


Fig. 4.2: Target Dendrimeric Chelating Agent

A peculiar symbolism has been established for amino acids based dendrimers, recalling that in use for protein. In the letter amino acids are written in left-right direction with the amino group on left and the carboxylic termination on the right. In the symbolism adopted for amino acid based dendrimers the substituent linked to the amino group are written on left end, followed by the amino acid type (in parenthesis) and the specification of the generation preceded by a capital G. Finally carboxylic substituents are written on the right end. The target molecule for instance is written Bz-(Glu)G3-OH, meaning a three generation Glutamic acid dendrimer with a benzoyl functional group linked to the terminal amine and carboxylic acid on the surface terminations.

4.3 Identification of the synthetic strategy

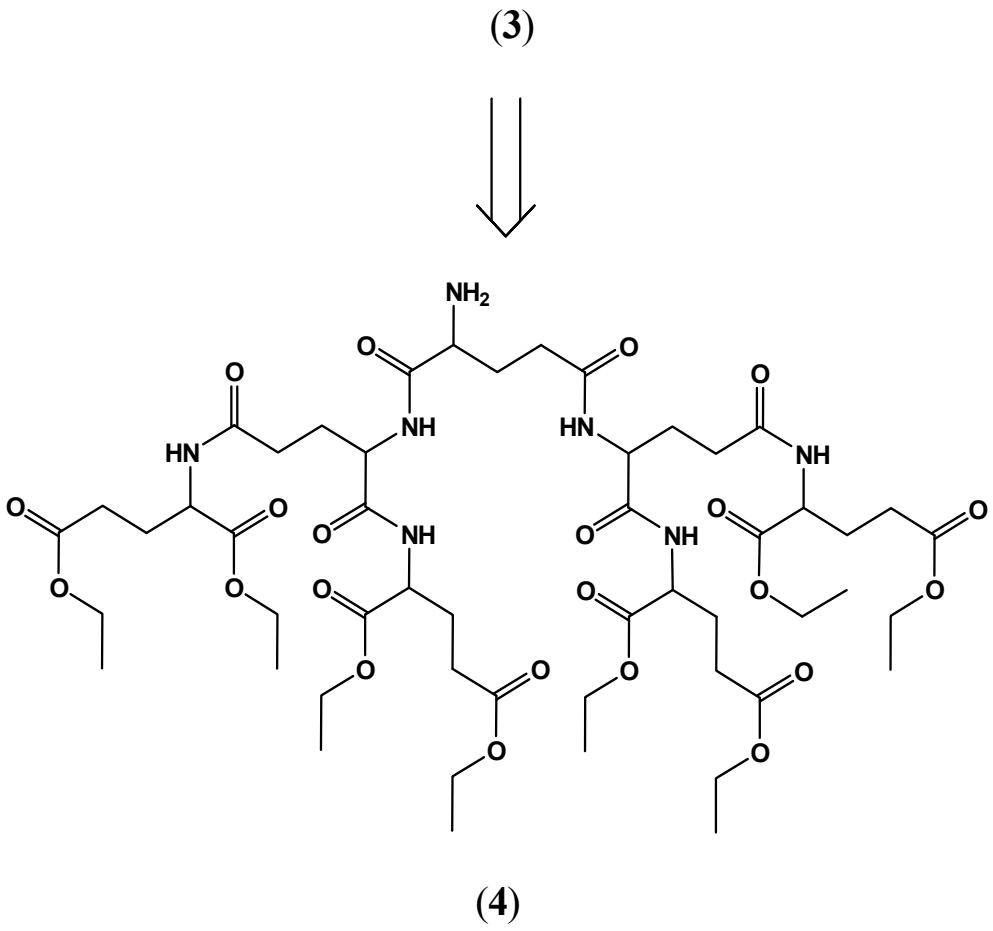
Once that the target molecule has been chosen, the disconnection approach (Schemes 4.7-4.10) was used to identify the better reaction sequence that lead to its synthesis.



Scheme 4.7: Retrosinthetic analysis of the target molecule.

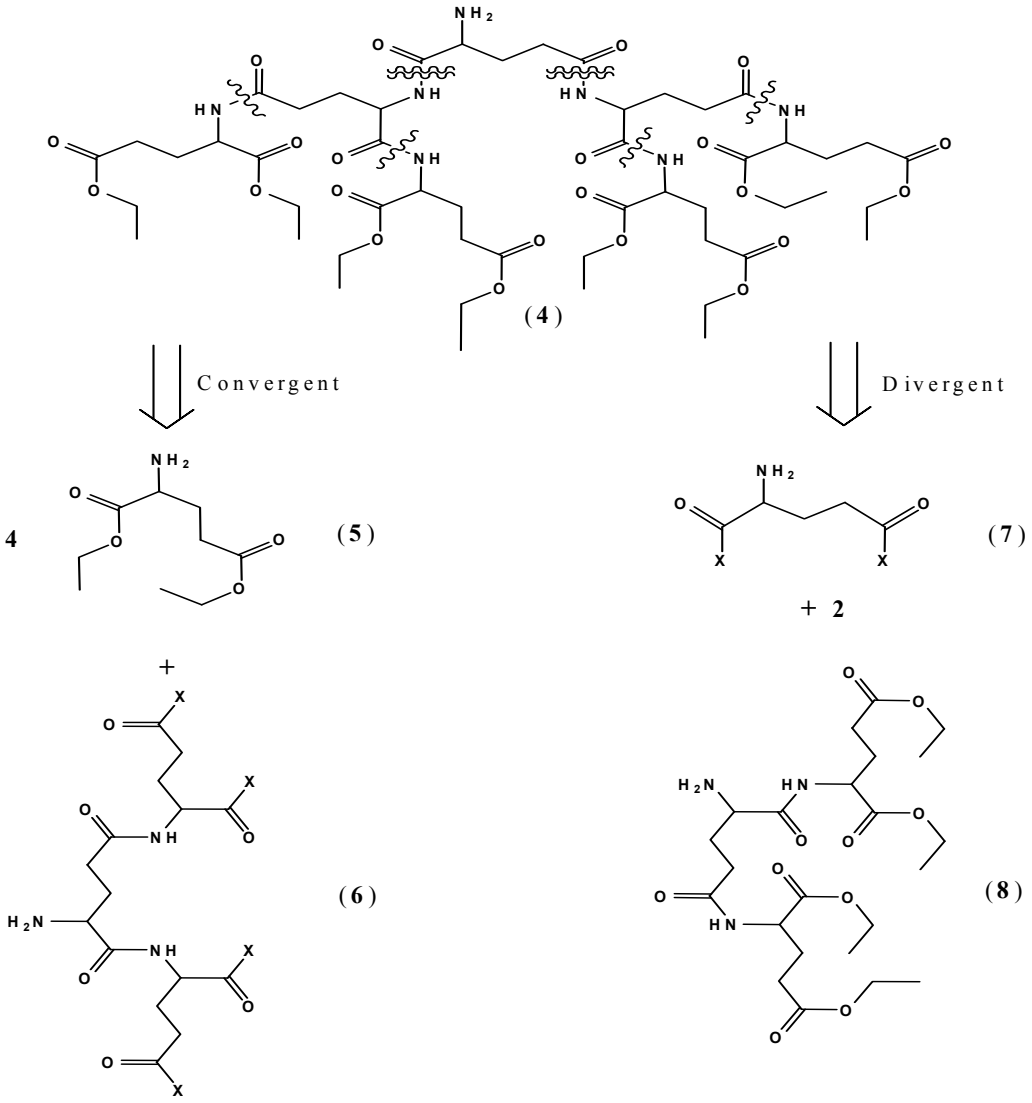
First step is the disconnection of the benzoic group, to leave an homogenous molecule composed only by glutamic acid monomers. This lead on one side to a benzoic synthon, that in the actual synthesis can be replaced by bezoyl chloride synthetic equivalent, and on the other to a third generation polypeptidic dendrimer with a free terminal amino group.

To avoid the formation of the mixed anhydride, acidic functionalities must be protected. The esteric functionality has been chosen for this aim because it is an excellent protective group and allows the use of diethyl glutamate (**31**), a commercially available product, as starting material.



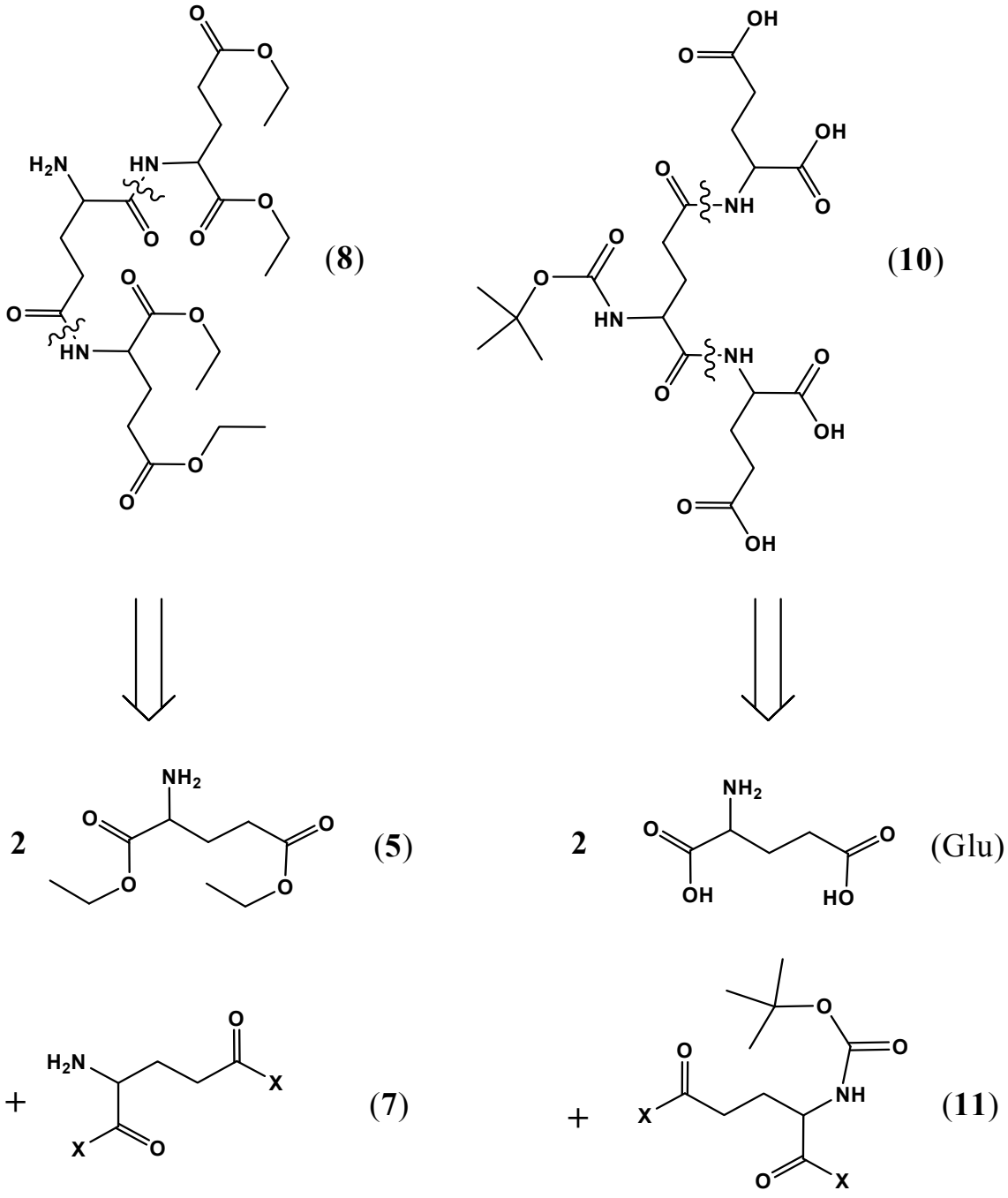
Scheme 4.8: Retrosynthesis of the target molecule

When a dendrimer is to be synthesized the first choice –except some very exotic procedures- is between convergent and divergent methods. The most sensible disconnection in a polypeptidic dendrimer is on the amidic bounds. In the following schemes double waved lines lead to convergent approach synthesis disconnection path while single lines lead to a divergent approach disconnection path.



Scheme 4.9: Convergent and divergent disconnection of H-(Glu)G3-OEt. Single XXX

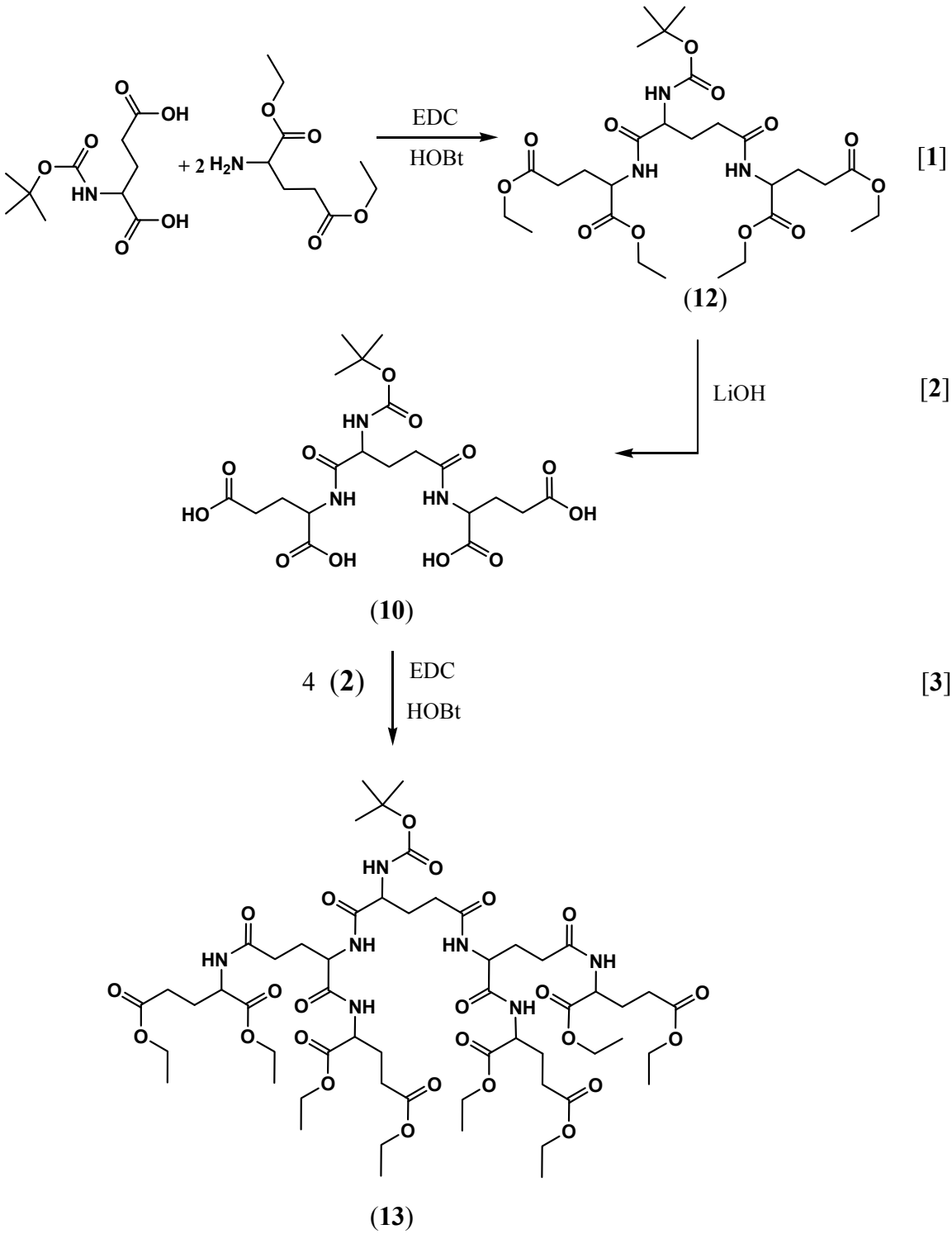
Product **31** is the commercially available diethyl glutamate. To find the synthetic equivalents of synthons H-(Glu)G2-X (**32**) and H-Glu-X (**33**) the generic X groups can be replaced by hydroxyl groups (a plenty of peptidization reactions between amines and carboxylic acids are well known),[42] and amino groups have to be protected. Di-*ter*-butyl dicarbonate (BOC)[43], that can be easily inserted and deblocked, is used in several commercially available products, will be used each time an amino group has to be protected in this reaction sequence. The used synthetic equivalent for product **33** is the commercially available Boc-Glu-OH . Product **36** and H-(Glu)G2-OEt (**34**) have to undergo to a further retrosynthetic step.



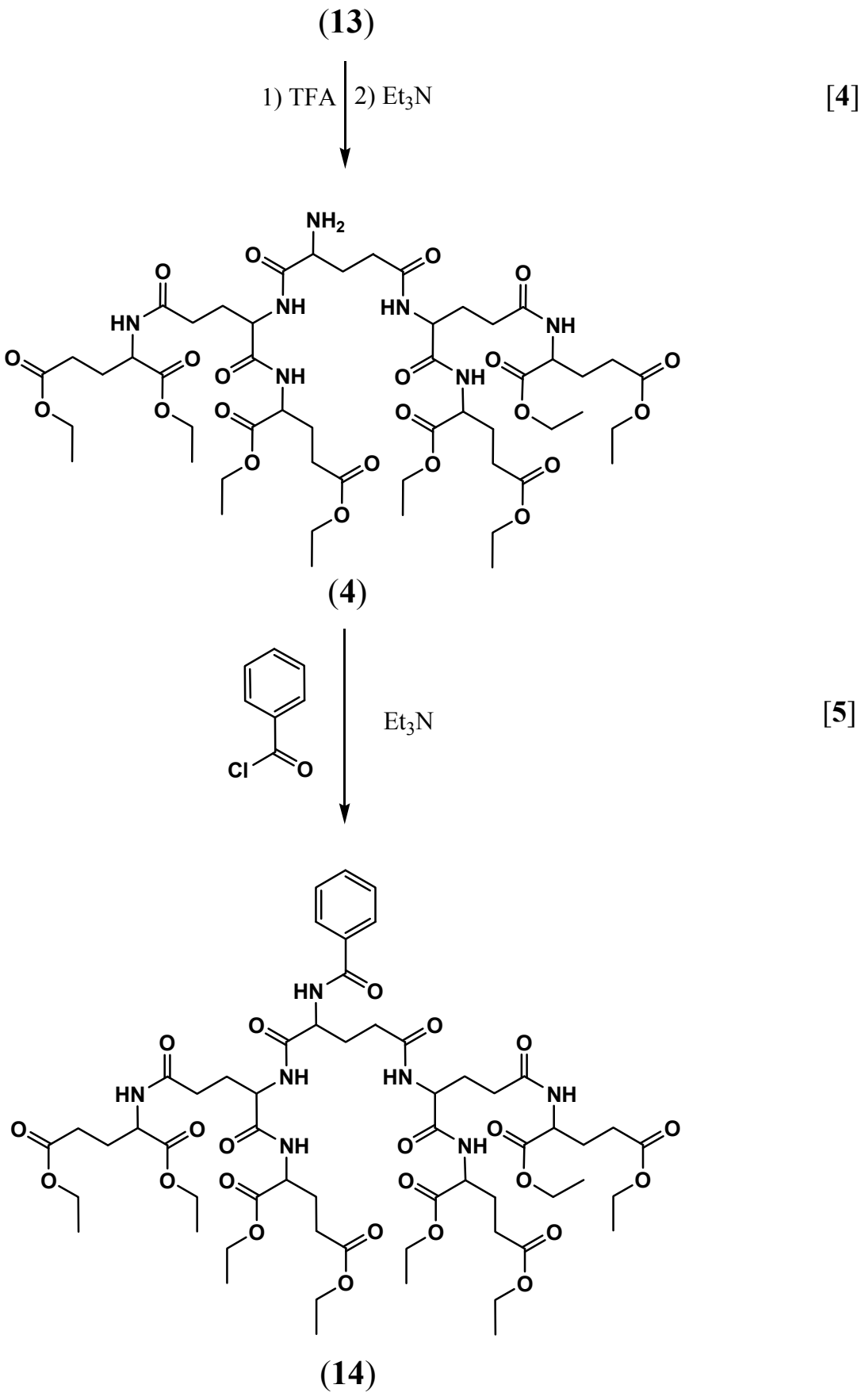
Scheme 4.10: Retrosynthesis of the target molecule

If, like in the previous retrosynthetic step, X groups in the synthons **33** and BOC-Glu-X (**37**) are replaced by hydroxyl groups and the amino group in **33** is protected using BOC, it becomes clear that they share a common synthetic equivalent, the already used commercial BOC-Glu-OH. Protecting carboxylic acids in Glu with ethyl esterification, it becomes also clear that **31** is its synthetic equivalent.

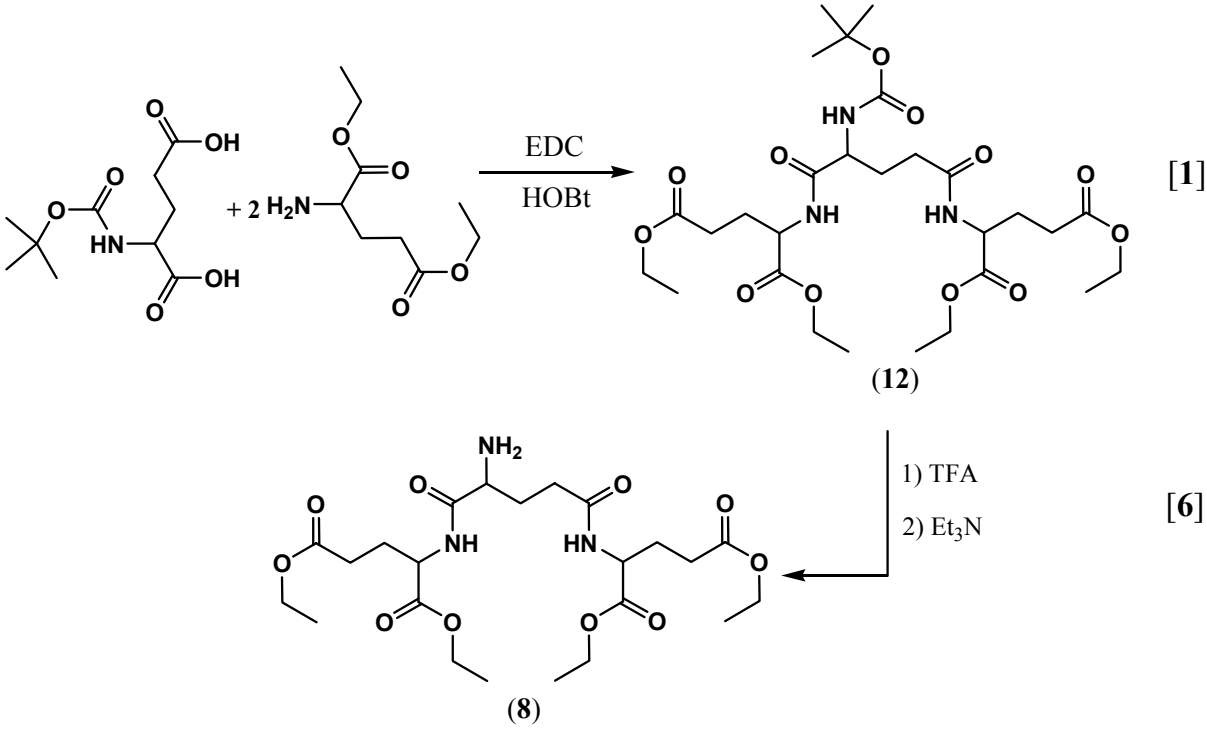
Two feasible paths for the synthesis of the target molecule starting from commercially available molecules have been identified by this retrosynthetic analysis. Corresponding convergent and divergent direct syntheses are shown respectively in schemes 4.11-4.14.



Scheme 4.11: Retrosynthesis of the target molecule

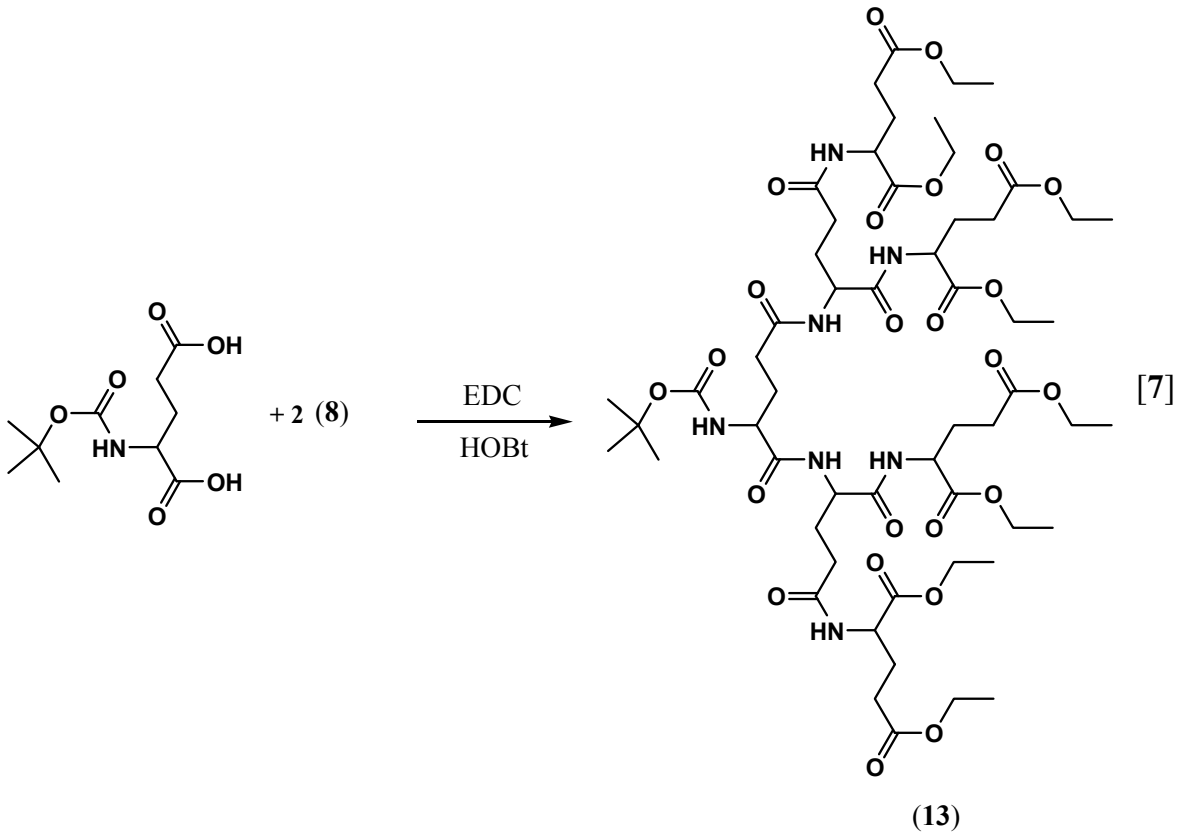


Scheme 4.12: Divergent Synthetic path to Bz-(Glu)G3-OEt



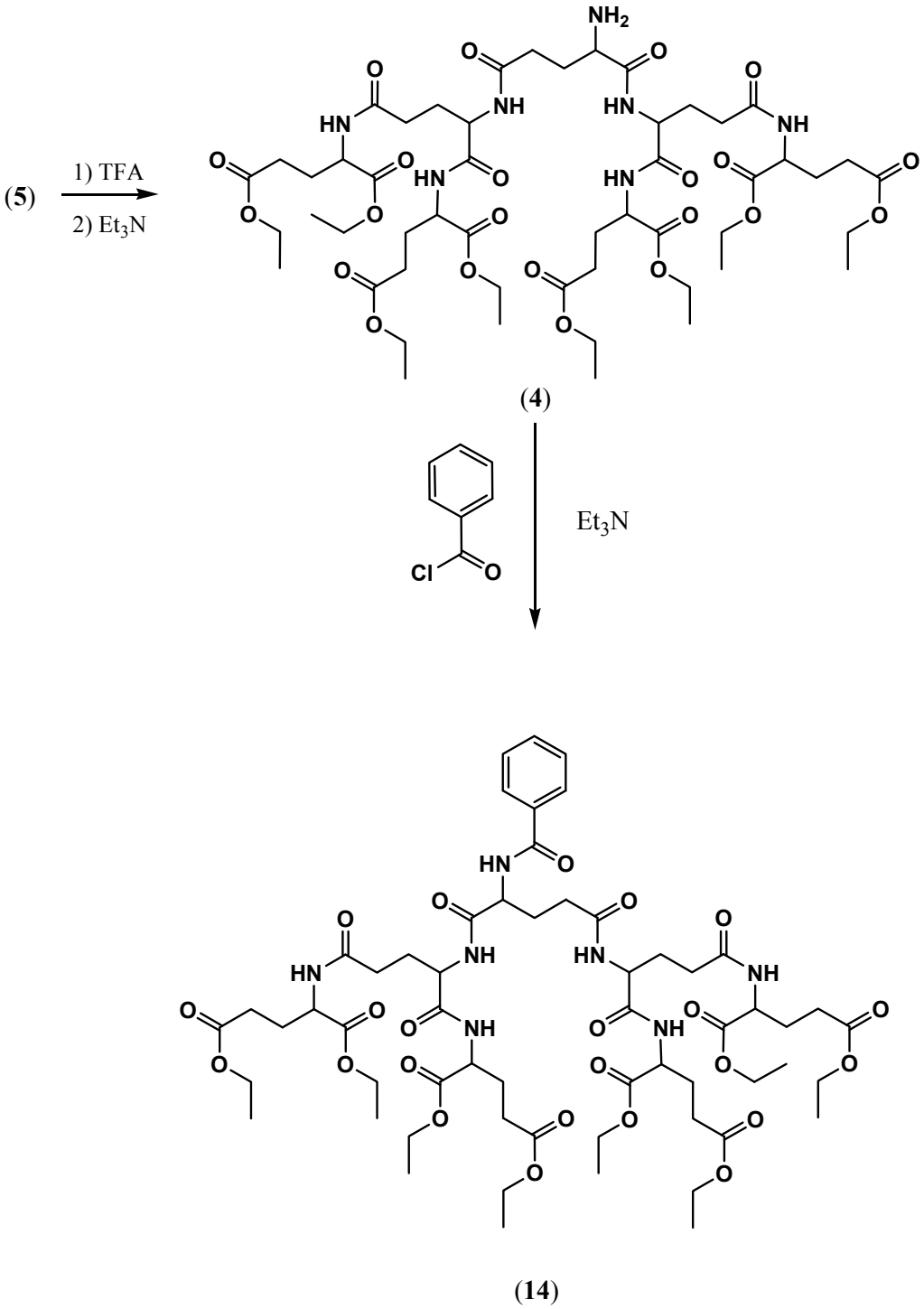
[1]

[6]



[7]

Scheme 4.13: Conergent Synthetic path to Bz-(Glu)G3-OEt



Scheme 4.14: Convergent Synthetic path to Bz-(Glu)G3-OEt

Multi-step reactions used for dendrimers are a kind of controlled polymerization, and like in polymerization processes we can identify initiation, propagation and termination reactions that take place in the reaction vessel, also in the case of dendrimer initiation, propagation and termination reaction

steps can be identified. Of utmost importance for efficient synthesis planning is the identification of a recursive "propagation" steps sequence, where generation n dendrimer reacts to give the homologue of generation n+1. In the divergent Synthesis this cycle is identified by growth step (reaction with monomer as in [**1**], catalyzed by EDC and HOBt) and deblocking (in this case of esteric protective groups with LiOH,[44] as in [**2**]). The convergent synthesis present a recursive cycle with the same growth step, but this time is the BOC protective group that is deblocked using first TFA[45, 46] and than triethylamine to neutralize the solution, as in reaction [**6**].

Both synthetic pathways can be followed and would lead to the target molecule, but convergent approach, that minimizes defects in the final structure and allow an easier recovery of the final product, is used. If the target molecule would have been an 6th or higher generation this approach would have not been possible because of steric hindrance around the core molecule.[38]

In conclusion this synthetic path lead to the preparation of the protected form of Bz-(Glu)G3-OH (Bz-(Glu)G3-OEt), with a global process yield of 34%. This product will be deblocked and its chelating properties toward Ac^{+++} assessed in a facility entitled to work with radioactive materials.

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Part III

Experimental Section

Chapter 5

Experimental Part

5.1 General Methods and Materials

5.1.1 Purification of solvents and reagents

All syntheses were carried out in air unless otherwise stated.

Chloroform, Methylene chloride used for synthesis were dried on Calcium hydride and distilled under nitrogen after the use.

5.1.2 Instruments

Thin-layer chromatography was performed with pre-coated silica gel plates (DC-Alufolien Kieselgel 60 F_{254}, Merk KGaA, Darmstadt, Deutschland).

pH measurements have been performed either with litmus 435140 (Carlo Erba, Rodano, Italy) or with a pH-meter Hanna pH tester checker 1 (Hanna Instruments, Woonsocket, RI).

Gaschromatographic analyses were performed on a Perkin-Elmer (Waltham, Massachusetts) 8500 or on a Perkin-Elmer Clarus 500 gaschromatograph equipped with a split-splitless injector and a FID detector, using a capillary column ZB-1 (15m*0.25 mm, film thickness 0.25 µm), and nitrogen as carrier gas.

Mass spectra were recorded either on an Agilent (Santa Clara, California) 5995A spectrometer, interfaced with an Agilent 5980 gas chromatograph equipped with a split-splitless injector and a ZB-1 column (30m x 0.25mm, film thickness 0.25 µm), using He as carrier gas; or on a Shimadzu Axima-CFR MALDI-TOF (Kyoto, Japan).

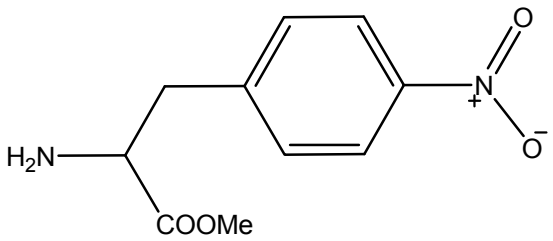
Uncorrected Melting points (mp) were determined on a Koffler Heizbank Reichert Thermovar 18.43.21.

Infrared spectra have been acquired on a Perkin-Elmer 1605 FT-IR

^{1}H and **^{13}C-NMR** spectra were recorded on a Varian Gemini-200 NMR spectrometer at the frequencies of 200 MHz for ^{1}H and 50 MHz for ^{13}C, or on a Varian VXR-300 at the frequencies of 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shift measurements have been determined at room temperature (~ 25 °C) unless otherwise stated and are referred to TMS or to solvent as internal standard.

5.2 Synthesis of PT-HEHA-CA

5.2.1 Synthesis of methyl 2-amino-3-(4'-nitrophenyl)propanoate



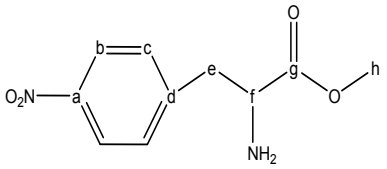
2-amino-3-(4'-nitrophenyl)propanoic acid (320 mg, 1.5 mmol) was suspended into 2,2-dimethoxypropane (22.5 cm³). Concentrate Hydrochloric acid (1.5 cm³ 36%) was added to this stirring mixture, and the reaction was

further carried on at room temperature for 17 h. During this time the solution darkened considerably.

The evaporation of the solvent and lither products at reduced pressure gave a yellow precipitate, that was dissolved in anhydrous Methanol and precipitated by the addition of anhydrous diethyl ether. After filtration the product was dried under vacuum and the oily product was recovered (260 mg, 1.5 mmol) in quantitative yield.

^{1}H-NMR (200 MHz, $CDCl_3$)**:** δ 8.23 (2H, d, *J*=0.09, *H*-Ph-CH_2); 7.48 (2H, d, J=0.04, NO_2-Ph-*H*); 4.48 (1H, t, J=0.03, -CH_2-C*H*(NH_2)-$COOCH_3$); 4.12 (2H, s, NH_2); 3.78 (3H, s, COOC*H*$_3$); 3.39 (2H, dd, -Ph-C*H*$_2$-CH(NH_2)-$COOCH_3$).

^{13}C-NMR (200 MHz, $CDCl_3$,)**:** δ 169.2 (g); 146.9 (d); 141.4 (a); 130.1 (c); 123.8 (b); 53.2 (f); 53.1 (e); 34.9 (h).

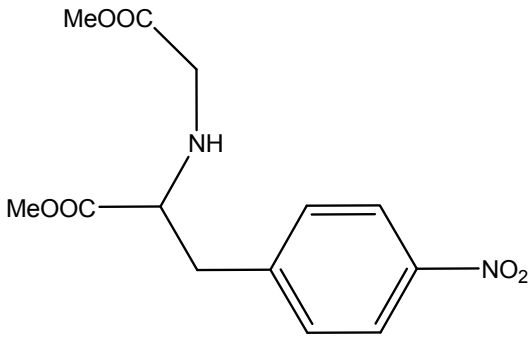


IR (KBr, cm^{-1})**:** 2919, 2673, 2626, 1743, 1604, 1513, 1491, 1450, 1347, 1237, 1181, 1145, 1105, 1058, 979, 947, 931, 865, 856, 842, 809, 750, 700, 652, 506, 489.

GC (140°C/5' @ (30°C/min) 280°C, solvent THF, column 15m, DB1, P=8 atm, Injector 220°C, Detector 240°C). t=7.83 min.

MS (m/z): 165 (50), 137 (8), 119 (20), 88 (100).

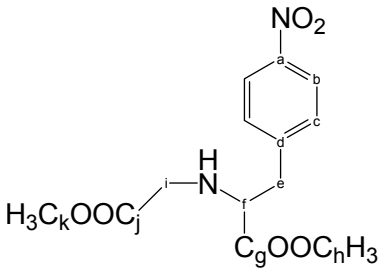
5.2.2 Synthesis of methyl 2-((methoxycarbonyl)methylamino)-3-(4'-nitrophenyl)propanoate



Methyl 2-amino-3-(4'-nitrophenyl)propanoate (500 mg, 1.8 mmol) was dissolved under Nitrogen atmosphere in 50 cm^3 of anhydrous THF. To the stirring solution were added three equivalents of Triethylamine (0.75 cm^3, 5.4 mmol) and three equivalents of methyl 2-bromoacetate (0.5 cm^3, 5.4 mmol). After stirring at room temperature (2.5 h) the yellow solution was washed several times with water. The phases were separated and the aqueous one washed with methyl acetate. After drying with sodium sulphate, the solution were concentrated under vacuum to give 533 mg (1.15 mmol) of a yellow oil with a 64% yield.

^{1}H-NMR (200 MHz, $CDCl_3$)**:** δ 8.18 (2H, d, J=0.05, *H*-Ph-NO_2); 7.42 (2H, d, J=0.05, *H*-Ph-CH_2); 3.72 (6H, s, O-C*H*$_3$); 3.65 (1H, t, J=0.03, -NH-C*H*(COOMe)-CH_2-); 3.42 (2H, d, J=0.01, -C*H*$_2$-COMe); 3.32-3.18 (2H, m, Ar-C*H*$_2$-CH-); 2.1 (1H, s, -N*H*-).

^{13}C-NMR (200 MHz, $CDCl_3$)**:** δ 173.6 (g); 172.0 (j); 147.1 (a); 145.0 (d); 130.2 (c); 123.7 (b); 61.6 (f); 52.2 and 52.0 (h-k); 49.0 (i); 39.2 (e).

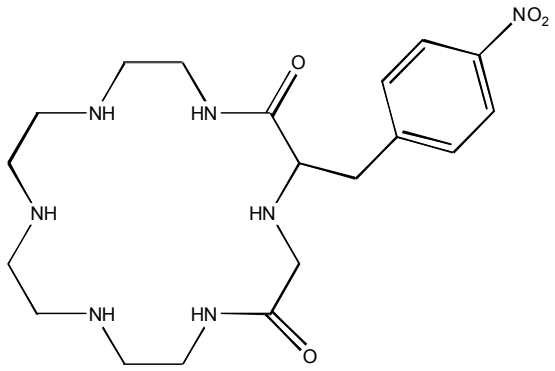


IR (KBr, cm^{-1})**:** 3455, 3336, 2948, 2850, 1734, 1600, 1519, 1432, 1346, 1206, 1109, 1011, 985, 855, 742, 699, 650.

GC (140°C/5' @ (30°C/min) 280°C (15'), solvent THF, column 15m, DB1, P=8 atm, Injector 220°C, Detector 240°C). t=10.15 min.

MS (m/z): 297 (M+1, 1), 237 (M-COOMe, 65), 160 (M-NO_2-Bz, 100), 100 (M-NO_2-Bz –COOMe, 50).

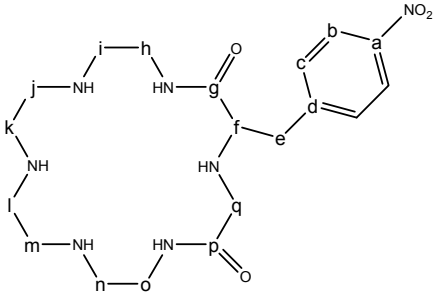
5.2.3 Synthesis of 3-(4'-nitrobenzyl)-2,6-dioxo-1,4,7,10,13,16-hexaazacyclooctadecane



To a stirring solution of sodium methoxide (2.2 cm^3 0.5 M, 1.1 mmol) in anhydrous THF (250 cm^3), a solution of 1,11-diamino-3,6,9-triazaundecane (0.2 cm^3, 1 mmol) in anhydrous THF is added, under Nitrogen atmosphere. After 1 h at the reflux of the solvent, 2-((methoxycarbonyl)methylamino)-3-(4'-nitrophenyl)propanoate (290 mg, 1.0 mmol) dissolved in anhydrous THF is added. After 96 h the solvent is evaporated at reduced pressure and the product suspended in Chloroform, filtered and purified by flash chromatography (Acetone/pentane = 20/80). The red-brick solid product (82 mg, 0.19 mmol) was recovered after evaporation of the solvents under vacuum with a 20% yield.

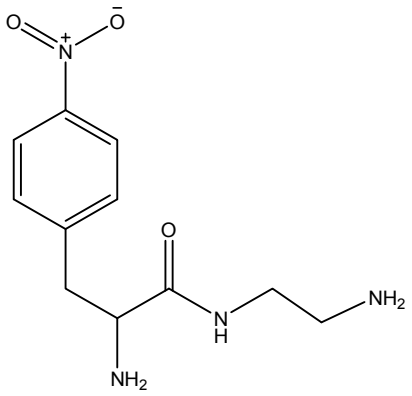
^{1}H-NMR (200 MHz, $CDCl_3$)**:** δ 8.16 (2H, d, J=0.05, *H*-Ph-NO_2); 7.54 (1H, s, -NH-CO-); 7.39 (2H, d, J=0.05, *H*-Ph-CH_2); 7.25 (1H, s, -NH-CO-); 3.92 (1H, t, J=0.04, NO_2-Bz-C*H*-(CO-)NH-); 3.51-2.79 (12H, m); 2.69 (8H, s, -NH-CH_2-); (3H, s, NH).

^{13}C-NMR (200 MHz, $CDCl_3$)**:** δ 170.4 (g); 168.8 (p); 145.3 (a); 144.9 (d); 128.0 (c); 120.3 (b); 62.8 (f); 50.4 (j-m); 49.9 (i,n); 49.6 (q); 44.1 (h,o); 36.7 (e).



IR (KBr, cm^{-1})**:** 3278, 2963, 2854, 1658, 1514, 1458, 1341

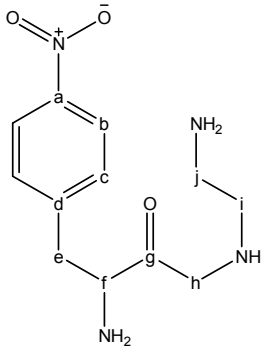
5.2.4 Synthesis of 2-amino-N-(2'-aminoethyl)-3-(4'-nitrophenyl)propanamide



A solution of 1,2-diaminoethane (7.0 cm^3, 130 mmol) is slowly added to a stirring solution of 2-amino-3-(4'-nitrophenyl)propanoate (450 mg, 2 mmol) in methanol (40 cm^3). The solution is stirred at room temperature for 18 h and the solvent is evaporated at reduced pressure to give brown solid product (460 mg) quantitatively.

^{1}H-NMR (200 MHz, $CDCl_3$)**:** δ 8.19 (2H, d, J=0.04, *H*-Ph-NO_2); 7.68 (1H, s, -NH-CO-); 7.41 (2H, d, J=0.05, *H*-Ph-CH_2); 3.67 (1H, m, NH_2-C*H*-CO-); 3.47 (2H, s, NH-CH_2-CO-); 3.39-2.75 (8H, m, $2CH_2$, $2NH_2$).

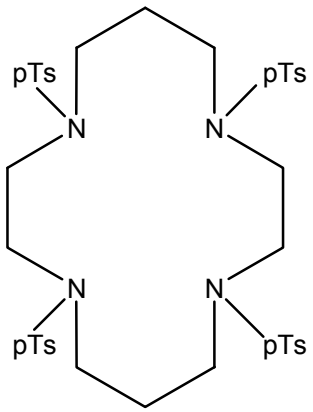
^{13}C-NMR (200 MHz, $CDCl_3$)**:** δ 173.7 (g); 147.2 (a); 146.0 (d); 130.4 (b); 123.9 (c); 56.3 (f); 42.0 (e); 41.6 (h); 41.1 (i).



IR (KBr, cm^{-1})**:** 3360, 2930, 2861, 1660, 1644, 1598, 1343, 1106, 857, 744, 700.

5.3 Synthesis of nitrogenated macrocyclic chelating agents

5.3.1 Synthesis of 1,4,8,11-tetraparatoluenesulphonyl-1,4,8,11-tetraazacyclotetradecane

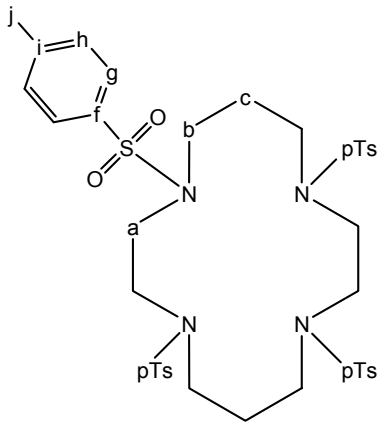


Paratoluenesulfonyl chloride (5.0 g, 25 mmol) dissolved in Chloroform (50 cm^3) was slowly added to a stirring basic aqueous solution (12 g NaOH in 50 cm^3 of water) containing 1,4,8,11-tetraazacyclotetradecane (1.0 g, 5 mmol) and a catalytic amount of Triton B.

After 18 h the reaction was interrupted, the phases separated and the product crystallized by addition of diethyl ether to the organic one. After filtration the white crystals were recovered with a 97.5% yield.

^{1}H-NMR (200 MHz, $CDCl_3$)**:** δ 7.79 (8H, d, *J*=0.05, *H*-Ph-SO_2); 7.42 (8H, d, J=0.05, CH_3-Ph-*H*); 3.30 (8H, s, N-C*H*$_2$-C*H*$_2$-N); 3.22 (8H, t, J=0.03, $(CH_2)_2$-C*H*$_2$-N); 2.52 (12H, s, -Ph- CH_3); 1.96 (4H, t, J=0.03, CH_2-C*H*$_2$-CH_2-).

^{13}C-NMR (200 MHz, $CDCl_3$)**:** δ 144.0 (i); 134.5 (f); 130.0 (h); 127.6 (g); 50.0 (a); 48.1 (b); 27.8 (j); 21.6 (c).



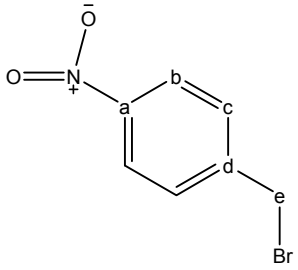
IR (KBr, cm^{-1})**:** 3066, 2922, 2869, 1597, 1459, 1341, 1159, 1090, 914, 814, 732, 549.

5.3.2 Synthesis of 1-(bromomethyl)-4-nitrobenzene

NBS (360 mg, 0.5 mmol) is added to a solution of p-nitrotoluene (276 mg, 0.5 mmol) in CCl_4 (100 cm^3) a solution of. After 3 h at the reflux of the solvent the precipitate is filtered and the solvent evaporated at reduced pressure. The reaction gave the product (89 mg, 0.4 mmol) with a 83% yield.

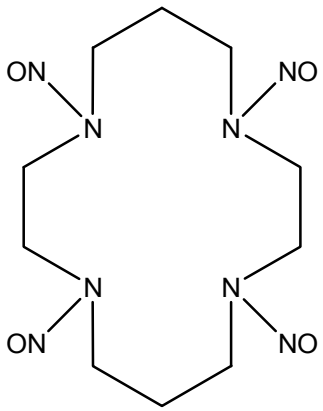
^{1}H-NMR (200 MHz, $CDCl_3$)**:** δ 8.25 (2H, d, *J*=0.05, *H*-Ph-NO_2); 7.59 (2H, d, J=0.05, CH_2-Ph-*H*); 4.54 (2H, s, CH_2Br).

^{13}C-NMR (200 MHz, $CDCl_3$)**:** δ 147.9 (a); 143.5 (d); 129.3 (c); 120.7 (b); 34.0 (e).



MS (m/z): 217 (M^+ +1, 6.3), 215 (M^+ -1, 6.4), 136 (100.0), 106 (12.2), 90 (23.9), 89 (28.4).

5.3.3 Synthesis of 1,4,8,11-tetra-N-nitroso-1,4,8,11-tetraazacyclotetradecane with NaOH



In 200 cm^3 of water, 1,4,8,11-tetraazacyclotetradecane (1.0 g, 5.0 mmol) is dissolved. The solution is acidified (pH = 2.5) with concentrated hydrochloric acid (1.6 cm^3) and cooled to 4 °C. A water solution of sodium nitrite (1.53 g, 22

mmol in 10 cm^3 of water) is slowly added. The pH is kept between 5.0 and 5.5 with the addition of glacial acetic acid. After 2h the precipitate, separated from the solution and washed with cold water, was dried. The product (1.14 g, 3.6 mmol) was recovered in 72% Yield.

^{1}H NMR (200 MHz, DMSO)**:** δ 4.68 – 3.34 (16 H, m, -NH-C***H***$_2$-CH_2-C***H***$_2$-NH-); 2.48 (4 H, m, -NH-CH_2-C***H***$_2$-CH_2-NH-, 1st conformer); 2.20 (4 H, m, -NH-CH_2-C***H***$_2$-CH_2-NH-, 2nd conformer); 1.89 (4 H, m, -NH-CH_2-C***H***$_2$-CH_2-NH-, 3rd conformer); 1.64 (4 H, m, -NH-CH_2-C***H***$_2$-CH_2-NH-, 4th conformer).
IR (KBr, cm^{-1})**:** 2955, 1450, 1366, 1339, 1311, 1272, 1139, 1022, 966, 885, 811, 789, 650, 555.
Mp = 211 °C

5.3.4 Synthesis of 1,4,8,11-tetra-N-nitroso-1,4,8,11-tetraazacyclotetradecane with Dinitrogen tetroxide (ipoazotite):

Ipoazotite, produced by extremely slow addition of oil of vitriol to sodium nitrite (22.3 g, 0.32 mmol), is bubbled into a water solution of 1,4,8,11-tetraazacyclotetradecane (218 mg, 1.1 mmol in 100 cm^3 water). After 4 h, when no more fumes are produced, the reaction is interrupted, the colourless precipitate is filtered, washed with cold water and dried.
By spectroscopy analyses it is identified as the target molecule. Yield: 227 mg, 0.7 mmol, 68%.

5.3.5 Synthesis of 1,4,8,11-tetra-N-nitroso-1,4,8,11-tetraazacyclotetradecane (**7**) via Amberlist and silica wet:

1,4,8,11-tetraazacyclotetradecane (1.73 g, 8.7 mmol) is dissolved Into a stirring reaction mixture composed by wet silica (21.7 g, 330 mmol of silica in 200 cm^3 water), methylene chloride (200 cm^3), Amberlist 15 (12.2 g) and sodium nitrite (6.26 g, 91 mmol). When no more fumes evolves (6 h) the reaction is interrupted and the mixture filtered. By soxhlet extraction (Acetonitrile for 16 h) only a poor quantity (104 mg, 0.5 mmol) of the original reactant (1,4,8,11-tetraazacyclotetradecane) is recovered.

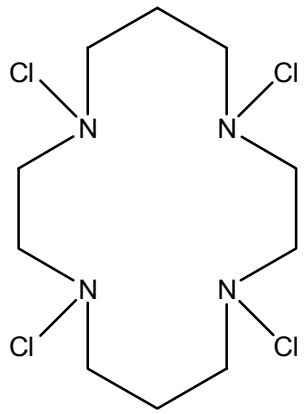
5.3.6 Synthesis of 1,4,8,11-tetra-N-nitroso-1,4,8,11-tetraazacyclotetradecane with trimethylnitrososilane:

Chlorotrimethylsilane (0.8 cm^3, 6 mmol) is add to a stirring solution of sodium nitrite (310 mg, 4.5 mmol) in methylene chloride (30 cm^3). After 30 minutes a pale yellow solution of 1,4,8,11-tetraazacyclotetradecane (200 mg, 1 mmol) in methyl chloride (10 cm^3) is carefully dropped into the stirring solution, followed by Sodium carbonate (2.0 g, 18 mmol). After 16 h the precipitate is isolated by filtration, washed with cold water and dried. At spectroscopic analyses the solid (230 mg, 0.73 mmol) is recognized as the target product with a 73% Yield.

5.3.7 Synthesis of 1,4,8,11-tetra-N-nitroso-1,4,8,11-tetraazacyclotetradecane with Chlorotrimethylsilane:

Chlorotrimethylsilane (0.8 cm^3, 6.0 mmol) is added to a stirring solution of sodium nitrite (400 mg, 5.8 mmol) and 1,4,8,11-tetraazacyclotetradecane (200mg, 1mmol) in methylene chloride (50 cm^3). After 19 h the reaction is stopped, the solid is filtered and washed with methylene chloride. After evaporation of the solvent under reduced pressure a colorless solid (165 mg, 0.52 mmol) identified by IR spectroscopy as the target molecule, is collected with a 52% Yield.

5.3.8 Synthesis of 1,4,8,11-tetraazacyclo-1,4,8,11-tetrachlorotetradecane

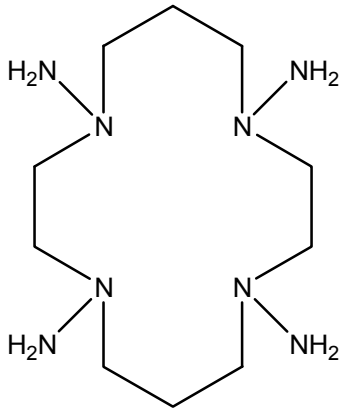


An aqueous solution of 1,4,8,11-tetraazacyclotetradecane (200 mg, 1mmol in 10 cm^3 H_2O) is added at room temperature Into an aqueous solution of NaClO (8 mmol in 10 cm^3 H_2O). After 1.5 h the precipitate is collected by filtration, washed with cold water and dissolved in acetone. After filtration the organic

phase was dried over anhydrous sodium sulfate and the solvent evaporated at reduced temperature, to leave a white solid (218 mg, 0.65 mmol) in a 65% yield.

^{1}H NMR (200 MHz, $CDCl_3$)**:** δ 3.31 (8H, s, -N-C***H***$_2$-C***H***$_2$-N-); 3.14 (8H, t, J=0.03, -N-C***H***$_2$-CH_2-C***H***$_2$-N-); 1.97 (4H, p, J=0.03 -N-CH_2-C***H***$_2$-CH_2-N-).
^{13}C NMR (200 MHz, $CDCl_3$)**:** 63.68 (-N-***C***H_2-***C***H_2-N-); 60.1 (-N-***C***H_2-CH_2-***C***H_2-N-); 26.2 (-NH-CH_2-***C***H_2-CH_2-NH-).
IR (KBr, cm^{-1})**:** 2967, 2878, 1455, 1433, 1344, 1283, 1022, 1044, 961, 911, 872, 783, 628, 561, 517.

5.3.9 Synthesis of 1,4,8,11-tetra-N-amino-1,4,8,11-tetraazacyclotetradecane in phase transfer conditions



Palladium-on-carbon at 10% (1.0 g, Fluka) is suspended into a solution of 1,4,8,11-tetra-N-nitroso-1,4,8,11-tetraazacyclotetradecane (316 mg, 1 mmol) in ethanol (10 cm^3), cyclohexene (0.5 cm^3, 5 mmol) and benzene (20 cm^3). After 2.5 h at the reflux of the benzene and 16 h at room temperature, the solid was separated by filtration and the solvents evaporated at reduced pressure to give a greenish oil (30 mg, 0.12 mmol) of the product in a 12% yield.
^{1}H NMR (200 MHz, $CDCl_3$)**:** δ 2.87 (8H, s, -NH_2); 2.60 (8H, s, -N-C***H***$_2$-C***H***$_2$-N-); 2.48 (8H, t, J=0.04, -N-C***H***$_2$-CH_2-C***H***$_2$-N-); 1.53 (4H, p, J=0.04 -N-CH_2-C***H***$_2$-CH_2-N-).
^{13}C NMR (200 MHz, $CDCl_3$)**:** 59.4 (-N-***C***H_2-CH_2-***C***H_2-N-); 58.0 (-N-***C***H_2-***C***H_2-N-); 22.0 (-NH-CH_2-***C***H_2-CH_2-NH-).
IR (KBr, cm^{-1})**:** 3472, 3406, 2959, 1655, 1639, 1541, 1508, 1301, 1257, 1192, 1022, 799, 668.

5.3.10 Synthesis of 1,4,8,11-tetra-N-amino-1,4,8,11-tetraazacyclotetradecane with Sodium borohydride:

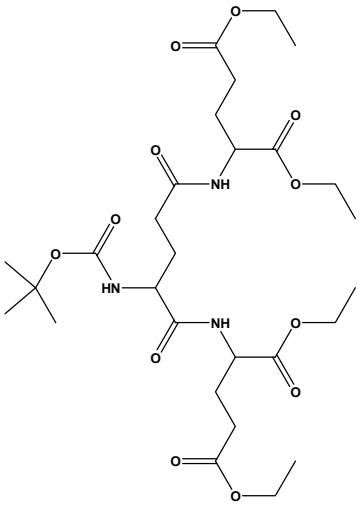
1,4,8,11-tetra-N-nitroso-1,4,8,11-tetraazacyclotetradecane (630 mg, 3 mmol) is suspended along with sodium borohydride (1.96 g, 51 mmol) in methanol (50 cm^3) at room temperature. After 3 h the reaction solution is brought at the reflux of the solvent for 40 h. The solution is filtered and the solvent is evaporated under reduced pressure. The solid dissolved in ethyl acetate was filtered and the solvent evaporated at reduced pressure. 560 mg of a white solid, that appeared to be the starting material, were recovered.

5.3.11 Synthesis of 1,4,8,11-tetra-N-amino-1,4,8,11-tetraazacyclotetradecane with Titanium(III) chloride:

1,4,8,11-tetra-N-nitroso-1,4,8,11-tetraazacyclotetradecane (660 mg, 2.0 mmol) is suspended along with Titanium(III) chloride (1.70 g, 34 mmol) in water (200 cm^3) at room temperature. After stirring (1 h) the mixture is cooled (3 h) and brought at room temperature for 19 h. The mucilage-looking solid is filtered and extracted with ethyl acetate. The organic phase is dried on anhydrous sodium sulphate. The solvent is evaporated under reduced pressure, giving rise to the greenish oily product (100 mg, 0.4 mmol) in a 20% yield.

5.4 Synthesis of dendrimeric BCA

5.4.1 Synthesis of BOC-G2-OEt



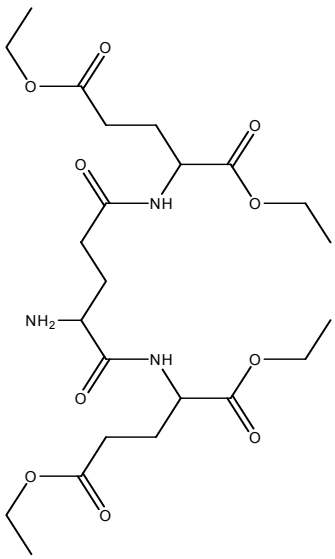
EDC (1.5 g, 8.0 mmol) was added to a stirring mixture of Boc-Glu-OH (0.9 g, 3.7 mmol) and HOBt (1.23 g, 8.0mmol) in CH_2Cl_2 (50 cm^3). At the same time in a separate flask H-Glu-OEt•HCl (2.25 g, 9.0 mmol) was dissolved in CH_2Cl_2 (10 cm^3) and Et_3N (0.6 ml, 8.0 mmol) and than added to the stirring reaction mixture.

After 53h the solution was washed with: saturated aqueous solution (10%) of sodium bicarbonate, aqueous solution (10%) of citric acid, saturated solution (10%) of sodium bicarbonate and finally water.

The organic phase was dried over anhydrous sodium sulphate, filtered and after the removal of the solvent in vacuo gave a white solid, that was dissolved in MeOH and purified by column chromatography (MeOH/$CHCl_3$ = 1/9). After evaporation of the solvents under reduced pressure the white solid product (1.70 g, 2.8 mmol), was recovered in a 76% yield.

^{1}H-NMR (200 MHz, $CDCl_3$) δ**:** 7.82 (1H, d, *J*=0.06 NH); 7.60 (1H, d, *J*=0.06 NH); 4.92 (1H, d, *J*=0.06 NH); 4.45 (2H, t, *J*=0.05, -NH-C*H*R-CO-); 4.12 (8H, q, -COO-C*H*$_2$-CH_3); 3.51 (1H, t, *J*=0.04, -NH-C*H*R-CO-); 2.41-2.22 (6H, m, -NH-(CH)-CH_2-C*H*$_2$-COO-); 2.20-2.04 (6H, m, -NH-(CH)-C*H*$_2$-CH_2-COO-); 1.33 (9H, s, Bu^t); 1.25 (12H, t, *J*=0.03 -COO-CH_2-C*H*$_3$).
^{13}C-NMR (200 MHz, $CDCl_3$) δ**:** 172.8, 171.6, 171.5, 171.3, 154.0, 79.5, 61.2, 59.5, 50.7, 50.5, 31.6, 31.2, 29.7, 29.4, 28.2, 27.3, 25.7, 25.9, 13.1.
IR (KBr, cm^{-1})**:** 3322, 2977, 2933, 1733, 1683, 1655, 1522, 1258, 1200, 1167, 655.
mp = 87 °C

5.4.2 Synthesis of H-G2-OEt

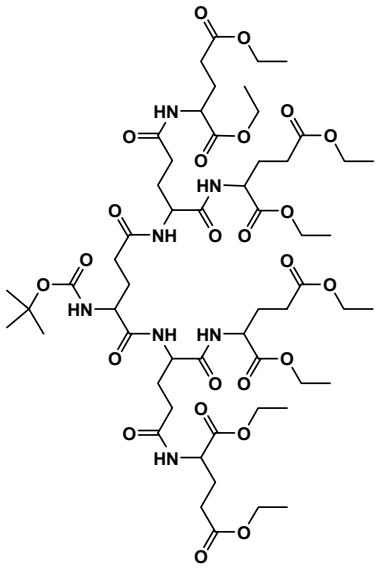


Trifluoroacetic acid (0.5 ml, 6.5 mmol) is added to a stirring solution of BOC-G2-OEt (1.51 g, 2.5 mmol) in CH_2Cl_2 (50 cm^3). After 2 h the solution is neutralized with Triethylamine (0.9 ml, 6.5 mmol) and washed with an aqueous solution of sodium bicarbonate (5%). The organic phase was dried over anhydrous sodium sulphate, filtered and the solvent removed under reduced pressure to give the product (1.22 g) in 96% yield.
^{1}H-NMR (200 MHz, $CDCl_3$)**:** 7.79 (1H, s, NH); 7.63 (1H, s, NH); 3.80 (2H, s, NH_2); 4.42 (2H, t, *J*=0.05, -NH-C*H*R-CO-); 4.15 (8H, q, -COO-C*H*$_2$-CH_3); 3.48 (1H, t, *J*=0.04, -NH-

C*H*R-CO-); 2.4-1.9 (12H, m, -NH-(CH)-CH_2-CH_2-COO-); 1.2 (12H, t, *J*=0.03 -COO-CH_2-CH_3).

^{13}C NMR (200 MHz, $CDCl_3$)**:** δ 172.8, 171.6, 171.5, 171.3, 61.2, 59.5, 50.7, 31.2, 29.4, 28.2, 25.9, 13.1.
IR (KBr, cm^{-1})**:** 3609, 3324, 3324, 2977, 2933, 1733, 1683, 1655, 1522, 1258, 1200, 1167, 655.
mp = 90 °C.

5.4.3 Synthesis of BOC-G3-OEt (**3**)



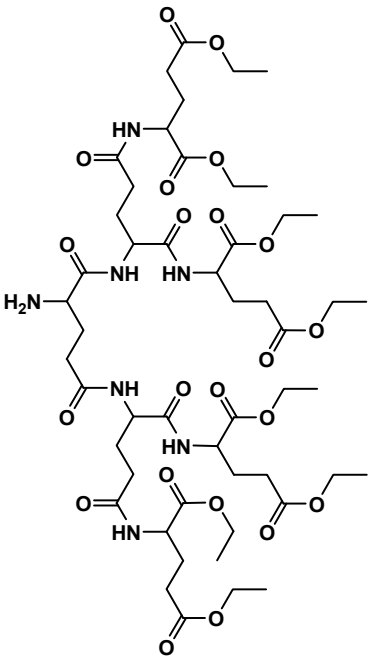
EDC (380 mg, 2 mmol) was added to a stirring mixture of Boc-Glu-OH (230 mg, 0.9 mmol) and HOBt (310 mg, 2 mmol) in CH_2Cl_2 (50 cm^3). At the same time in a separate flask H-G_2-OEt (1.34 g, 2.2 mmol) was dissolved in methylene chloride (10 cm^3) and than added to the stirring reaction mixture. After stirring (40 h) the reaction is stopped and the solution undergoes the same workup used for generation G2.

The organic phase is dried on anhydrous sodium sulphate and filtered. The crude was purified by column chromatography using a Chloroform/Methanol = 95/5 solution. Removing the solvents under reduced pressure the waxy product (820 mg, 0.65 mmol) is obtained in 65% yield.

^{1}H NMR (200 MHz, $CDCl_3$)**:** δ 7.88 (3H, d, J=0.04, NH); 7.66 (3H, d, J=0.04, NH); 5.08 (1H, d, J=0.04, NH); 4.8-4.6 (7H, m, -NH-C***H***R-CO-); 4.32-4.06 (16H, m, -CO-O-C***H***$_2$-CH_3); 2.53-2.32 (16H, m, -NH-CH-C***H***$_2$-C***H***$_2$-COOEt); 2.32-2.14 (6H, m, -NH-CH-C***H***$_2$-CH_2-COO-); 2.04-1.84 (6H, m, -NH-CH-CH_2-C***H***$_2$-COO-); 1.43 (9H, s, Bu^t); 1.33-1.21 (24H, m, -CO-O-CH_2-C***H***$_3$).

^{13}C NMR (200 MHz, $CDCl_3$)**:** δ 188.6, 172.7, 171.1, 154.0, 79.5, 62.2, 60.6, 53.5, 51.5, 32.3, 30.7, 30.5, 29.3, 28.4, 27.0, 26.8, 14.1.
IR (KBr, cm^{-1})**:** 3324, 2984, 2928, 1738, 1683, 1650, 1529, 1264, 1250, 1204, 1168, 653.
MP = 110 °C.

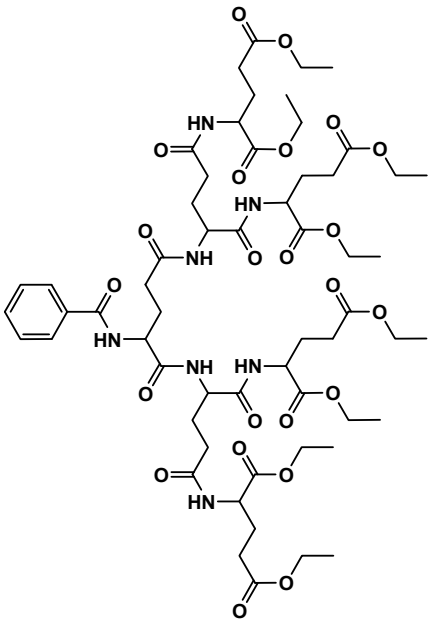
5.4.4 Synthesis of H-G3-OEt (**4**)



Trifluoroacetic acid (1.0 cm^3, 13 mmol) is added to a stirring solution of BOC-G3-OEt (540 mg, 0.43 mmol) in Methylene chloride (50 cm^3). After 2 h the solution is neutralized with Triethylamine (1.8 cm^3, 13 mmol) and washed with an aqueous solution of sodium bicarbonate (5%). The organic phase was dried over anhydrous sodium sulphate, filtered and the solvent removed under reduced pressure to give a pergameneous product (500 mg, 0.44 mmol) in quantitative yield.

^{1}H NMR (200 MHz, $CDCl_3$)**:** δ 7.87 (3H, d, J=0.04, NH); 7.64 (3H, d, J=0.04, NH); 4.7-4.5 (7H, m, -NH-C***H***R-CO-); 4.30-4.04 (16H, m, -CO-O-C***H***$_2$-CH_3); 3.93 (2H, s, NH_2); 2.50-2.30 (16H, m, -NH-CH-C***H***$_2$-C***H***$_2$-COOEt); 2.30-2.12 (6H, m, -NH-CH-C***H***$_2$-CH_2-COO-); 2.02-1.83 (6H, m, -NH-CH-CH_2-C***H***$_2$-COO-); 1.31-1.18 (24H, m, -CO-O-CH_2-C***H***$_3$).
^{13}C NMR (200 MHz, $CDCl_3$)**:** δ 188.9, 173.0, 171.4, 62.4, 60.8, 53.8, 51.7, 32.5, 31.0, 30.5, 29.6, 27.2, 27.1, 14.3.
IR (KBr, cm^{-1})**:** 3613, 3324 ,3324, 2980, 2935, 1735, 1685, 1658, 1525, 1260, 1205, 1170, 657.
mp = 114 °C

5.4.5 Synthesis of Benzoil-G3-OEt (**5**)



Benzoyl chloride (1.0 cm³, 0.8 mmol) diluted in Methylene chloride (10 cm³) is slowly added to a stirring solution of H-G3-OEt (310 mg, 0.3 mmol) in Methylene chloride (60 cm³) and Triethylamine (1.0 cm³, 1.3 mmol). After 16 h the solution is washed with a sodium hydroxide solution (5%) and then purified with a flash chromatography (Ethyl acetate/hexane = 20/80, then Methanol).

After evaporation under reduced pressure a white waxy solid (260 mg, 0.21 mmol) is obtained in 70% yield.

^{1}H NMR (200 MHz, DMSO)**:** δ 8.02 (2H, d, J=0.04, orto); 7.58-7.35 (3H, m, meta and para); 7.02 (3H, s, N-H); 6.74 (3H, s, N-H); 5.65 (1H, d, J=0.04, N-H); 4.72-4.60 (7H, m, -NH-C***H***R-CO-); 4.17-4.00 (16H, m, -CO-O-C***H***$_2$-CH$_3$); 2.43-2.36 (16H, m, -NH-CH-C***H***$_2$-C***H***$_2$-COOEt); 2.26-2.06 (6H, m, -NH-CH-C***H***$_2$-CH$_2$-COO-); 2.04-1.81 (6H, m, -NH-CH-CH$_2$-C***H***$_2$-COO-); 1.23-1.108 (24H, m, -CO-O-CH$_2$-C***H***$_3$).

^{13}C NMR (200 MHz, DMSO)**:** δ 172.9, 172.6, 172.4, 169.8, 135.0, 132.1, 130.0, 128.7, 61.3, 60.7, 54.5, 52.0, 35.5, 32.7, 30.6, 28.9, 26.8, 14.7.

IR (KBr, cm^{-1})**:** 3433, 3322, 3055, 2978, 2922, 2355, 2333, 1744, 1733, 1716, 1698, 1681, 1651, 1602, 1558, 1540, 1523, 1402, 1276, 1200, 1171, 722, 672.

mp = 124 °C