Synthesis and biological application of glyco- and peptide derivatives of fullerene C60

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Abstract:

Fullerenes have attracted considerable attention for their possible use in human therapy. Pure C_{60} is soluble only in some organic solvents, but this could be overcome by chemical modifications. This review investigates the derivatization strategies and biological applications of fullerene C_{60} by using polar "active" molecules as sugars and amino acids/peptides that allow the increase of solubility in water.

The effect of glycosylation on biological activity of fullerene can be divided in indirect and direct action. The "indirect action" of sugars correlates with their ability to make fullerene soluble in water but glycosylation can be also exploited for the target delivery; accordingly, glyco-derivatives of fullerenes have been investigated in PDT (photodynamic therapy), as inhibitors of in HIV-1 protease inhibition or against neurodegenerative diseases.

The "direct action" involves fullerenes conjugated with sugars having a defined therapeutic role and the "multivalency" is the properties that ensure a good biological activity of glycofullerene derivatives. Increasing the sugars attached to fullerene intensifies the multivalency needed to efficiently use these glycosylated nanoparticles as potential ligands for receptors and enzymes that mediate the infection of viruses and bacteria (e.g. *E. Coli*, Ebola or Dengue viruses) for the infection.

Also, amino acids-derivatives of fullerenes have been studied as anti-infective agents (against viruses such as cytomegalovirus and HIV), thanks to their immunological properties; derivatives as fullerenol or by linking tuftsin on a C_{60} core could be exploited as immunogenic nano-carriers. Alternatively fullerene conjugated with amino acids or peptides is investigated

in the treatments of pathologies that request new approaches (Alzheimer, cancer, mixed connective tissue disease, lupus).

Keywords: Fullerene; C_{60}; Carbohydrate, Peptide, Synthesis, Biological Application

Contents:

1 Introduction

In 1985 was discovered C_{60} Fullerene, a new carbon allotrope defined as a soccer ball shape pure carbon structure. This new material due to its size and composition has attracted attention of scientist belonging to different fields and homologous with higher number of carbons were also discovered.

Fullerenes are generally represented by a formula Cn, where n is the number of carbons that makes up the cage but until 1990 fullerene structure remained a matter of theory because the spectroscopy analysis was the only method to detect it. After some years also experimental studies confirmed this structure (by using analytical instruments as DCC, mass spectrometry and NMR)[1].

Since C_{60} is the most representative and the most studied compound within the fullerene family, in this review the word fullerene is always attributed to C_{60}. This is the smallest stable fullerene, has high symmetry, as confirmed by ^{13}C-NMR analysis and studies performed on

crystalline C_{60}[2]. The diameter is 10.34 Å. There are two different types of bonds: the 5,6 bond that is a single bond of 1,45 Å length between pentagon and hexagon; the 6,6 that is a double bond of 1,38 Å between two hexagons that correlates with the fullerene reactivity (Figure 1).

Solubility is an important parameter to evaluate fullerene for possible chemical transformations and reactions. Ruoff R.S. et al. [3] checked the solubility of C_{60} in different solvents: it is completely insoluble in polar solvents, sparingly soluble in alkanes, appreciably soluble in aromatic solvents.

These data were also confirmed by computational studies[4]. Thus, pure C_{60} is highly hydrophobic and soluble only in organic solvents, in particular in aromatic ones. This is incompatible with a possible pharmacological application. Moreover, the lack of solubility in aqueous solvent leads to the consequent formation of aggregates when C_{60} is used in water.

This problem can be solved with chemical or supramolecular approach: by using cyclodextrins, by co-solving C_{60} with polyvinylpyrrolidone (PVP) in chloroform or by using artificial lipids membrane[5,6].

However, the most common method regards modifications of surface by addition of molecules that change the physical and chemical property of the fullerene. Depending on the choose of the attached compounds, the derivatization increases the solubility in aqueous solvent and reduces aggregation opening to a possible application in medicinal chemistry.

Even if fullerene was discovered more than thirty years ago, it is still considered a “new material” because chemical modification and the introduction of fullerene derivatives in biomedical application is recent[7–9]. Consequently, the toxicity is one of the characteristics that are still under investigation. It seems that the toxicity decreases with increasing of functionalization of fullerene[10]. The pure C_{60} toxicity is due to three factors[11]:

- Its dominant apolar character that collate with its ability to penetrate and merge with biological membrane.
- The propensity to form aggregates that can have an impact mainly on the environment [12,13].
- The capacity of reacting with a lot of biological relevant compounds.

Accordingly, Colvin et al. hypothesized that toxicity decreases as much as fullerene derivatives are soluble in water [10], as confirmed by other studies [14–16].

Actually the toxicity depends on several features as dose and the method of administration but also the size of fullerene (C_{60}, C_{70} etc..) and the compounds used for its derivatization, [17]. So the crucial aspect is conducting a specific toxicity assay on the final product of interest[18,19].

2 Chemical modification

The geometry, the high symmetry and shape of fullerene are at the base of its reactivity. Its particular shape forces carbons to have no planarity changing the hybridation of sp2 orbitals [20]; this leads to a sort of deactivation of the aromatic nature inducing a different reactivity of fullerene with respect to a normal aromatic compounds. Thus, It's better to use the term "pseudo-aromaticity" for fullerene that is considered an electronegative molecule, proper for addition at 6,6 bond and redox reactions.

The addition can be obtained in different positions: 1,2 position (for no bulky reagents) and 1,4 position (principally for bulky reagents because of their steric hindrance)[21]. (Figure 1)

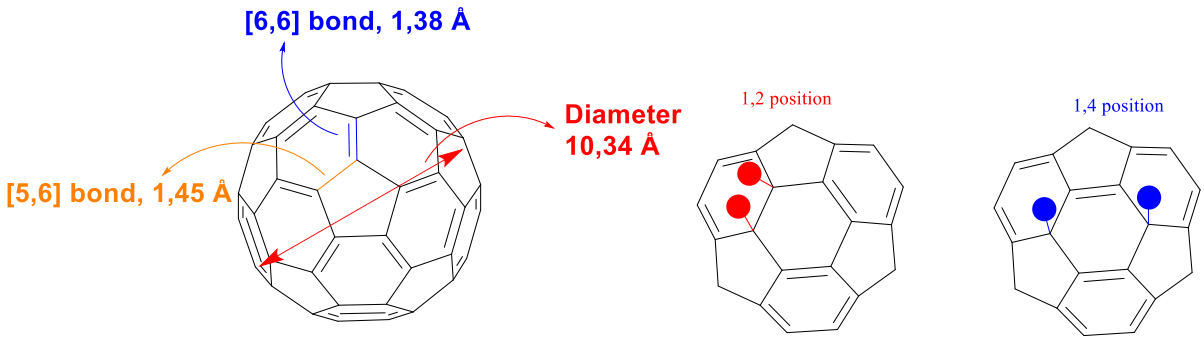
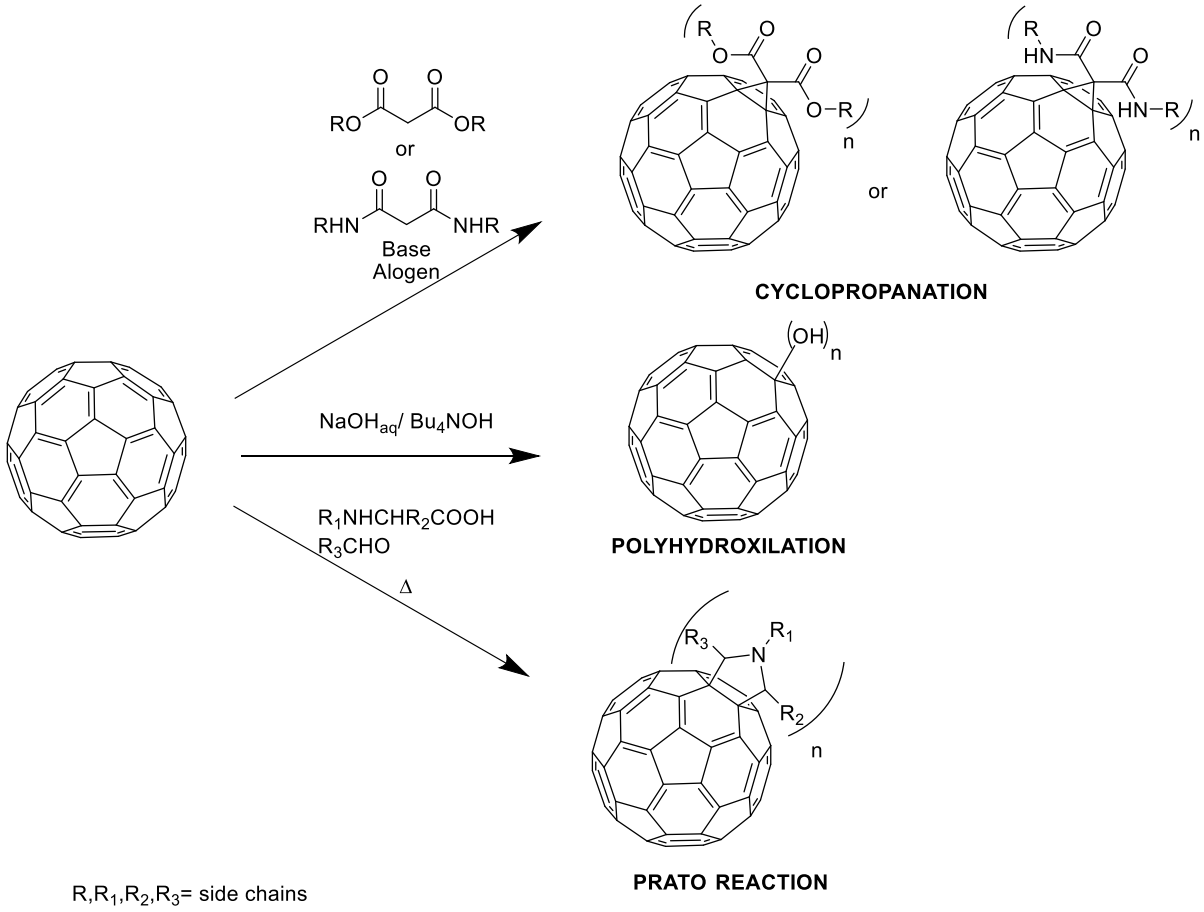


FIGURE 1: Bond lengths and C_{60} reactivity positions

2.1 Main strategies of conjugation

Different strategies have been explored to make fullerene suitable for pharmaceutical application. In order to make fullerene soluble in water and aqueous solvents, a lot of different functional group have been used (OH,-NH_2, -COOR). There are three main synthetic approaches to obtain these derivatives (Scheme 1): the cyclopropanation, polyhydroxylation and cycloaddition azomethine ylides (known also as Prato reaction)[22].



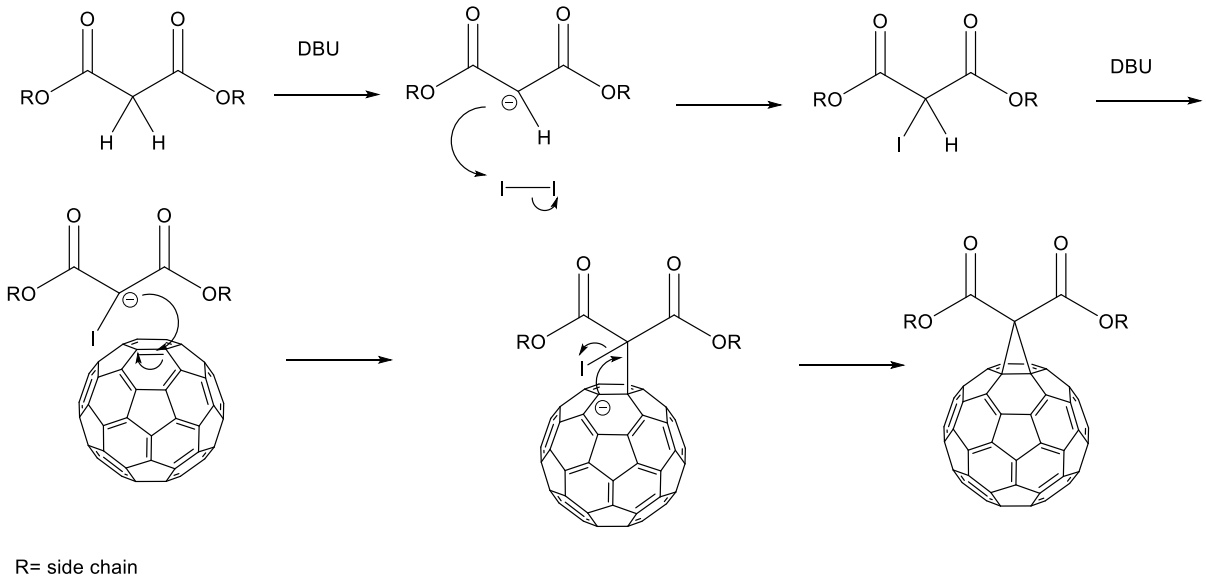
SCHEME 1: Main synthetic approaches

The cyclopropanation strategy is the most used. It can be obtained by different methods as the addition of frees carbenes, the terminal addition of diazo compounds and the nucleophilic cyclopropanation with addition-elimination mechanism. The last one is the most common strategy (best yields) and it's also known as Bingel reaction[23] discovered in 1993. Bingel described a kind of Michael reaction between an α-halo carbanion and fullerene followed by intramolecular substitution of halogen in order to form a methanofullerene[24]. Initially NaH was used as base for the reaction, afterwards also other bases were employed (pyridine, triethylamine and LDA) [25].

In order to improve the yields, some other strategies were tested, such as a one-step reaction, performed using α-halomalonates generated *in situ* (in presence of I_2 or CBr_4) and DBU (1,8-diazabicyclo[5.4.0]undec- 7-ene) as base[26–29]. The use of I_2 is preferable in mono-addition

(maximum three groups conjugation), otherwise tetrabromomethane can be used for higher adducts[30].

The mechanism is always the same: the base removes an acid proton from malonate forming a carbanion which reacts with the halogen building an α-halomalonate. Again, the base removes the other malonate acid proton and the new carbanion attacks the electron deficient double bond of fullerene. The carbanion just formed on fullerene removes the halogen by intramolecular nucleophilic substitution (Scheme 2).



SCHEME 2: Bingel reaction mechanism

The different strategies proposed for fullerene derivatization were considered for developing different products. For example by using Prato reaction peptides can be introduced on fullerene: Aroua S. et al., starting from Bis-*t*-butyl ester, prepared a scaffold for obtaining water-soluble fullerene derivatives such as peptide or PEG conjugates[31].

Otherwise by using cyclopropanation it is possible to functionalize fullerene with two different groups in order to derivatize it with two different molecules: one group can be propargyl, therefore suitable for reacting with azido group by click chemistry; the other one is a thiol maleimide that can be used to link also amino acids or peptides *via* Michael addiction [32].

There are also other functionalizing methods based on some specific characteristics of fullerene. They include radical additions, metal complex formations, oxidations and reactions with electrophiles[33].

2.2 Conjugation of carbohydrates

Conjugation of fullerene with carbohydrates for biological application has been already investigated[34]. The main way to link sugars with C_{60} is through a multiple cyclopropanation: six malonate ester chains having azido terminal groups are bonded in one step on fullerene by Bingel reaction. Now it is easy to link carbohydrates (mainly monosaccharides) by click chemistry, once sugar has a terminal propargyl group. It works also vice versa, with propargyl on fullerene chain and azide on the carbohydrate[35].

However, the CuAc (Cu(I) alkyne–azide cycloaddition reaction) (Figure 2**A**) is not the only way to link sugars once malonate chain is linked to C_{60}. Ramos-Soriano et al.[36] designed a cyclooctyne fullerene hexakis adducts suitable for cupper free click chemistry (SPAAC)[37] (Figure 2**B**). Otherwise, through cyclopropanation is possible to link just one malonate ester chain already presenting the carbohydrates [38] (Figure 2**C**).

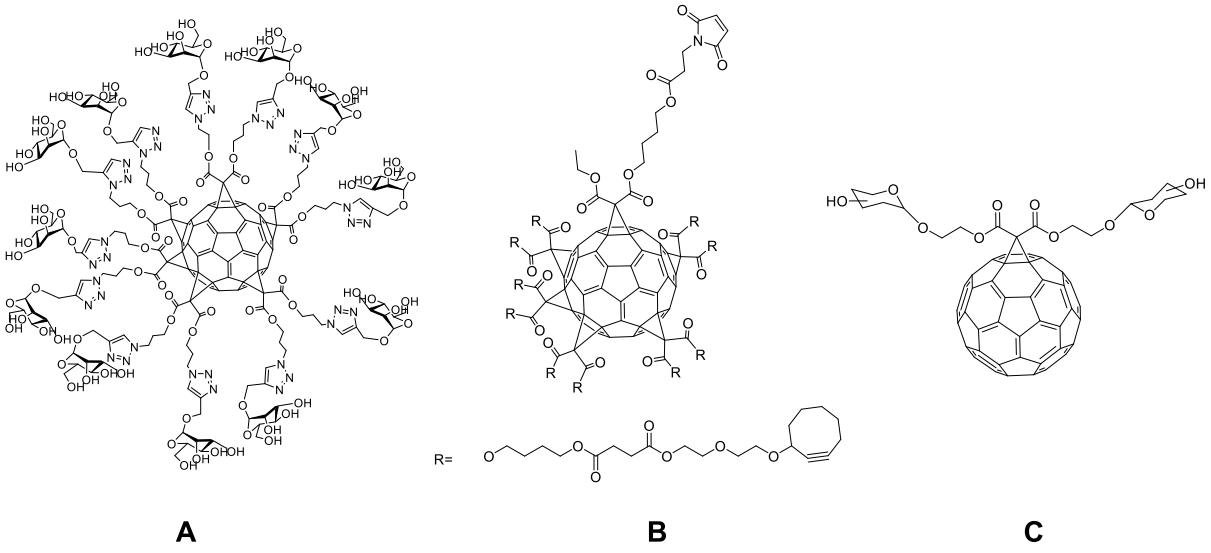


FIGURE 2: Sugar conjugation *via* malonate: A,B) Multiple Bingel cyclopropanation ; C) Single cyclopropanation

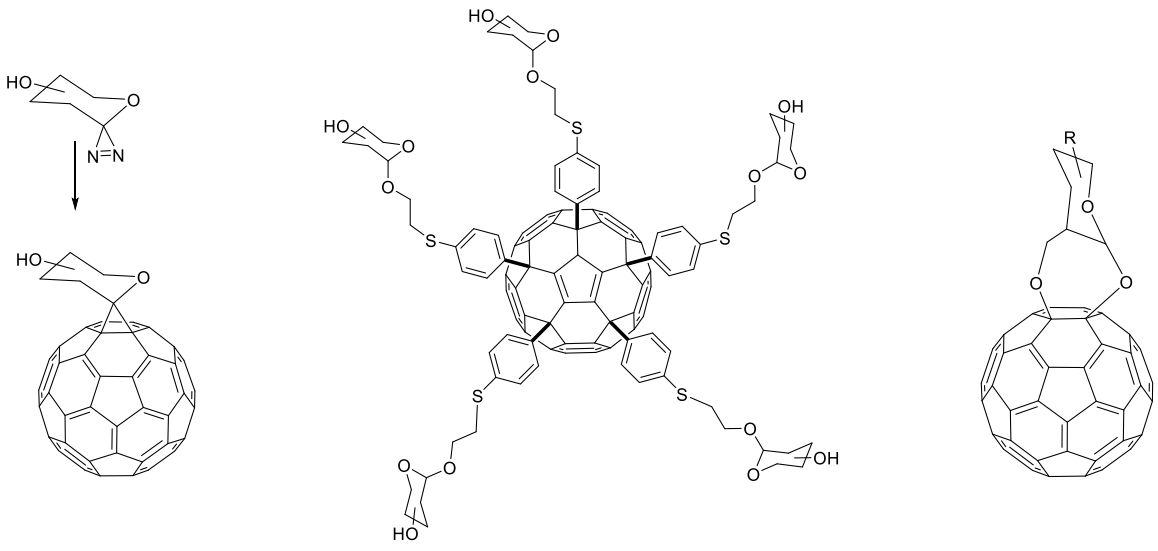
According to literature there are also other approaches to conjugate sugars (Figure 3). The first is the cyclopropanation mediated by carbene addition (Figure 3**A**). In this case the

reaction between C_{60} and glycosylidene carbenes, derived from diazirine, leads to spiro-linked C-glycosides with good yields. The reaction is enantioselective[39–41].

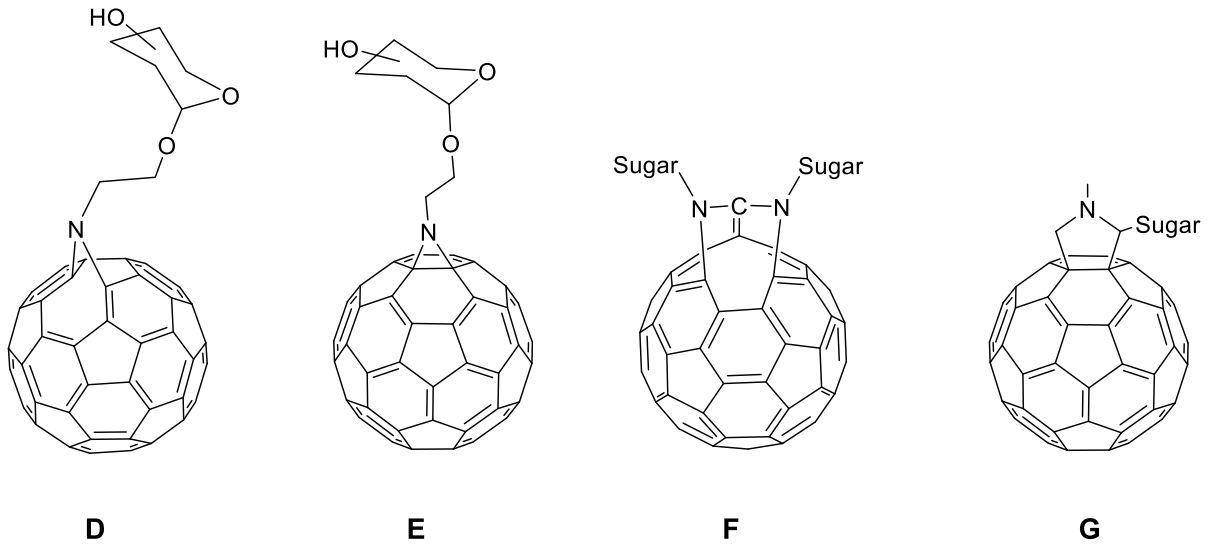
There is also a method suitable for linking in one-step five carbohydrates, by using a thiolate/alkyl halide coupling reaction (Figure 3**B**): in the first step the pentathiol fullerene is prepared, followed by nucleophilic substitution reaction of thiolate with bromoalkyl glycosides. The aqueous solvent, in which these reactions take place, eliminates the protecting groups and accelerates the reaction[42]. A particular method uses iron salt and oxidant to link directly the sugar through two hydroxy groups (Figure 3**C**) [43].

The sugar conjugation can also occur between an anomeric alkyl-azido group and the double bond of fullerene *via* 1,3-dipolar cycloaddition[44]: for the monoadducts obtained theoretically there are four possible isomers, but the most common are 5,6-open[45] (Figure 3**D**) (adduct preferred by alkyl azide) and 6,6-close (Figure 3**E**) (preferred by acyl azide). It is also possible obtain bis-adduct involving the all-carbon framework of fullerene[46] (Figure 3**F**).

Another conjugation reaction involves a mixture of sarcosine, sugar aldehydes and C_{60} in reflux[47]. 1,3-dipolar sugar azomethine ylide intermediate is formed as firs step and after it is linked to fullerene (attacks 6,6-ring junction) forming a fulleropyrrolidine glycoconjugate (Figure 3**G**).



A **B** **C**



D **E** **F** **G**

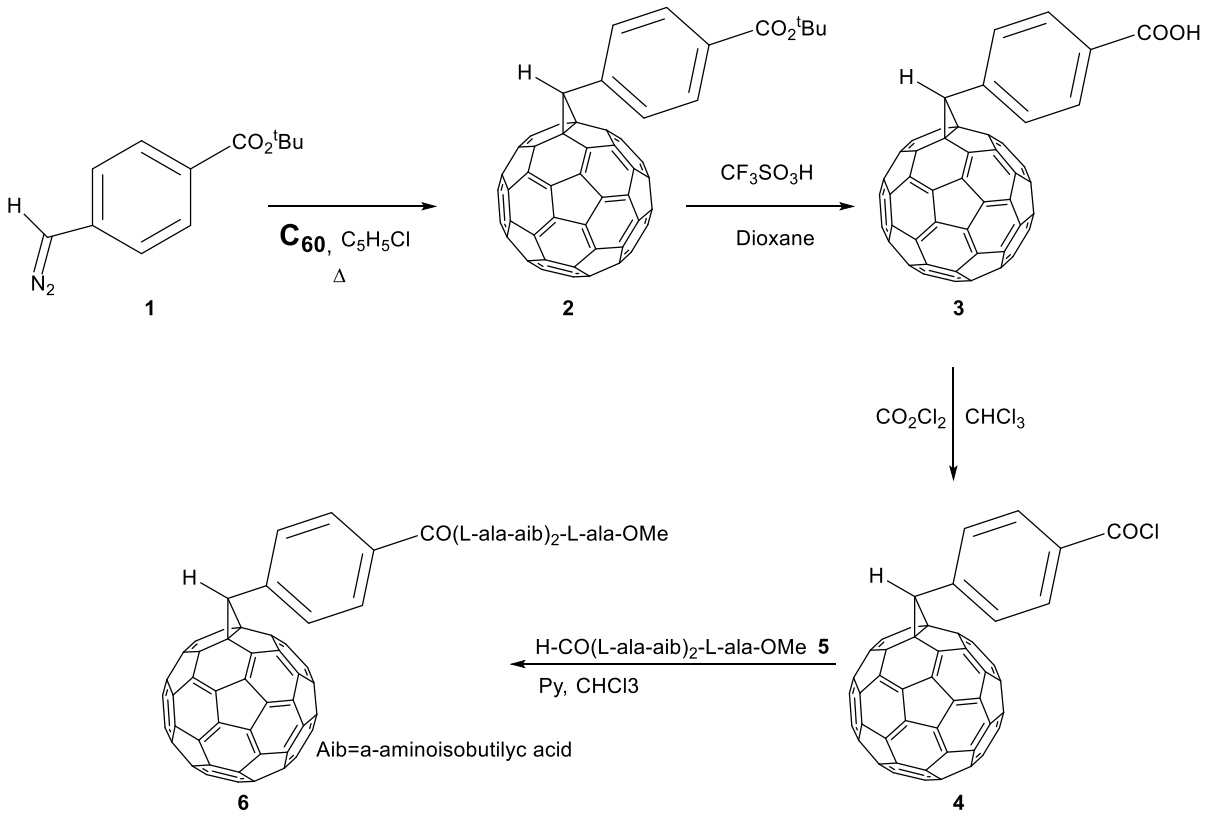
FIGURE 3: Sugar conjugation: A) Carbene addition ; B) thiolate/alkyl halide coupling reaction C) Iron salt and oxidant reaction; D,E,F) *via* Azide; G)sarcosine, sugar aldheydes and C_{60} reaction.

Selecting the appropriate approach, different kinds of sugars can be linked to fullerene, for example cyclodextrins[48] or carbohydrates (as mannose)[49].

2.3 Preparation amino acids and peptide-fullerenes conjugates

Amino acids and peptides are another class of compounds with biological interest that once linked on fullerene could be exploited for several pharmaceutical applications. The preparation of this type of fullerene derivatives could be classified depending on the chemical reactions used for the coupling: cycloaddition, nucleophilic addition and radical reaction[50,51].

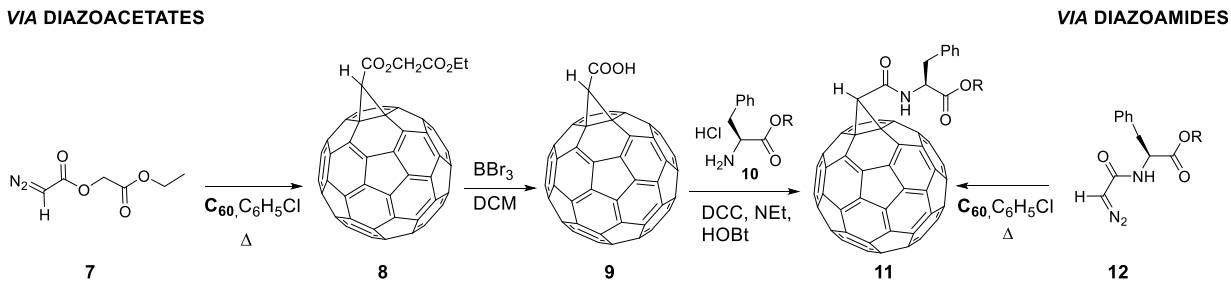
For the synthesis of the first fullerene-peptide conjugate [52] in 1993, it was employed a [2+1] cycloaddition reaction[53] by using a diazomethane derivative **1** that once deprotected to a carboxylic acid **3** was coupled to a pentapeptide to form the first fulleryl derivative **6** (Scheme 3).



SCHEME 3: First peptide conjugation

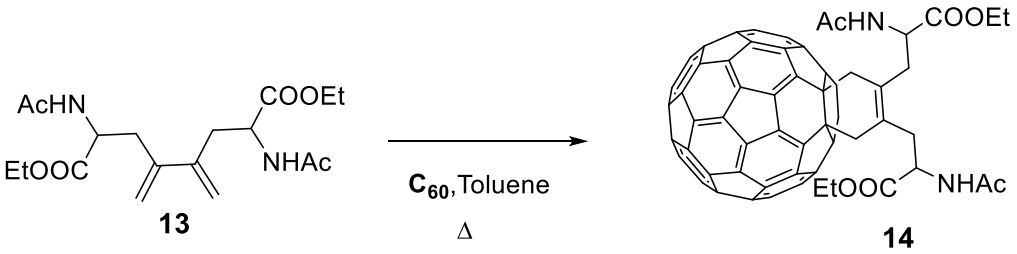
Then more efficient attempts followed, by using directly diazoacetates and diazoamides to obtain peptide-fullerenes derivatives by reducing the number of steps[54,55].

Diazoacetate **7** once linked on C_{60} (**8**) is deprotected to carboxylic acid **9** and then coupled to amino acids by using standard condition (DCC and TEA). Instead by using a diazoamide **12** is enough a single step, a direct addition, to obtain compound **11** (Scheme 4).



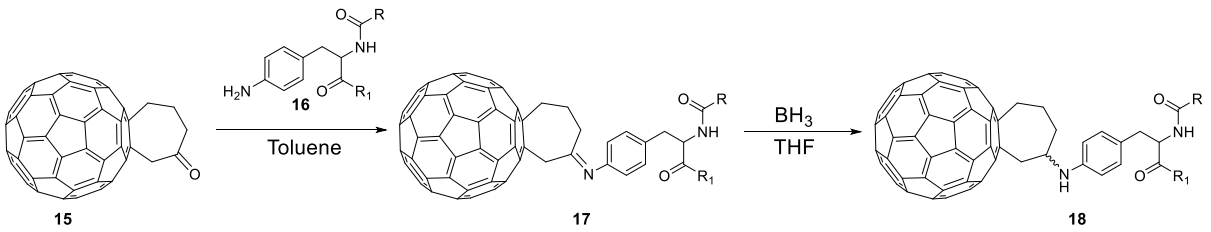
SCHEME 4: [2+1] cycloaddition by diazoacetates and diazoamides

Another cycloaddition reaction largely employed for the preparation of these conjugated compounds is the Diels-Alder ([4+2] cycloaddition). A functionalized diene **13** reacts with the electron-deficient double bond presents on C_{60} to provide compound **14**. Different diene derivatives were used in order to synthesized fullerene derivatives with amino acids or peptides [56–58] (Scheme 5).



SCHEME 5: Dies-Alder reaction for preparation of peptide-fullerene derivative

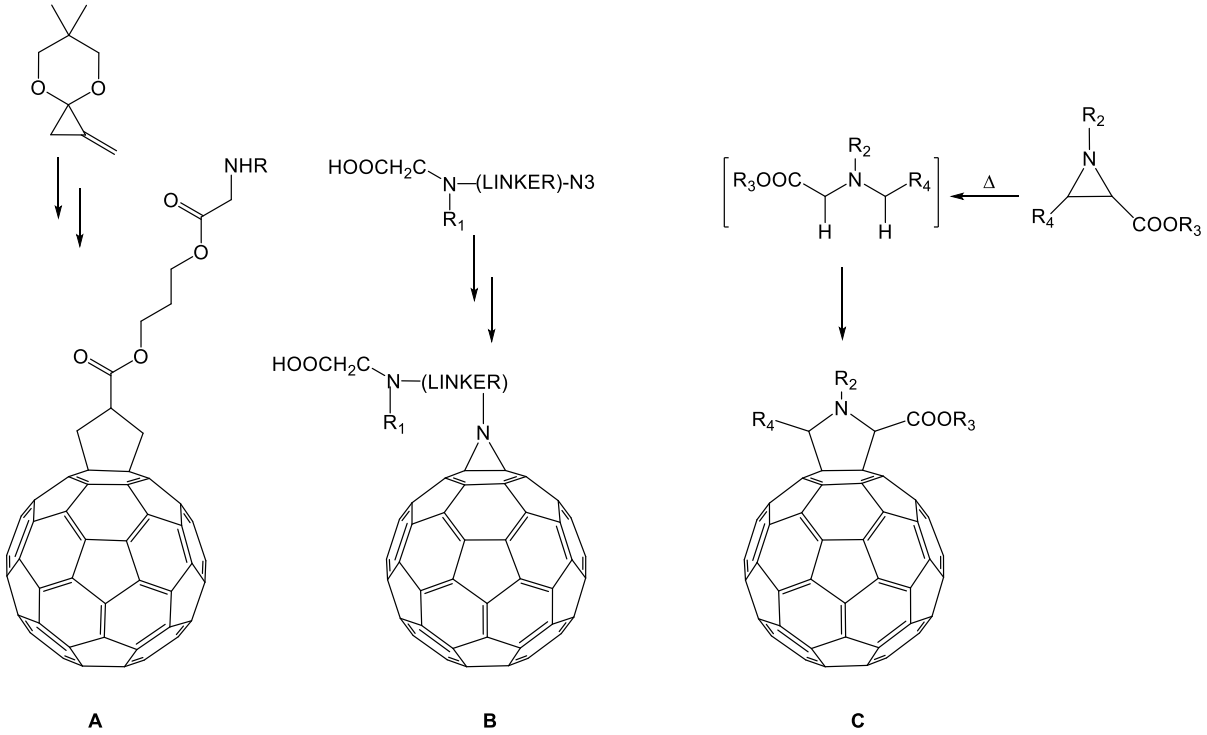
Yang et al. reported the synthesis of the 1,2-(4'-oxocyclohexano) fullerene **15** via Diels-Alder reaction that was used for linking amino acids such as phenylalanine **16** or lysine analogues[59,60]. For example, the 4-amino phenylalanine derivatives **16** forms the imine **17** that is then reduced to obtain a carbon-nitrogen stable bond (the amines **18**).



SCHEME 6: Preparation of phenylalanine derivatives

Also the [3+2] cycloaddition (1,3 dipolar cycloaddition) is useful for the preparation of fullerene-conjugated compounds obtained by linking amino acids or peptides. Compounds with different structures can be obtained depending on the reaction involved (Scheme 7). The reagent could be a methylenecyclopropanone ketal to form fulleryl amino ester derivatives after hydrolysis of the ketene acetal and a DCC-coupled esterification[61] (Scheme 7**A**). Alternatively, organic azides (the linker group) can be used to yield fulleroaziridines derivatives [62–64] (Scheme 7**B**); finally, azomethine ylides (via Prato reaction)[65–67] can be used for the synthesis of fullerenoprolines (fpr) and its derivatives[68] (Scheme 7**C**) and

other peptide-fullerene derivates such as conjugated products presenting GABA [69], or porphyrin moieties[70], as well as proline-rich fullerene[71] and intermediates used in solid-phase synthesis of peptides [31,72].



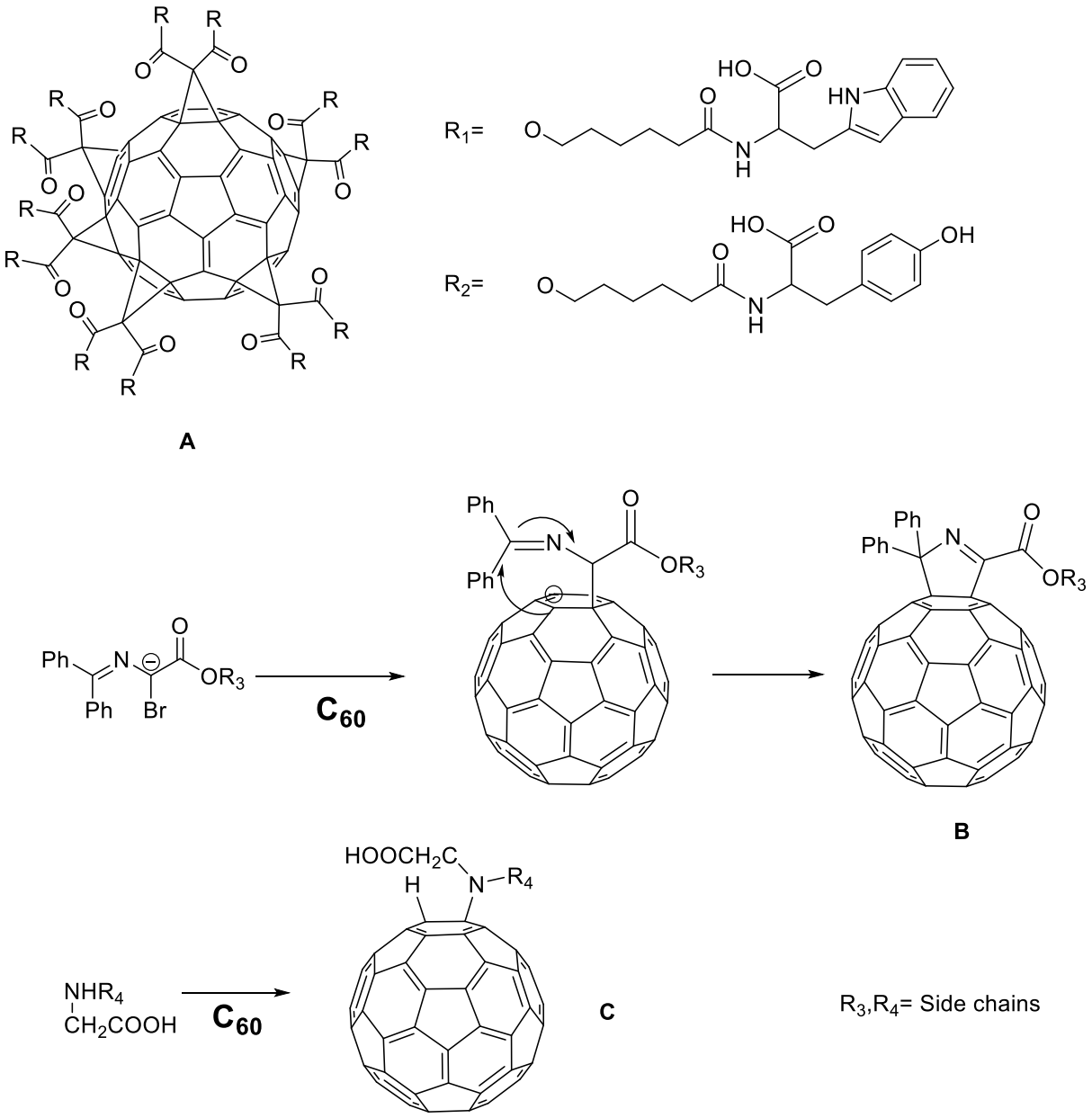
R,R_1,R_2,R_3,R_4= side chains

SCHEME 7: Different type of [3+2] cycloaddition

Cycloaddition is not the only way to obtain amino acids or peptides derivatives, indeed another approach involves nucleophilic addictions.Bingel belongs to this class of reactions, Ruiz-Santaquiteria et al.[73] exploited it to obtain a hexa-adduct fullerene containing tryptophane or tyrosine (Scheme 8**A**).

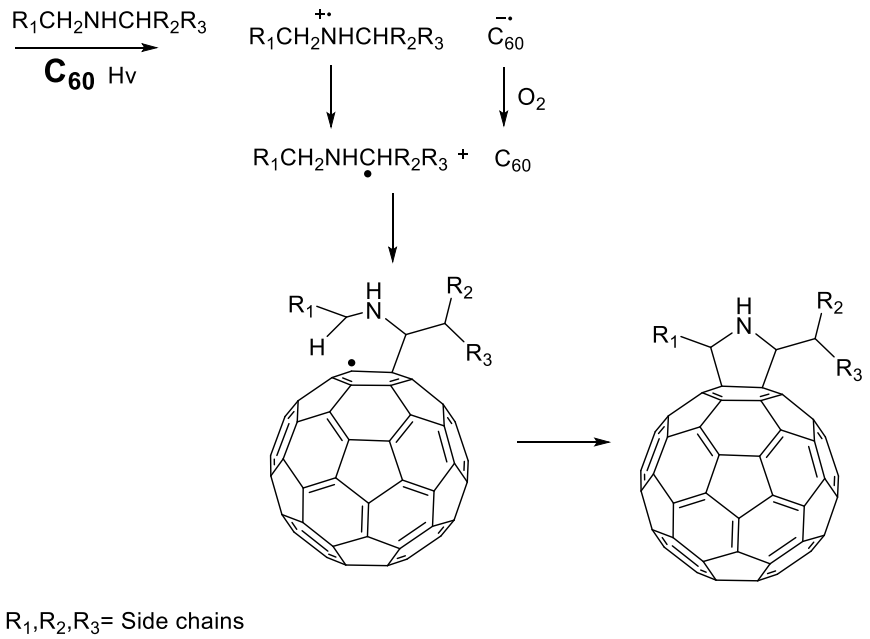
Instead, Burley et al.[74] and Ball et al.[75] have reported a different mechanism of this famous reaction, using *N*-diphenylmethyleneglycinate esters as reagents. Indeed the 60-fullerenyldihydropyrroles (Scheme 8**B**) are the products of these type of reaction.

Also, primary and secondary amines seem able to undergo nucleophilic addition with C_{60} double bond. Romanova et al. linked on fullerene amino groups belonging to different amino acids (Scheme 8**C**) by means of this approach [76,77].



SCHEME 8: Syntheses by nucleophilic addiction

Photolysis reaction is another method used for the synthesis of peptide-fullerenes derivatives [78]. The mechanism is reported in Scheme 9; the photoreaction starts with the formation of α-carbon radical that reacts with fullerene. Moreover, some reactions as [3+2] cycloaddition mediated by azide could be activated by photochemical or thermal stimuli[63].



SCHEME 9: Photoreaction for coupling amino acids and peptides with fullerene

Resuming, the strategies of functionalization of C_{60} reported in literature involve different part of the amino acid structure in the conjugation[79]. Thus, amino acids can be directly linked *via* the α-carbon as well as involving its amino or carboxyl group. In this context, fullerenyl amino acids conjugates, mainly synthesized by Diels-alder reactions or [3+2] cycloaddictions, were used in the peptide synthesis as a non-natural amino acid and therefore fullerene was introduced in a peptide sequence.

3 Terapeutic application of C_{60} derivatives

Functionalised derivatives of fullerene can be considered for biological application because of its therapeutic characteristics and physical and chemical properties that make fullerene derivatives perfect candidates as nanomaterial for drug delivery and as bioactive molecule for specific diseases. First of all it's interesting to evaluate the fullerene characteristics that make C_{60} effective for biological and therapeutic applications.

The mains properties that should be considered for drug delivery application are the following: size (it is considered a nanomaterial since its dimension is around 0.7-1 nm), high surface area that is also regular and reproducible, solubility in water when it is properly derivatized, reversible aggregation and possibility of covalent conjugation.

Moreover we can't forget also the chemical properties that make fullerene a molecule of relevant therapeutic prospective: C_{60} has the ability to be either a reactive oxygen species (ROS) generator and a radical scavenger depending on the presence or absence of light[80]. Under light exposition C_{60} is able to generate ROS by two pathways: *via* energy transfer reaction (from the triplet excited state of C_{60}) and *via* electron transfer reaction (from the C_{60} radical anion)[31]. Conversely in the dark, it has an exceptional capacity for radical scavenging, indeed it's also called "radical sponge"[81].
Such features make fullerene a very good candidate for photodynamic therapy (PTD)[82] , for antioxidant therapy or in diagnosis technics[83].

3.1 Therapeutic applications of sugar conjugated of C_{60}

Focusing on applications of glycoconjugated fullerene, we can discern two groups based on the role of sugars. There are applications where carbohydrates have an indirect action because they make fullerene water soluble as required for pharmacological applications and, in some cases, also mediate target delivery of fullerene (the active pharmaceutical agent).
On the other hand, there are applications where sugars have a biological role by mediating the interaction with specific therapeutical targets (Direct action).

3.1.1 Indirect action

As previously discussed, one of the main possible fullerene employments is in PDT. In this context, one important application of PDT is in cancer therapy. An improved selectivity of the sugar-fullerene derivatives towards cancer cells can lead a great efficiency and a low frequency of side effects.
Accordingly, Mikata et al.[84] in 2003 demonstrated that sugar-fullerene derivatives exhibit phototoxicity against HeLa cells (std cancer cells) as confirmed in 2010 by Otake et. al.[82].
The glycoconjugated derivatives of C_{60} (with one or more glucoses linked on fullerene) are more specific against cancer than healthy cells. So, these compounds showed a tumour-selective cytotoxicity both in vivo and vitro tests, that may be ascribed to an increased presence of GLUT (glucose transporters) in cancer cells.

Later, other scientists designed glycoconjugated derivatives that resulted even more active towards cancer cells. Yano et al.[85] discovered that the type of conjugation influences the activity, Glucose-C_{60} conjugate (**19**, Figure 4) exhibits higher toxicity than Glc-azafulleroid (**20**, Figure 4). Instead Serda et al.[86], based on the fact that the selectivity depends on the high presence of GLUT, designed a compound with six linked sugars, the hexakis-glucosamine C_{60} derivative **21** (sweet-C_{60}) in Figure 4, that showed high selectivity and strong cytotoxic activity once illuminated by light, above all in pancreatic stellate cells in which **21** is predominately accumulated. The same research group[87] in 2020 discovered also that these kind of glycofullerenes can be considered inhibitors of non-receptor tyrosine kinase (cytosolic enzymes able to regulate cell growth, proliferation, differentiation, adhesion, migration and apoptosis). Baranska et. al.[88] in 2021, always based on the selectivity due to high GLUT presence and the non-toxicity of these carbon carrier in absence of light, confirmed the potentiality of Sweet-C_{60} as vehicle for the treatment of pancreatic cancer.

The effect of fullerene glycosylation was also confirmed when Narumi et Al.[89] carried out a study showing that a glycoconjugated TEG-C_{60} (**22**, Figure 4, a fullerene conjugated with a malonamide chain bearing two glycosylated tetraethylene glycol, TEG) could be a promising approach to increase the photodynamic activity of C_{60}. Moreover, it seems that it acts in aqueous medium forming micelles by hydrophobic interaction between C_{60} units that exposes on the surface the glycosylated TEG chains (the portion responsible of the tumour-targeting and the biocompatibility).

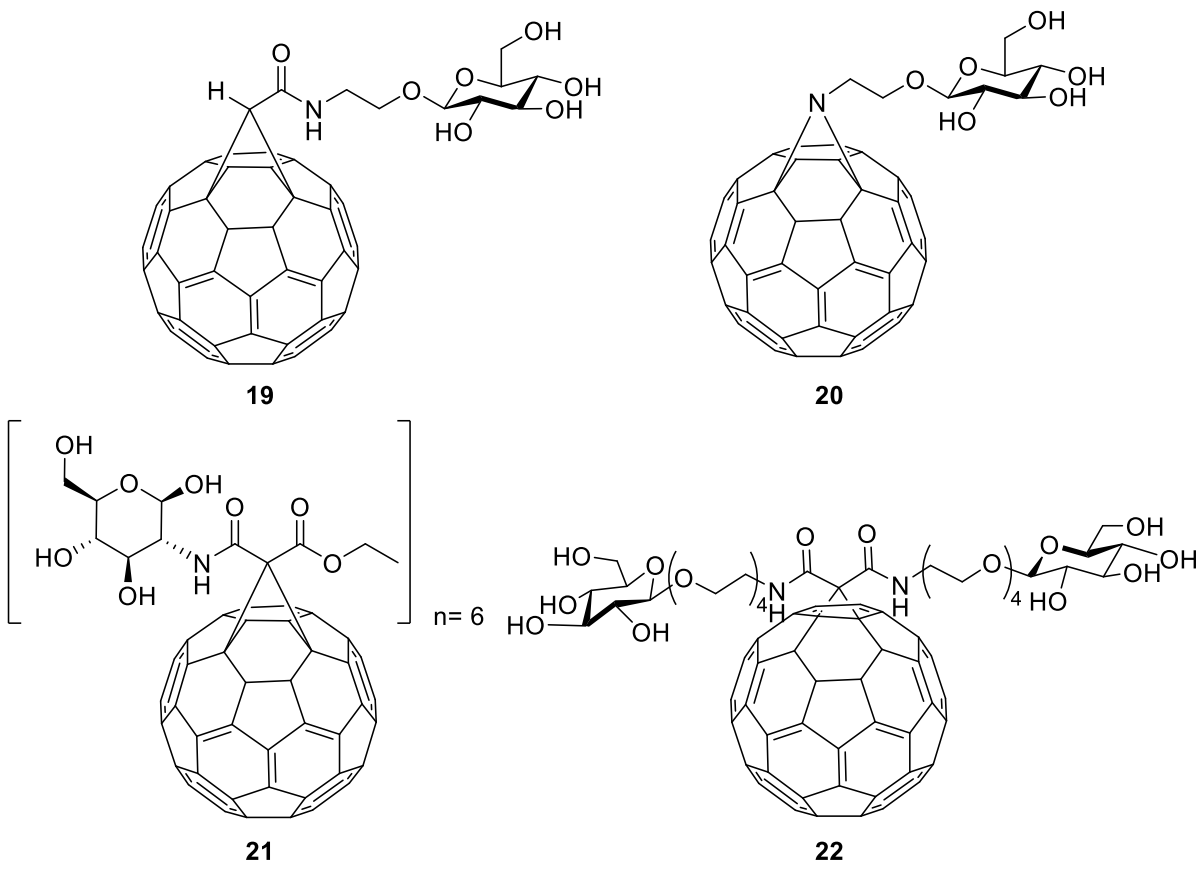


FIGURE 4: Compound **19** (Glc-pendant C_{60}) and Compound **20** (Glc-pendant azafulleroid) Compound **21** (hexakis-glucosamine C_{60} derivative), Compound **22** (Glc-TEG-C_{60})

About improving PDT activity of fullerene derivatives as photosensitiser, it has been shown that the use of specific oligosaccharides, such as cyclodextrins, helps this function in addition of making fullerene water soluble[90].

Considering the biological effect of fullerene under light irradiation, other possible applications may be envisaged. First of all, as antiviral agent as demonstrated by Zhu et al.[91] that investigated the potential use of C_{60} as anti-influenza virus agent (with mechanism still unknown), once conjugated with cyclodextrins (thanks to the increase in water solubility). In particular, two types of cyclodextrins were employed : α-CD, composed by six glucose subunits, and γ-CD by eight glucoses. It was observed that only γ-CD C_{60} conjugates gave antiviral activity hypnotizing that this could be due to some γ-CD specific properties (different from alpha type): a) it has higher water solubility than α-CD, making C_{60} even more hydrophilic and b) it allows less aggregation between C_{60} conjugates and therefore generates more singlet

oxygen [92]. Although the IC_{50} values of C_{60} conjugates with γ-CD are half compared with Oseltamivir (that was considered as positive control), they exhibit an enough anti-influenza virus activity to consider this class of compounds for further optimizations.

Another antiviral application concerned fullerene alone or linked with some amino acids is the inhibition of HIV-1 protease by photoirradiation[44,93], but when the conjugation is with sugars a better activity is obtained. Because of the simultaneous presence of a hydrophobic centre and hydrophilic chains, the C_{60}-glycoconjugate interaction with the HIV enzyme (HIV-1 protease) is reinforced by different hydrogen bonds.

HIV-1 protease is an important target since it is essential for the replication of the virus. Tanimoto et al.[94,95] discovered that fullerene could degrade HIV-1 protease under photo irradiation, thanks to the production of $^1O_2$. Indeed, the cleavage of this enzyme protein backbone is carried out by ROS or radical species.

The use of sugar-fullerene derivatives improves this effect: 1) sugars increase water solubility and 2) it was demonstrated that fullerene-sugar hybrid compounds such as **23** (Figure 5) have high affinity for this enzyme and its inhibitory activity increased of 6.7 fold under light irradiation.

The same research groups evaluated the activity of conjugate **24** (Figure 5) against $Aβ_{42}$ peptide (amyloid β) that is involved in Alzheimer disease (AD)[85,96]. This molecule has affinity with the amyloid peptide and is able to degraded it, always by photo-irradiation and the production of ROS species. Also, oligomers of the $Aβ_{42}$ peptide can be degraded under long-wavelength UV radiation, without any additives (such as metals or reducing agents as norally used).

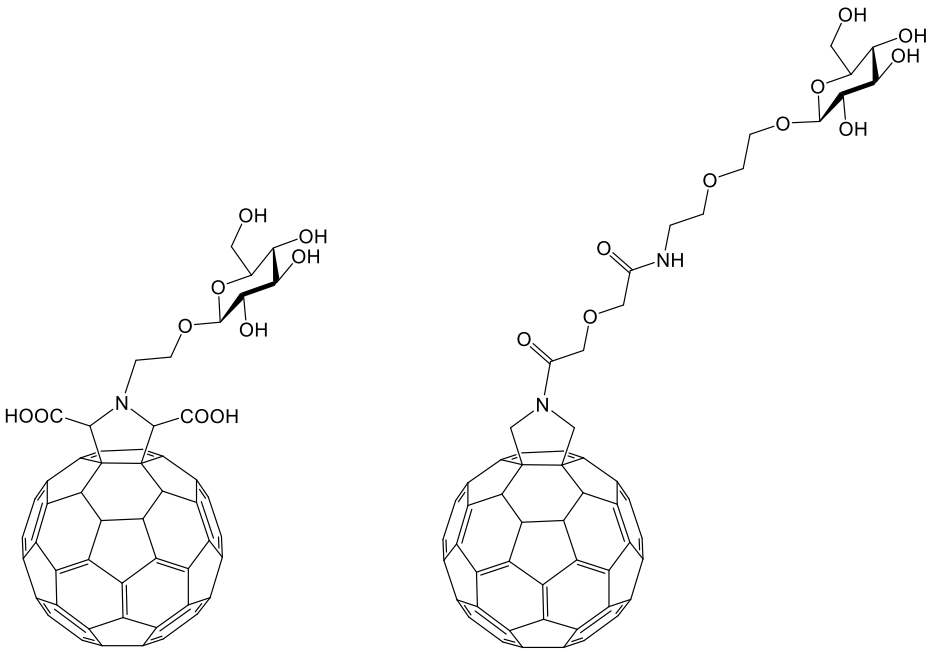


FIGURE 5: Compound **23** (used against HIV-1 protease) and Compound **24** (used against amyloid β)

Once in the dark, fullerene photosensitizer changes its properties and becomes an antioxidant agent. Always talking about neurodegenerative diseases, antioxidants could be used in some pathologies where the triggering effect is the oxidative stress. Horie et al.[97] measured the capability of two type of glycoconjugated fullerene derivatives (the first with one sugar-chain and the other with two sugar-chains) to inhibit the free radicals coming from lipid peroxidation remarking that these compounds act as radical scavenger with an antioxidant activity comparable to phenols.

Moreover in order to increase this activity, Kop et al.[98] suggested to formulate these polysaccharides-fullerene products with hydrophobic materials (such as cholesterol) in order to form nanoparticles and observed that antioxidant activity increases improving the hydrophobicity of the formulation.

An unusual application always related to antioxidant properties of fullerene is in the treatment of shock. Shock is a syndrome characterized by an insufficient tissue perfusion. Since it seems that ROS is one of the causes involved in shock parthenogenesis, fullerene, used as a radical sponge, can reduce the irreversible cellular damage, but only the conjugation with polar molecules as sugars enables the use in-vivo[99].

A recent study[100] investigated fullerene, according to its good radical scavenger properties, in the treatment of particulate matter (PM) that induces skin diseases and pulmonary and cardiovascular disfunctions. Indeed, PM is involved in skin inflammation and in the activation of ROS. Hexa-adduct fullerenes presenting twelve sugars (to increase C_{60} water solubility) resulted protective against ROS productions and so valid candidates for PM therapy. It seems that all inflammatory consequences due to PM could be reduced by pre-treatment with this kind of glycofullerenes.

3.1.2 Direct action

We are used to thinking of a monovalent "lock and kay" type of binding between a biological target and its ligand, but this kind of interaction is typically weak when the ligand is an oligosaccharide.

For this reason, some kind of protein (for example lectins) benefits from multivalency for their biological activity. The multivalency, multiple epitopes that interact with multiple cells receptors, is a mode of molecular recognition involved in important biological processes mediated by oligosaccharides, above all in the cell-cell adhesion such as the interaction of host cells with pathogenic microorganisms[101]. The multivalent presentation of carbohydrates enhances the affinity and selectivity by an effect also known as " cluster glycoside effect"[102].

The main classes of receptors involved in this type of recognition are lectins. In the last years, since this category of protein is important for the adhesion of virus, bacteria and pathogens to host cells, specific molecules have been investigated to block the infection by inhibiting the sugar receptors interaction using multivalent compounds[103,104].

FimH (a bacterial adhesin belongs to *E. Coli*)[105], and PA-IL (a bacterial lectin from *Pseudomonas aeruginosa*)[35] were the first examples of lectins that were inhibited by this multivalent approach and above all by using dodecavalent-adducts of fullerene presenting 12 sugar units linked through six malonate chains (called also hexa-adduct with regards to number of linker chains).

Indeed, it has been demonstrated through assays such as SPR (surface plasmon resonance), ELLA (enzyme-linked lectin assay), HIA (hemagglutination inhibition assay) and ITC (isothermal titration calorimetry) that the affinity of these dodecavalent glycoconjugate of C_{60}

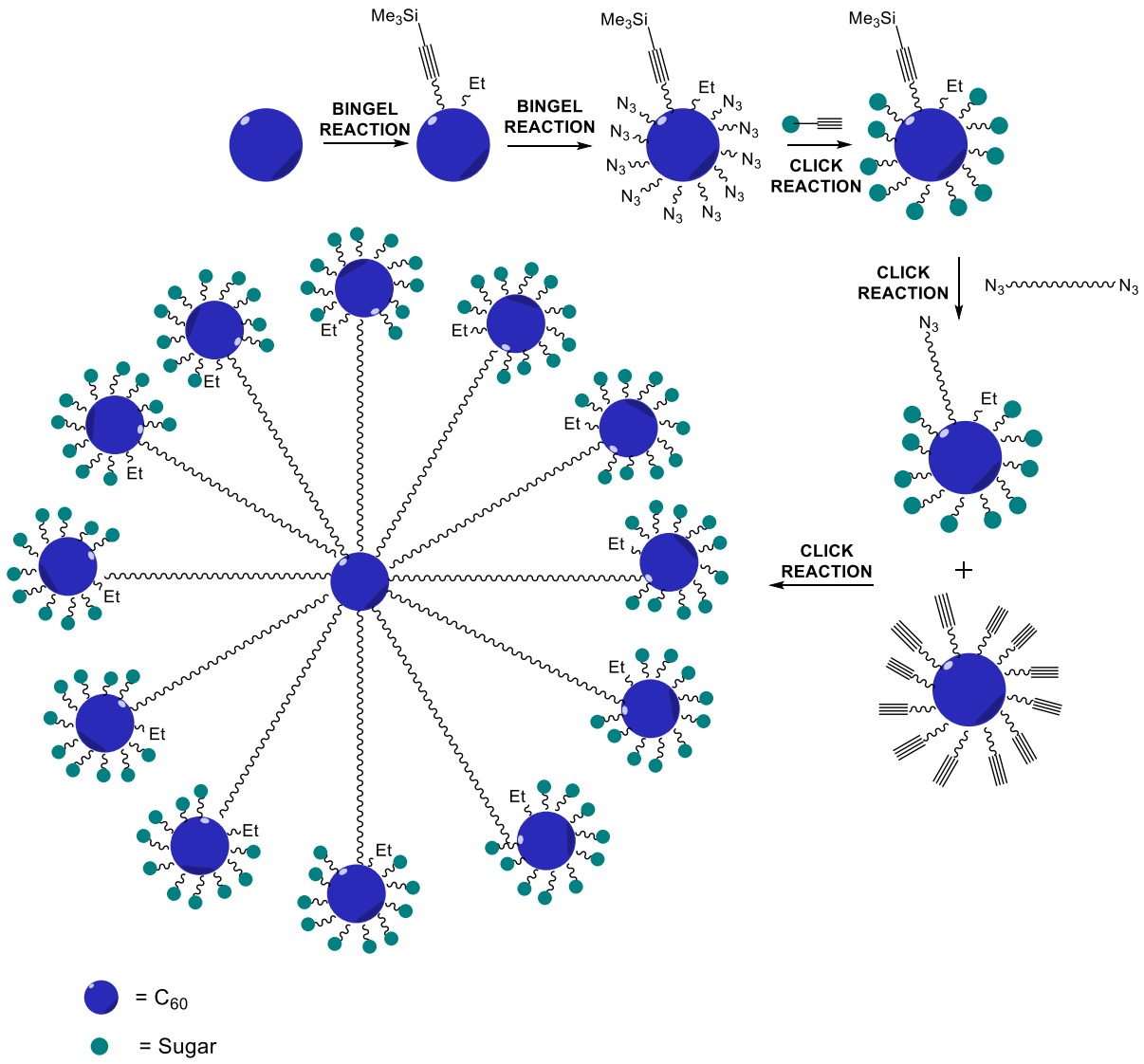
is 12000-fold higher compared to the free monosaccharides, and they can be considered as potential competitors in order to avoid the pathogen binding that leads to the beginning of the infection[106]. Further studies demonstrated that a good inhibition is promoted by a right spatial distribution of carbohydrates involved in the interaction with lectins [107].

Another group of lectins involved in cell-cell adhesion is DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin). Some viruses target DC-SIGN (a C-type lectin receptor) to enter to the host cell and start the infection, so antagonists of this receptor can stop the infection process.

One of the first applications was against Ebola virus. Luczkowiak et al. were the first to prove the efficiency of dodecavalent-adducts of fullerene (compound **25**, figure 6) presenting 12 mannoses linked on C_{60} through six malonate chains, as anti-Ebola agent, highlighting that compounds with same valency but longer spacers could be more active[108]. This was confirmed by Engstrom et al. [109] that demonstrated as a spacer extension (as for compound **26** figure 6) makes glycofullerene derivatives more dynamic and flexible, favouring the binding [110].

This work has certainly inspired subsequent research in the same topic. In 2015, Munoz et al.[111], found a methods to synthesize giant globular multivalent glycolfullerenes acting as potent infection-inhibitors against Ebola virus. First of all, it necessary to emphasize the great synthetic challenge to build these fullerene sugar balls. This research group was able to synthesize a fullerene derivative presenting 120 mannoses, consisting in fullerene-based dendritic structure (with C_{60} at the branching points as well as at the core) functionalized with carbohydrates. They assembled 12 sugar-coated fullerenes (each one with 10 carbohydrate groups) to a central fullerene scaffold in only one synthetic step based on the click chemistry (Scheme 10).

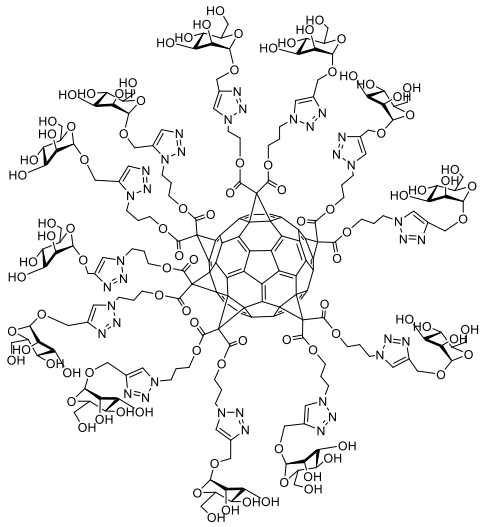
The combination of Bingel reaction and click chemistry resulted a successfully approach[112].



SCHEME 10: Synthetic steps for fullerene sugar ball (120 sugars)

These giant C_{60} sugar balls (such as compound **27**, Figure 6[111]), called tridecafullerenes, were tested as inhibitor of Ebola virus. They resulted very efficient with a IC_{50} in the sub-nanomolar concentration range (0,000667 µM).

The glycoconjugates obtained from C_{60} by linked only 12 or 36 sugars (compounds **25** and **26** in figure 6) were also investigated but showed lower activity compared with tridecafullerenes **27**. The first one (**25**) have a good inhibitor activity of 2 µM (micromolar range) meanwhile compound **26** reaches an IC_{50} of only 68 µM[108]; thus, the activity dramatically improves with compound **27** that expose 120 mannoses-units [113].



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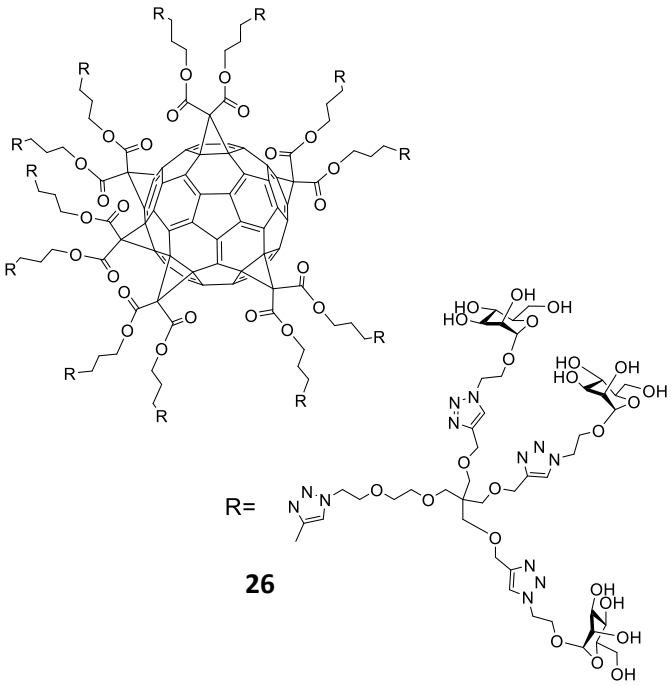

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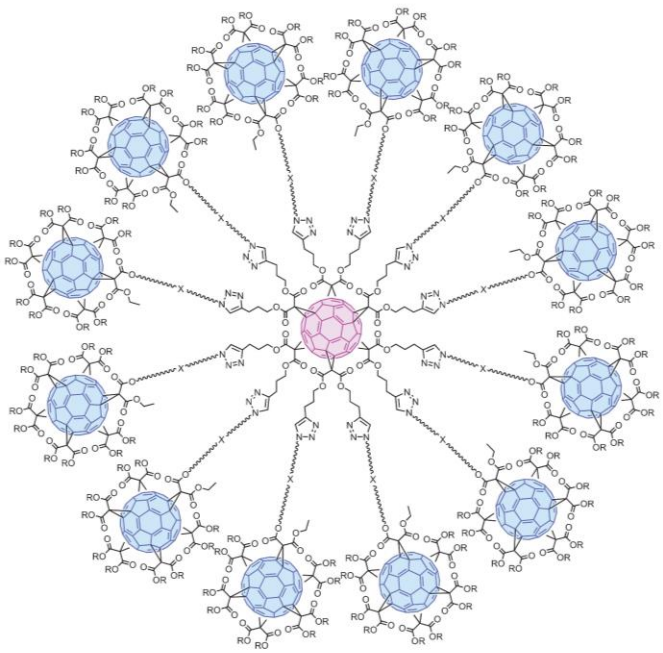

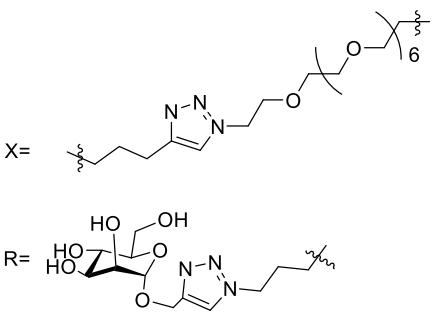

27

FIGURE 6: Hexa-adducts of C_{60} and its multivalent derivatives.

Other viruses also target the same lectin to start the infection. Ramos-Soriano et al.[114,115] further increased the number of sugars linked to a fullerene based scaffold through a new synthetic strategy, the SPAAC (Strain-promoted azide-alkyne cycloaddition) that avoids the

use of cupper catalyst, synthesized a fullerene ball of 360 disaccharides (**28**, in Figure 7). They chose man-α1,2-man disaccharides because they have higher affinity with DC-SIGN , 3-4-fold respect to monosaccharides. This compound was tested against ZIKV (Zika virus, a positive single-strand RNA virus transmitted by mosquitos) and DENV (Dengue virus) and resulted a very good inhibitor of the infection with IC_{50} in the picomolar range. Since there is not any therapy for ZIKV, this study opens a door on a new potential treatment of ZIKV based on inhibition of carbohydrate-protein binding.

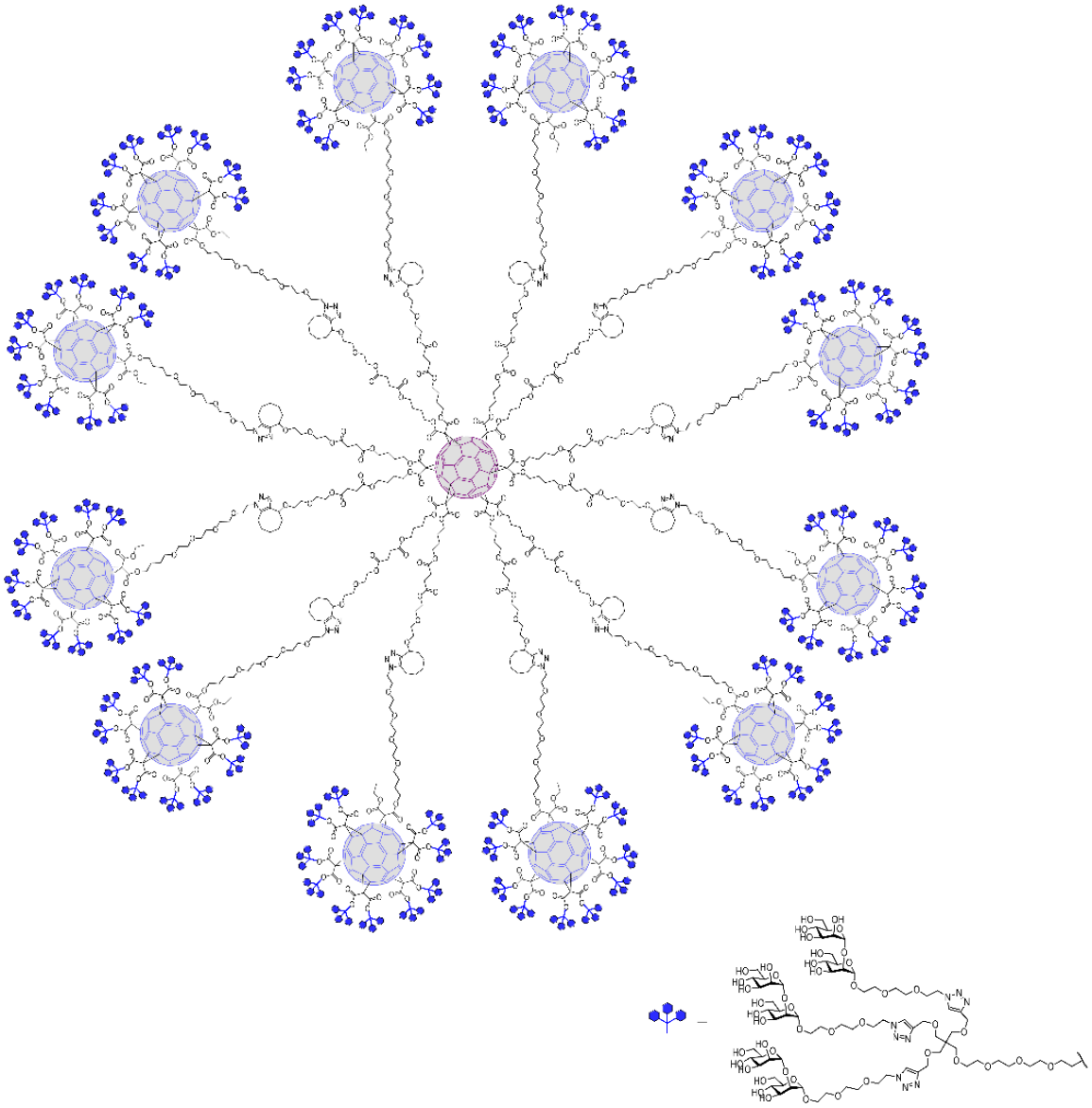

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FIGURE 7: Potential antiviral agent against ZIKV and DENV with 1,2 binding disaccharides having higher affinity with DC-SIGN[114]

Lectins usually show multiple carbohydrate-binding pockets able to establish multiple simultaneous interactions promoting cross-linking between host cell and pathogen. So, it was logical to think about the use of multivalent compounds as anti-viral or -bacterial agents.

On the contrary, it would not be intuitive to adopt the same approach for enzymes such as glycosidases and glycosyltransferases, which generally only bind one oligosaccharide substrate with high selectivity and affinity as they have a single substrate-binding site. However, contrary to what expected, even these important enzymes involved in viruses and bacteria pathogenesis, are subjected to a counterintuitive effect called "multivalent inhibition effect"[116].

Glycosidases are ubiquitous enzymes that cleave glycoside anomeric bonds in glycoconjugates and oligosaccharides. They have a main role in many biological processes including pathogenic processes. For this, its inhibitors could be attractive as anti-viral, anti-cancer and anti-HIV agents but also for the therapy of diabetes[117] and Alzheimer disease[118]. Again, multivalent (multiligand) compounds proved to be highly active.

Compain et al.[119] proved the inhibitory activity of a sugar-fullerene derivative linking twelve sugar units of 1-deoxynojirimycin (DNJ) by means of six malonate linkers. Thus, the fullerene conjugate **30a** (figure 8) showed an enhanced activity compared to DNJ alone (a well-known glycosidases inhibitor and inhitope for several enzymes of this class). This was the first result that introduced the relevance of multivalency also for inhibition of glycosidases. In 2013 Risquez-Cuadro et al.[120] further studied the mechanism of multivalency inhibition of glycosidases. This research group used as inhitope motifs a) 1-amino-5N,6O-oxomethylydenenojirimycin (1N-ONJ) and b) its C2 epimer 1-amino-5N,6O-oxomethylydenemannnojirimycin (1N-OMJ), two sp^2-iminosugars with ''mismatching'' (a) and ''matching''(b) structure relationships with α-mannosidase. In this study, through a lectin (PNA)/glycosidase competitive assays, it was investigated the mechanism of multivalency inhibition and the relevance of the non-glycone sites for this activity; a comparison of the effect induced by simple monovalent iminosugars **29a,b** and fullerene sp^2-iminosugar balls **30b**,**c,d** (figure 8) demonstrated that the matching monovalent sugar **29b** has a better inhibitory potency compared with the mismatching inhitope **29a**; but when these sugars are involved in the formation of multivalent compounds, they exhibit similar activity. Thus, inhitopes when used as single compounds are, as expected, not recognized by mismatching

glycosidases, because their structure is different from the substrate specific for these enzymes. However, mismatching sugar derivatives become active as inhibitors towards nonspecific enzymes when involved in multivalent compounds.

It seems that two different binding modes are involved for mono and multivalent inhibitor. For the first one, it is important the high affinity of the sugar moity with the binding site, in a typical key-lock model; instead, multivalent inhibitors activity seems also depend on interaction with non-glycone binding sites. Non-glycone binding sites with lectin-like abilities are probably implicated in this process and the presence of matching inhitopes can cooperate, but it is not fundamental for its inhibitor activity.

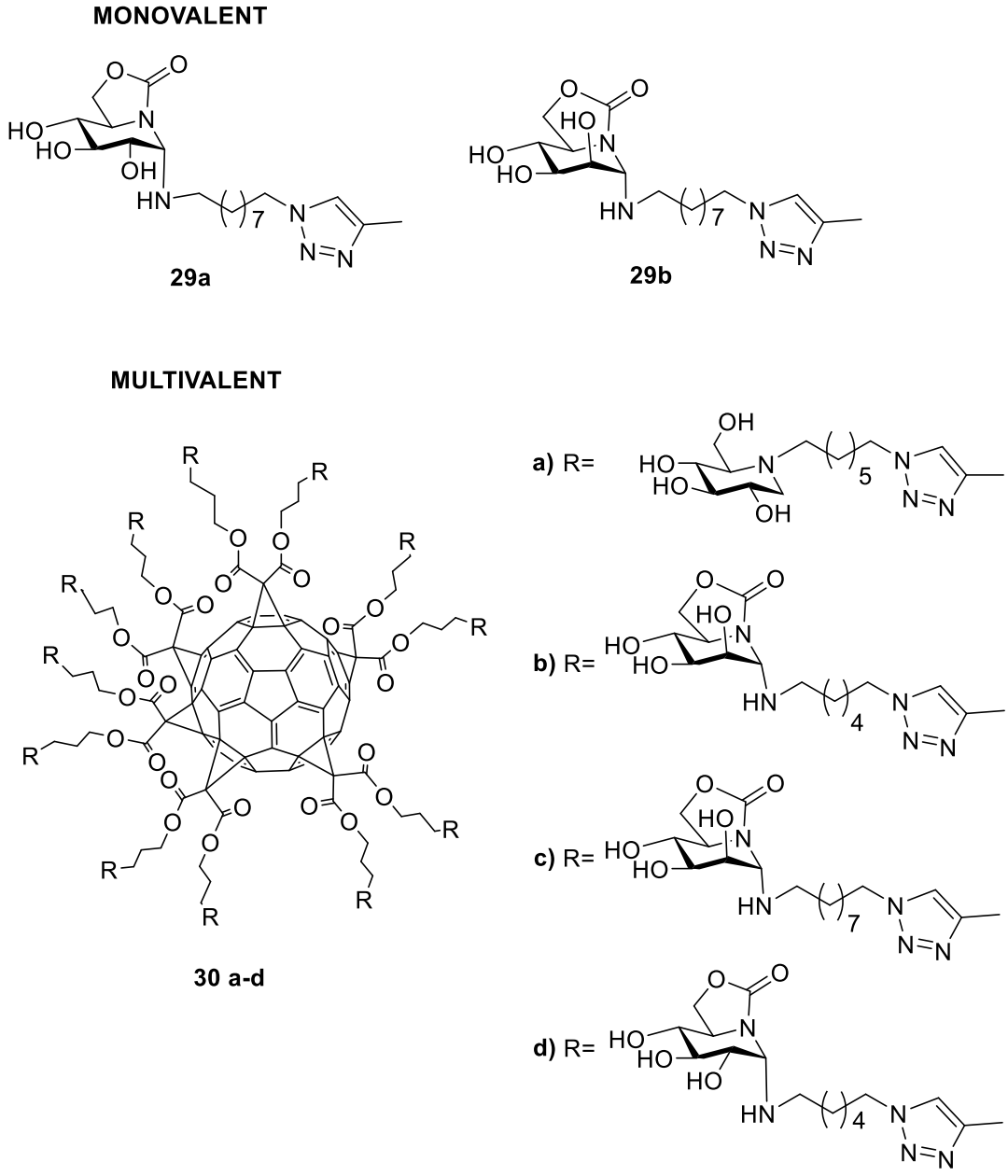


FIGURE 8: Monovalent and multivalent inhitopes of glycosidase

This hypothesis was confirmed by Flos et al.[27] that investigated this topic in detail, by preparing homovalent fullerene derivatives **31a-c** containing 12 identical sugars and heterovalent products **32a-c** linking 10 sugars and a single sp2-iminosugar inhitope moiety (figure 9) and evaluated their inhibitory potencies on 3 types of glycosidases: Jack bean α-mannosidase (with an open active site and easily accessible), yeast maltase and isomaltase (with a "narrow" active site) and β-galactosidase (having an "open-to-narrow flexible" site).

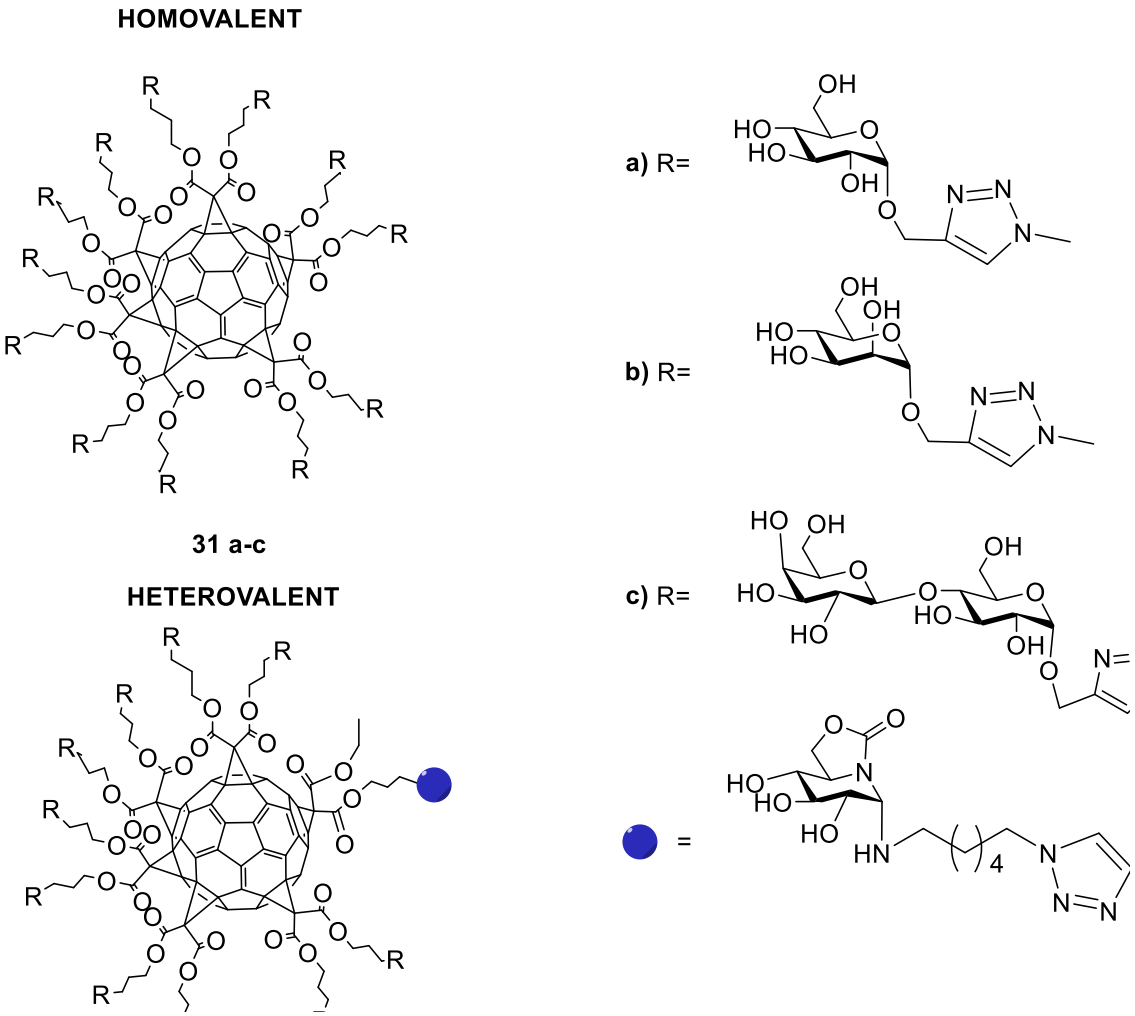


FIGURE 9: Homo and heterovalent fullerenes

In this study, it was confirmed that the multivalent carbohydrate fullerene conjugates (**31** and **32**) exhibit dual glycosidase and lectin binding properties. This is because non-glycone regions

of glycosidase with lectin-like behaviour are certainly involved in the inhibition, so if these sugar balls can interact with lectins they can also interact with these regions.

Moreover, homo and hetero-valent fullerenes show different mechanism but the different sugar moieties (hetero valency) in compounds **32a-c** do not affect their inhibitory activity.

As described above the glycosidases considered in this study have a different catalytic pocket topology and accessibility which affects the type and the potency of the inhibition. Its seems that compounds **31a-c** and **32a-c** are not able to inhibit α-mannosidase, maybe because this enzyme requires sugars having an affinity for the "open" active site; on the contrary, **31a-c** and **32a-c** are potent inhibitors for the other glycosidases acting as competitive inhibitors for enzymes with "narrow" active site and uncompetitive or mixed-type for β-galactosidase (Table1).

Subsequent studies focused on finding more effective inhibitors and, as for lectin antagonists, the increase of multivalency leaded to an augmentation of the inhibitor activity. Trinh et al.[121] demonstrated that synthesizing the giant molecule **33a** composed by a fullerene core with 120 peripheral iminosugars (figure 10) similar to Munoz's compound reported in figure 6 but with sp2-iminosugars, a great inhibitory activity was obtained towards Jack bean α-mannosidase (underling that a high valency is necessary for the inhibition of this "open" site enzyme). The size of these molecules seems to be the main factor allowing a good inhibitory activity also towards β-galactosidase and β-mannosidase, with a IC_{50} in the nanomolar range. The inhitope density of multivalent compounds plays an important role, Nierengarten et al.[122] compared dendrimers **33b,c** composed by 36 iminosugars (figure 10) and product **33d** with 108 iminosugars (figure 10). Also 12- and 120- valent C_{60} iminosugars were included in this study. This work confirmed the relevance of the multivalency in the inhibitory activity, but added an additional factor, the density. The combination of these two aspects lead to a very potent inhibitor.

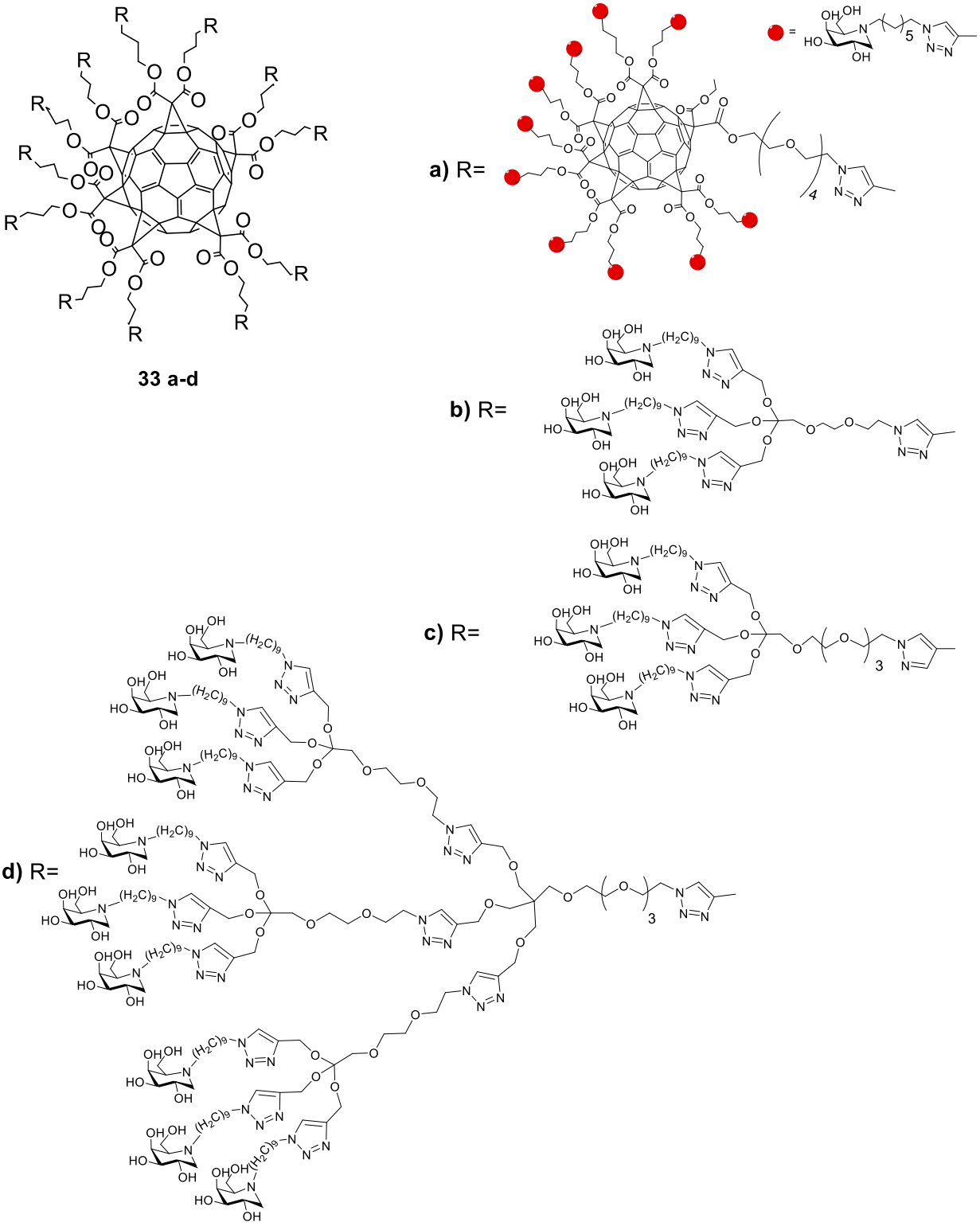


FIGURE 10: More efficient glycosidase inhibitors

TABLE 1: Values Ki (μM). Inhibition is competitive excepted **31b,c**. [a] uncompetitive inhibition, [b] mixed type (both Ki from competitive and uncompetitive inhibition). N.a. (no data available). N.I. (no inhibition detected)

	30a	**30b**	**30c**	**31b**	**31c**	**33a**	**33b**	**33c**	**33d**
α-Glucosidase:									
Maltase	18	67	104	0.9	9.4	N.a.	64	22	0.24
Isomaltase	10.5	25	193	5	65	N.a.	N.a.	N.a.	N.a.
Amyloglucosidase	N.a.	N.a.	N.a.	N.a.	N.a.	0.14	0.53	0.86	0.61
β-Glucosidase	247	65	24	N.a.	N.a.	87	25	224	6
α-Galactosidase	84	78	104	N.a.	N.a.	44	42	103	11
β-Gaòactosidase	0.15	0.085	0.66	1.3[b] 2.3[b]	7.1[a]	60	27	50	8.6
α-Mannosidase	N.a.	N.a.	N.a.	320	N.I.	0.0018[b] 0.0042[b]	0.069	0.0064	0.0072

The Glycosyltransferases are other enzymes subjected to multivalent inhibition. They catalyse the regio- and stereo- selective transfer of a carbohydrate from a donor to an acceptor. As glycosidases, also this class of enzyme is involved in key biological steps and could be considered as a good target for development of new therapy of several diseases (diabetes, cancer, viral infections etc..). Durka et al.[123] showed for the first time the efficacy of the multivalent glycofullerenes (12 sugars) as glycosyltransferases inhibitors. These compounds inhibit Heptosyltransferase WaaC (enzyme essential for biosynthesis of bacterial lypopolisaccharides) in the low micromolar range (IC_{50}=7–45 μM) showing a much better activity compared to monomeric glycosides (IC_{50} above 400 μM). The mechanism is still unknown but Tikad et al.[124] provided more information. Glycosiltransferases display two recognition sites, one for the acceptor and the other for the donor. They discovered that regardless the inhitope affinity for the acceptor or donor site, the multimers interact only with the acceptor binding site (more exposed and easily accessible). Moreover, the fullerene scaffold was found to positively influence the affinity, and this leads to increase the inhibitor activity.

Accordingly, a new generation of efficient inhibitors was prepared by the synthesis of some clickable keto-deoxyoctulosonate derivatives (**34 a-c** in figure 11) that were easily conjugated with fullerene. These Kdo sugars are multivalent compounds much more active as inhibitor and affine for glycosyltransferase than the precedent glycofullerene derivatives resulting in a new potential class of glycosyltransferase inhibitors that could inspire further studies to reach therapeutic applications for cancer or HIV.

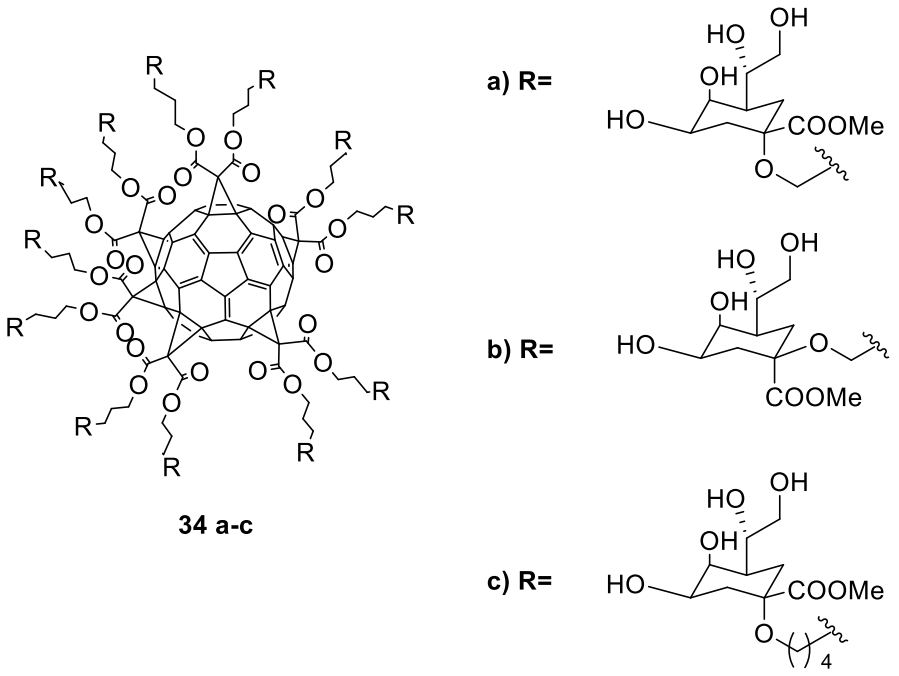


FIGURE 11: kdo-fullerene derivatives

The last possible application, for the moment at a preliminary stage, contemplates the possibility that the glycofullerenes can interfere with the action of certain toxins.

For example shiga toxin or shiga-like toxin by *E. Coli*, which can lead to serious consequences such as kidney damage, enters into mammalian cells through a process mediated by oligosaccharides recognition with the toxin receptor of human cells that is the glycolipid Gb3 α-D- Gal(1→4)β-D-Gal(1→4)β-D-Glc(1→O-ceramide). Antagonization of this recognition could be a valid therapeutic strategy and the use of multivalent inhibitors seems to be a possible approach[125]. One candidate could be a glycofullerene prepared by a copper-catalyzed [3 + 2] cycloaddition reaction and bearing five oligosaccharides (compound **35** in figure 12) as a Gb3-trisaccharide. The spatial orientation of these five P^k trisaccharides of Gb3 leads to a tighter binding with model of shiga-like toxin (Figure 12)[126].

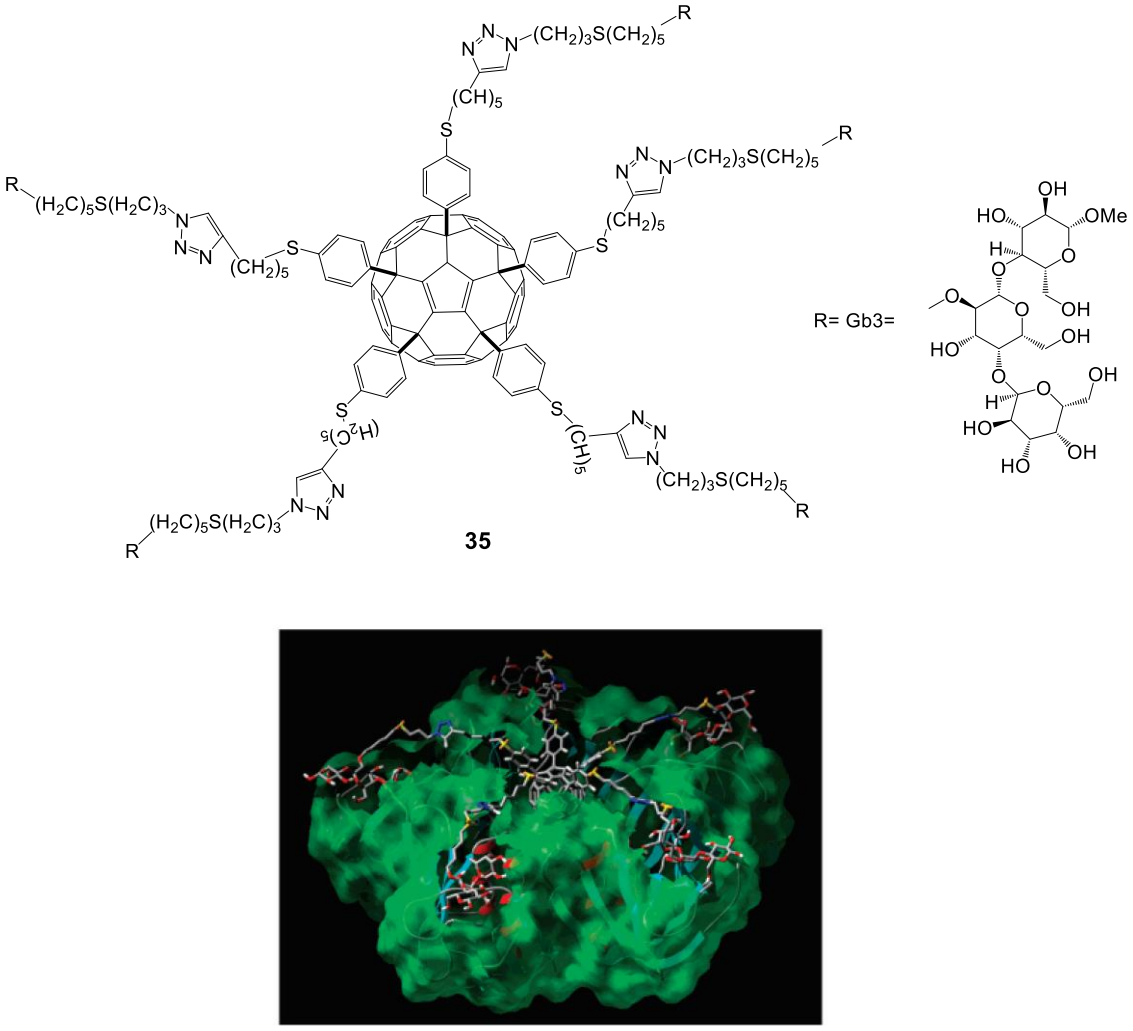


FIGURE 12: Fullerene conjugated with Gb3-trisaccharides. It fit very well with a model of shiga-like toxin

Another toxin that exploits carbohydrate-protein binding to enter in the host cell is ricin-toxin, belonging to *Ricinus communis* family. Also in this case, a fullerene conjugated with sugars, the bis(β-lactosyl)-fullerene, can be a tool for detecting and decontaminating from this deadly toxin[127].

3.2 C_{60} peptide derivatives

The C_{60}-peptide represents a possible example of bioconjugate that could be exploited for different biomedical applications. As for sugars, the conjugation of peptide with fullerene provides structural diversity, but also charge, flexibility and specific recognition properties [128].

Several medical areas were investigated for the application of these fullerene derivatives and the main applications concern the development of new anti-infective agents and immunological new tools.

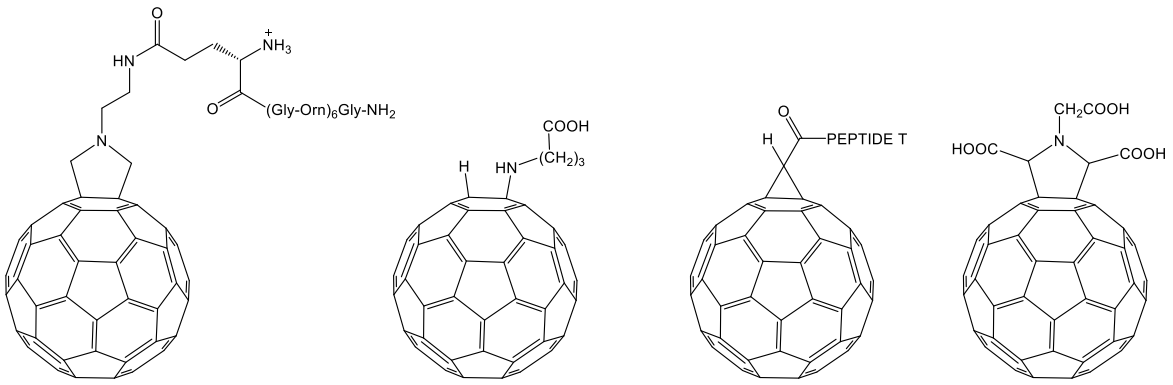
3.2.1 Anti-infective properties

Generally, the amphiphilic nature of antimicrobial peptides (with cationic charges) is essential for their antibacterial activity; indeed, the hydrophobic residues interact with the lipidic bilayer, instead the polar and charged residues are in exposed on the membrane towards the external aqueous environment. The presence of a hydrophobic core in the C_{60}-peptides promotes interaction with the membrane, helping the peptide to achieve its target. Pellarini et al. [72] and Pantarotto et al.[129] studied some peptide-fullerene derivates (**36** in Figure 13) that showed remarkable effect against gram-positive bacteria (e.g. *S. Aureus*) but their activity was reduced in *E. coli*, gram-negative bacteria, maybe because of the aggregation or the large dimension of C_{60} that prevents the penetration of peptide to the LPS-rich membrane of gram-negative bacterium.

Some of these derivates (**37** in Figure 13) have also antioxidant properties to be exploited for developing potential anti-viral products[51]. Kotelnikova et al.[130] established a correlation between the cytomegalovirus infection (CMVI) infection and the lipid peroxidation; thus they proved that amino acids-fullerene with antioxidant activity could be a new generation of drugs for the treatment of herpes virus infections, specifically against CMVI.

The treatment HIV was also investigated with peptide derivatives of fullerene. Friedman et al. in 1993 published a computer graphic simulation where C_{60} seems to be perfectly hosted by the hydrophobic cavity of HIV-1 protease[131]. Further works[132,133] supported this hypothesis, indeed was demonstrated that some peptide/amino acids derivatives (**38** and **39** in Figure 13) resulted inhibitors of HIV-1 protease.

Recently, Dostalova et. al[134] studied the conjugation of an antimicrobial peptide, maximin H5 and its derivatives (isolated from Asian toad *Bombina maxima*) with C_{60}, generating another example of product with antiviral activity that can be modulated by the amount of peptide linked on the fullerene surface.



36 **37** **38** **39**

FIGURE 13: Peptide-*fullerene with anti-infective properties*

3.2.2 Immunological properties

In the last few years, nanomaterials started to be considered as good tool in development of efficient adjuvant/delivery systems for vaccination, thanks to their several advantages as biocompatibility, size, surface properties and the possibility to introduce modification. Thus preliminary studies were made also in a possible application of fullerene for this type of therapeutic approach [135,136]: the potential use of fullerene derivatives nano-material in vaccination can be promising since C_{60} has a vast surface and a size that makes its products well recognized by APC cells and suitable to penetrate through cells walls and endothelium. Moreover, fullerene has a regular and reproducible surface compared with other nanomaterials commonly used for pharmaceutical application (as metals or polymers).

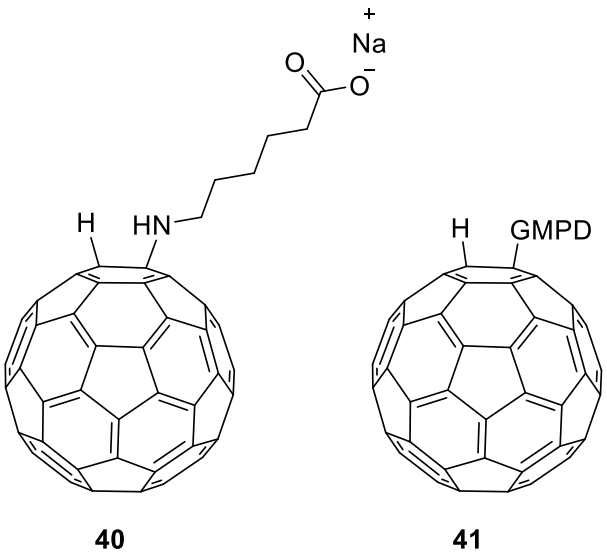
In addition, the size and shape of possible aggregates in water are reversible and fluxional[137]. Another advantage is the possibility to link the molecule of interest by covalent conjugation to obtain stable derivatives.

However, fullerene has some limitation for its use in nanomedicine because its size is not optimal (C_{60} has a size around 1nm while the optimal size should be around 20 nm). This concern can be addressed using fullerenes with size higher than C_{60} and/or designing surface modification that improves the final size (as previously reported for the derivative obtained by conjugation with sugars).

In addition, the design of these derivative could be performed in order to induce the formation of stable micelles with a core of fullerene that expose the antigens on the surface[89].

Some studies showed the efficiency of different C_{60} derivatives as adjuvant allowing the design of a new generation of vaccines. Ramon et al.[138] defined "adjuvant" a substance that produces a more robust immune response in combination with a specific antigen respect to the antigen alone. So, the role of the adjuvants is to increase the response, allowing the use of smaller doses of vaccine and accelerating the immunization process. Adjuvants could help through one of the following mechanism to enhance the immune response: assisting the antigen delivery in the site of injection; stimulating productions of cytokines and chemokines; promoting the cellular recruitment and promoting the antigen uptake and presentation by APC[139].

According to Masalova et al.[140], fullerene conjugated with aminocaproic acid or N-acetylglucosaminyl-N-acetylmuramyl-dipeptide (GMDP), **40** and **41** (figure 14) could be valid adjuvants thanks to their ability to increase IgG production. A group of mice was injected with pNS4 (antigenic peptide of Hepatitis C virus) without adjuvants, another group with formulations containing known adjuvants such as Freund's complete adjuvant (FCA) and aluminium hydroxide (HA) and a third group combining the antigen with the C_{60} derivatives **40** and **41**. The results indicated the superiority of these C_{60} derivatives respect to the other adjuvants thanks to their higher solubility in water, the long-term effect obtained, and their ability to cause more rapid antibodies formations (a wider spectrum).

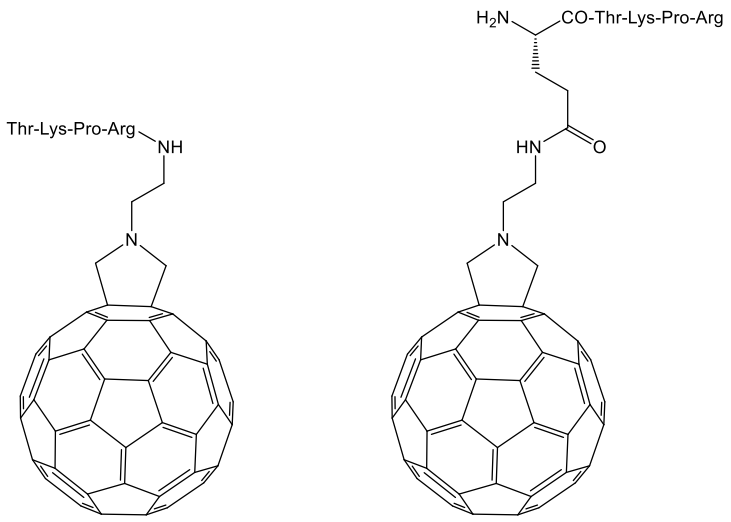


40 **41**

FIGURE 14: Compounds developed by Masalova et al.

Subsequent studies showed that multi-hydroxylated fullerenes (fullerenols) could be an useful component for the preparation of new vaccines for serious viral infections such as HIV[141] and hepatitis C [142]. In addition, it was demonstrated these fullerenols have the ability to preserve the antigenic portion (peptide or DNA encoding for the antigen) from enzymatic degradation.

Another work that confirmed the possible use of fullerene as nano-carrier for designing efficient adjuvants was published by Xu et al.' [143]. Two fullerene-tuftsin conjugates (**42** and **43** in figure 15) were compared with the non-conjugated tuftsin (a tetrapeptide, Thr-Lys-Pro-Arg, with immunostimulatory activity released by proteolysis from the IgG Fc-domain), showing a significant enhancement of the immunostimulant activity of the peptide induced by its conjugation with fullerene(increased presence of phagocytes, major chemotaxis activity in macrophages stimulated with this conjugates). Therefore, the presence of fullerene led to two important and favourable consequences: 1) it protects the amino acids from enzyme degradation prolonging the half-life of tuftsin; 2) thanks to the nano size of fullerene, the conjugates preferentially enter in the mononuclear phagocyte system improving the bioavailability of the antigen.



42 **43**

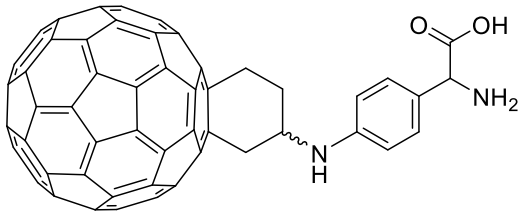
FIGURE 15: C_{60}-Tuftsin derivatives

Therefore, all of these studies support the idea that fullerene could be a good candidate for the design of innovative nano-vaccine by using fullerene derivatives as adjuvant for the formulation of antigens and/or as a carrier for covalent conjugation of immunogenic or antigenic peptides. However, few studies have been published on this subject and it deserves further investigation.

3.2.3 Other applications

Several other applications have been studied for peptide-fullerenes conjugation products. The antioxidant properties of these fullerene derivatives were exploited as anti-infective agents, but also against other pathologies such as cardiovascular disorders, neoplastic diseases, inflammations where ROS production seems to be involved in their pathogenesis[144]. The presence of peptide helps the solubility and decreases aggregation of C_{60}, increasing its antioxidant potential with respect to the non-conjugated fullerene [145].
On the other hand the linking of a peptide portion with fullerene could be exploited also for boosting cell penetration of peptide with pharmacological relevant activities. Yang et al.[60] used a fullerene-phenylalanine derivative (Baa, compound **44** in figure 16) as building block in the synthesis of cationic peptides. These products use the strong hydrophobic properties of C_{60} as a passport for the intracellular delivery of the peptide into neuroblastoma cells, HEK

293 and Hep G (where the peptide alone cannot enter). Two cationic peptides were incorporated, the nuclear localization sequence (NLS) forming H-Baa-Lys(FITC)-Lys-Lys-Arg-Lys-Val-OH and a poly-lysine peptide forming H-Baa-Lys(FITC)-Lys_8-OH. Also an anionic peptide (Lys(FITC)Glu_4Gly_3Ser-OH) was tested proving that its uptake is lower in comparison with the cationic ones.



44

FIGURE 16: Fullerene-phenylalanine derivative

In addition, it seems that peptide derivatives of C_{60} could be exploited in different diseases. For example Xiao et al.[146] reported that FT-C_{60} (fullerene conjugated to a peptide, cFLFLF, that binds to formyl peptide receptor-1) reduces inflammation and in particular could be used against pain in treatment of the degenerative disc disease.

Other pathologies considered for the treatment with peptide derivatives of fullerene are the Alzheimer disease[147], mixed connective tissue disease (MCTD) or systemic lupus erythematosus (SLE)[71], and also in cancer therapy (e.g. as targeting molecule or in diagnostic)[148,149].

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