Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
SCIENZE CHIMICHE

CICLO XXVI

COORDINATORE Prof. Carlo Alberto Bignozzi

Photoinduced hydrothiolation and hydrophosphonylation of alkenes and alkynes

Settore Scientifico Disciplinare CHIM/06

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ABSTRACT

Hydrofunctionalization of terminal double or triple bonds have become classical ligation tools for facile assembly of building blocks into larger molecules especially because comply Sharpless' prerequisite to be considered "click-chemistry" reactions. In particular the free-metal photoinduced radical thiol-ene (TEC) and thiol-yne (TYC) couplings are well-known to be atom economy, high-efficient, catalyzed only by light and completely regioselective. If both TEC and TYC have already been studied on simple substrates as linear terminal alkenes or alkynes, only few researches have been carried out about particular molecules as protein, peptides and endo-glycals. Hydrothiolation of protein, peptides and aminoacids has been studied to obtain a double different substitution of the peptidic scaffold with one carbohydrate and one marker (fluoresceine or biotine) using TYC. A new technique to synthesize S-disaccharides has been developed starting from different glycals and thio-glucose, demonstrating the efficiency of TEC on internal internal double bonds.

The successful work about hydrothiolation of different substrates has pushed us to study both hydrophosphonylation of alkenes and alkynes starting from the same conditions of TEC and TYC reactions. The different reactivity of the functionalization agent (thiol or *H*-phosphonate) has resulted in different conditions for the addition to double bonds, but not in a loss of efficiency o regioselectivity. On the other hand the addition on a triple bond has resulted to be ineffective and to stop at the internal double bond intermediate. A thiol-ene coupling on this intermediate, formally a vinyl phosphonate, gives equilibration to E form of the alkene without traces of hydrothiolation adducts.

It is well known that multivalent effect is a key factor in supramolecular chemistry and it governs many biological interactions, in particular in the relationship between pathogenic microorganisms and their host that involves protein–glycan recognition. The affinity of a multivalent cluster is highly dependent on the combination of the carbohydrate head with the cluster core and the spacer between them; several families of multivalent bioactive molecules have been developed by a large numbers of groups all around the world using disparate synthetic techniques. Thiol-Ene (TEC) and Thiol-Yne (TYC) couplings have been chosen as ligation tool for the synthesis of a variety of multivalent biomolecules containing carbohydrates or peptidic termini supported on different bio-inactive clusters as dendrimers and the rigid silica cube known as POSS (polyhedral oligomeric silsesquioxane). Both TEC and TYC are highly efficient, regioselective and atom economy reactions that, moreover,

permit us to avoid purification problems due to metal catalysis or to use large excess of reagents to have a complete substitution on the central core. In fact all the reactions have been carried out with success, high yield and without by-products of any kind. Affinity toward specific target of this large library of compounds has been tested by Enzyme-Linked Lectin Assay (ELLA) and results from good to excellent have been found in all classes of compounds.

SOMMARIO

L'idrofunzionalizzazione di doppi o tripli legami terminali è divenuta negli ultimi anni un classico metodo di coupling per la sintesi di architetture molecolari complesse tramite building blocks, specialmente perché soddisfano i requisiti di Sharpless per essere considerate reazioni di click-chemistry. In particolare le reazioni radicaliche fotoindotte non metallo catalizzate tiol-ene (TEC) e tiol-ino (TYC) sono molto note per essere efficienti, regioselettive e catalizzate solo dalla luce. Mentre sia la TEC che la TYC sono state già studiate su molecole semplici come alcheni o alchini terminali lineali, solo pochi studi sono stati condotti su substrati complessi quali peptidi, proteine o glicali contenenti doppi legami interni al ciclo zuccherino. L'idrotiolazione di proteine, peptidi e aminoacidi è stata studiata per ottenere una doppia sostituzione sullo scheletro peptidico con uno zucchero ed un marker (fluoresceina o biotina) usando la reazione tiol-ino (TYC). Una nuova tecnica per la sintesi di S-disaccaridi è stata sviluppata partendo da differenti glicali e dal glucosio-tiolo, dimostrando cosi l'efficienza della tiol-ene (TEC) anche sui doppi legami interni di tipo enol-etereo.

Il successo ottenuto nel lavoro sull'idrotiolazione di substrati particolari ci ha spinti a provare l'idrofosfonilazione sia di alcheni che di alchini partendo dalle stesse condizioni della tiol-ene e della tiol-ino. La diversa reattività dell'agente funzionalizzante (tiolo o fosfonato) comporta diverse condizioni necessarie per l'addizione selettiva ed efficiente al doppio legame. D'altra parte l'addizione su tripli legami è risultata inefficace e ha prodotto solo l'intermedio con il doppio legame interno; una reazione tiol-ene su questo intermedio, formalmente un vinil fosfonato, produce equilibrazione verso la forma E dell'alchene senza tracce di idrotiolazione.

È noto che l'effetto multivalente è un fattore chiave nella chimica sopramolecolare e governa molte interazioni biologiche, in particolare nella relazione tra microorganismi patogenici ed i loro target che coinvolge il riconoscimento proteine-glicocalice. L'affinità dei cluster multivalenti è altamente dipendente dalla combinazione di carboidrato con il core del cluster e lo spaziatore tra essi; numerose famiglie di molecole multivalenti bioattive sono state sviluppate da molti gruppi di ricerca in tutto il mondo usando svariate tecniche sintetiche. Tiol-ene (TEC) e tiol-ino (TYC) sono state scelte come metodo di accoppiamento per la sintesi di una varietà di biomolecole multivalenti contenenti estremità zuccherine o peptidiche supportate su cluster bio-inattivi come dendrimeri ed il cubo rigido di silicio noto come POSS (polyhedral oligomeric silsesquioxane). Sia la tiol-ene che la tiol-ino si sono dimostrate reazioni altamente efficienti e regioselettive che, inoltre, hanno permesso di evitare problemi di purificazione da metalli o di dover usare un largo eccesso di reagenti per ottenere una

completa sostituzione sul core centrare. Infatti tutte le reazioni sono state condotte con successo, con rese alte e senza sottoprodotti di alcun genere. L'affinità verso target specifici è stata misurata attraverso test ELLA (Enzyme-Linked-Lectin-Assay) e si sono ottenuti risultati sempre molto buoni se non eccellenti per ogni classe di composti testati.

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1. INTRODUCTION

To better understand the scope of this doctoral thesis it is necessary to explain the importance of the hydrothiolation and hydrophosphonylation of alkenes and alkynes. The following biological introduction is a short overview of some physiological and pathologic aspects that head all the subsequent chemical research.

1.1. Glycosylation in biological systems

Glycoproteins, glycolipids and glycoconjugates in general play a key role in a wide range of biological processes, indeed glycosylation of a specific molecule can directly modulate its physiological functionality.[1] Polysaccharides (or glycans) are essentials for glycoprotein folding, cellular homeostasis, immune regulation and are involved in several pathological conditions. An increased molecular and structural investigation of glycan role has resulted in development of therapeutics and novel targets for drug design (Figure 1).

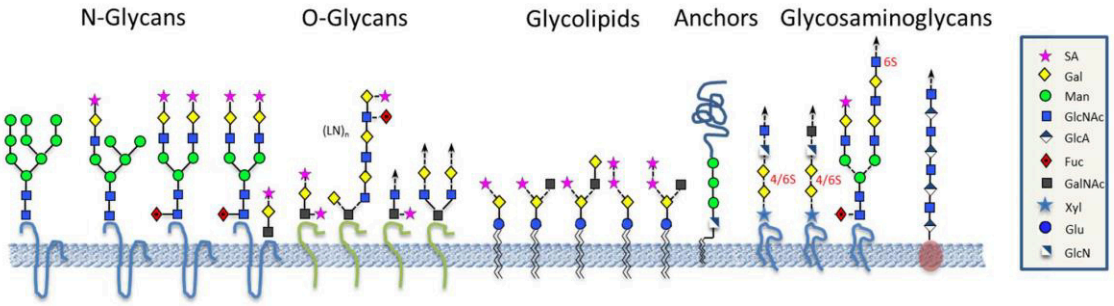


Figure 1: Classes of glycomolecules: *N*-glycans (left) and *O*-glycans (centre-left) are supported on trans-membrane proteins, glycolipids (centre), anchors (centre-right) and glycosaminoglycans (right) are supported on lipids, globular or membrane proteins. Functionality of these molecules is related to the last 2 or 3 sugar units. (see legend on the right)

It is well-established that glycans are synthesized under a not strictly controlled enzymatic regulation, so the polysaccharides chains linked to amino-acidic or lipidic sequences are presents in multiple forms and generate a variety of glycoforms with the same non-carbohydrate part and different saccharidic part. These can differ substantially for their biochemical properties and functions.[2] This difference is due to the large array of sequentially and competitively acting biosynthetic enzymes located throughout the endoplasmic reticulum and Golgi apparatus.[3] Glycoforms exert biological influence through their physicochemical properties to stabilize protein to form intrinsic components of extracellular matrix or because they are glycan-binding protein (GBPs) targets; this interaction is fundamental in cell

communication and trafficking. As example in multicellular systems immunology, embryo development and glycoprotein quality control are driven by GBPs.[4]

Glycosylation step is crucial for biosynthesis as much as for metabolic system. Indeed one of the most common metabolic process is the glycosylation of a compound to increase its hydrophilicity and to favour its excretion. The right understanding of these mechanism becomes clearly fundamental in order to better design new chemical tools for diagnostic and therapeutic purposes.

On the other hand, cellular homeostasis and host-pathogen interactions are the main aspects of glycoforms pathological involvement; many bacteria, viruses or microbial agents make use of cell glycans during critical early steps in their invasion of host tissue. Indeed, terminal host glycans (last two or three units) have been discovered to be the focal points of various invasive strategies by pathogens like influenza virus and parasites (Figure 2).[5] In rheumatoid arthritis, cystic fibrosis, Wiskott-Aldrich syndrome and in AIDS, protein glycosylation results altered. Furthermore in chronic diseases aberrant glycosylation can be an effective diagnostic and prognostic tool, although it is not clear at what point of the pathology this process is acquired.[6] In addition to these well-appreciated roles, carbohydrates are cast in a variety of interesting settings as glycoconjugates antibiotics, antitumor agents and cardiotonic glycosides.[7]

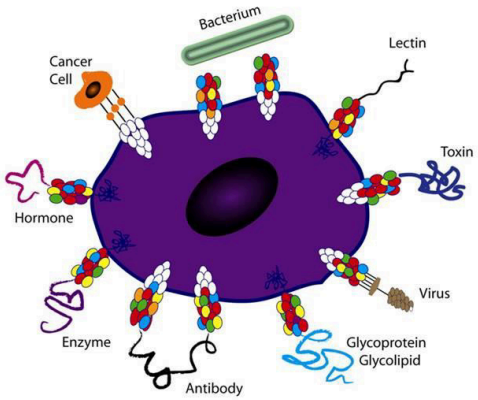
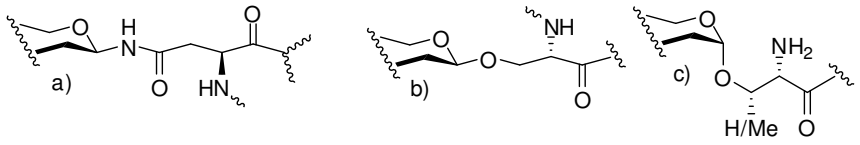


Figure 2: Cell-cell and cell-pathogens interactions mediated by glycoconjugates

1.2. Glycosidic Bonds: from natural to artificial

It is well-known that *O*- and *N*-glycoconjugates are the most common compounds in these systems, mainly for the aptitude to form and to break bonds quickly through hydrolytic enzymes.[8] *N*-glycosidic bond is usually linked to asparagine side chain, whereas *O*-glycosidic one is on serine or threonine chain (Scheme 1). *N*- and *O*-glycosylation take place in different

ways: the first starts only on a asparagine residue in a specific sequence that after is cut in a pentasaccharide used as base for further glycosylation, the second one, on the contrary, starts with a 2-acetamido-2-deossi-D-galactose (GalNAc) linked to serine or threonine. After that, different tissue-specific glycosyltransferases add other monosaccharides to obtain *O*-glycosylated protein, abundant also as tumour-linked antigens.



Scheme 1: different types of glycosidic bonds **a)** β-*N*-glycosidic (Asn) **b)** β-*O*-glycosidic (Ser) **c)** α-*O*-glycosidic (H = Ser; Me = Thr)

Disposition to form or to break effortless these linkages is essential for biological systems, e.g. protein glycosylation, addition of sugar chains to amino-acid sequences primarily by *O*-bonds, is a key step for their functionality and quaternary structure, *O*-polysaccharides, like glycogen, are used as sugar deposit and have to be ready to release quickly monosaccharides on request, *O*-phosphate groups are continuously relocated from one molecule to another as, mainly, energy carrier. It is clear that pivotal role of glycoforms in human physiology and pathology drives medical and pharmaceutical research to find new compounds to be used as diagnostic or prognostic tools; in this direction *O*-bonds weakness is evidently a delicate point and replacing native oxygen (or nitrogen) with different atoms in order to obtain stronger linkages can be an useful solution. The search for methods to synthesize new molecules with stronger bonds in the place of native oxygen glycosidic ones has been a wide research field for organic chemists, in particular those involved in carbohydrates chemistry. Substitutions of natural *O*-linkage with sulphur or carbon have driven to isostere molecules, namely compounds with same structure and geometry except for the linking atom.[9]

Sulphur represents one of the first choices to substitute oxygen because it belongs to the same chemical group and it has same valence, but it is more stable to enzymatic hydrophilic attacks. Carbon bonds permit to remove acetal motif from glycoconjugates and therefore give stronger molecules in respect to either oxygen- or sulphur-bearing ones (Scheme 2).

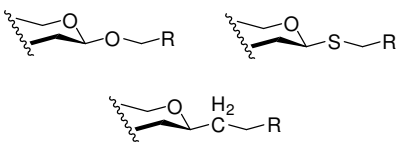


Figure 3: *O*-linked glycoside and its *S* and *C* isosteres

If the same structure with same geometry and same properties is a chemical possibility, finding new architectures that change in somehow the spatial configuration of the bond without affecting the physiological, therapeutic or diagnostic features is another way to obtain more candidate molecules to test. In this case a single atom can be replaced by a group with same pharmacological properties and the new molecules are called bio-isosteres. Isosteres and bio-isosteres are very important for structural and functional studies about glycoconjugates, so they have become a significant challenge for organic chemists that are called to find methods to synthesize them in the most efficient way as possible, as is required by pharmacologist to have larger libraries to study with a controlled cost.

1.3. Click-Chemistry and Bio-orthogonal Reactions

Enzymatic catalysis of glycoconjugation is surely more efficient and selective than synthetic methods usually are. This is the reason why one of organic chemists' goals should be to find better ways to obtain the same compound with less wastefulness of energy, material and money.

In 2001 Nobel Prize Winner B. Sharpless highlighted a set of criteria to be fulfilled by chemical reactions to be named as "click chemistry" reactions in order to serve as useful ligation tool for facile assembly of molecular building blocks into larger constructions.[10]

The complete set of criteria is very complicated to obtain all together:

> *"The reaction must be modular, wide in scope, give very high yields, generate only inoffensive by-products that can be removed by nonchromatographic methods, and be stereospecific (but not necessarily enantio-selective). The required process characteristics include simple reaction conditions (ideally, the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed, and simple product isolation. Purification–if required–must be by nonchromatographic methods, such as crystallization or distillation, and the product must be stable under physiological conditions."*

Despite that, it is important to find new methodologies to satisfy one or more of these parameters, in the spirit to improve step-by-step more syntheses as possible. As Sharpless cites reactions to use as "ligation tool" became clear the opportunity to link together this approach with the research of new coupling reactions to obtain glycoconjugates.

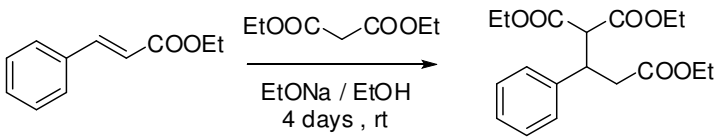
In the past two decades click chemistry has developed a lot, giving new synthetic tools for a variety of substrates, but the search is only at the beginning so it remains fundamental to keep

in mind essential criteria of this field: efficiency, selectivity, easy purification and higher yield as possible without by-products. Reaction bio-orthogonality, that is the modification compatibility with its biological function, plays moreover a crucial role in the choice of the method. Is quite obvious that to study biological molecules they have to be obtained by processes in which they do not lose their properties.[11] Unfortunately, only few chemical reactions are able to satisfy both principles and the majority of them concerns transformation of amines or azido groups. The drawback of these processes is the non-selectivity to different nucleophiles or electrophiles and the presence of metal catalysts that can leave some traces also after purification.[12–15]

1.4. Hydrofunctionalization: an overview

The main goal of all these principles and theories is to generate complex molecules by joining together small units with heteroatoms links (C-X-C). In this direction an intersection between biological purposes and chemistry aims can be found in the study of some alkenes or alkynes hydrofunctionalization. The addition of nucleophiles (X-H) to multiple carbon-carbon bonds have been of prime importance in synthetic organic chemistry, because this essentially simple reactions allow the formation of a new heteroatom linking bonds in an atom efficient manner.[16]

Michael addition represents the oldest reactions known and remains one of the most common way to add a nucleophile to an activate double bond with high efficiency in order to synthesize bulk, fine chemicals or building blocks for larger architecture (Scheme 3).



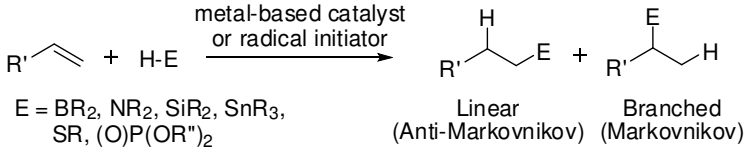
Scheme 3: Original Michael reaction

However Michael addition is possible only across activated double bonds, like alkenes with an electron-withdrawing substituents (e.g. carbonyl groups); this lack of reactivity is clearly due to electrostatic reason, since both non substituted reagents are electron-rich.[17] Inactivated alkenes have been targeted by chemists during decades for their role as important carbon feedstock, employed for synthesis of commodity chemicals as high MW polymers (ethylene,

α-olefins).[18] For these reasons, the development of efficient and selective methods for the elaboration of inactivated alkenes still remains an important challenge in both organic synthesis and homogeneous catalysis.[19] Notable methods for the elaboration of inactivated alkenes in both small and large scale synthesis include oxidation to form carbonyl compounds, epoxides or diols, [20–22] metathesis to form acyclic, carbocyclic or heterocyclic alkenes,[23–25] and hydrofunctionalization to form functionalized alkanes.[26,27]

Special interest has been given to nucleophilic attack across inactivated terminal alkene by a carbon-heteroatom (or carbon) containing molecule. Thinking to the two partners as termini of two synthetic blocks, it is clear the importance to achieve this kind of reaction with high yields, efficiency and selectivity. Necessity for activators has pushed research into a variety of them including radical initiator, strong Lewis or Brønsted acids, metals and electrophiles, but many of these transformations have suffered from myriad limitations including poor selectivity, limited scope, and/or poor functional group compatibility.[28,29] These reactions have traditionally required activation of the alkene also with a stoichiometric amount of an electrophile such as Hg(II) known to be very toxic and not-user-friendly. Apart from these extreme examples, metal catalysis and radical activation are the most common methods for addition.

One of the first examples was reported by Brown in the middle of the last century[30] about hydroboronation in presence of aluminium trichloride. The enormous synthetic utility of this reaction[31] is due to the easy conversion of the transient organoborane into a variety of useful compounds including alcohols, amines, alkyl halides. Other hydrofunctionalization reactions like hydroamination,[32–36] hydrosilylation,[37,38] hydrostannylation,[39] and hydrothiolation[40] have been studied by various research groups, finding out a large set of conditions to obtain the desired products. Depending on catalyst, substituents on olefin and nucleophile and conditions these reactions can give both linear and branched product, but for biological linking purpose result quite obvious that linear compounds are extensively preferred (Scheme 4).



Scheme 4: Hydrofunctionalization of alkenes

Metal catalysis has been widely studied for all these reactions mainly because, in association with a chiral agent, can give high enantioselectivity. On the other hand, metal traces, even in catalytic amount, are often difficult to remove from final products. A large amount of work has been carried out, particularly in the field of polymer chemistry, to improve these methodologies.

Radical chemistry, otherwise, has started to emerge as a new, metal-free, technique to obtain same results with higher efficiency, better yield and fewer purification problems. For instance free-radical hydrothiolation has recently gained an additional value as ligation tool for peptide and protein glycosylation and is considered an exemplary case of click chemistry.[41,42] Aiming to hydrofunctionalize terminal alkenes, radical thiol-ene coupling (TEC) has been chosen as starting point for this doctoral work. Its appreciable properties and characteristics make this "old" reaction still very innovative nowadays, if watched with click-chemistry criteria and focusing on bio-molecules targets. Furthermore, a new thio-glycosidation method has been optimized on glycal substrates in order to obtain disaccharides similar to the natural ones.

Our methodological investigations moved also on a totally different nucleophile, passing from thiols to phosphonates. Hydrophosphonylation of alkenes and alkynes by metal-free radical reaction has been reported only once in the literature using thermal activation, but never under photoinduced conditions.

The importance of hydrothiolation has been underlined as a fundamental tool to build multivalent sugars useful to test carbohydrate binding properties toward specific biological targets (lectins). Different types of scaffold have been tested as "core" to be functionalized with sugars, aminoacids or small peptides to obtain large libraries.

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SECTION I

Methodological Studies on Hydrothiolation and Hydrophosphonylation

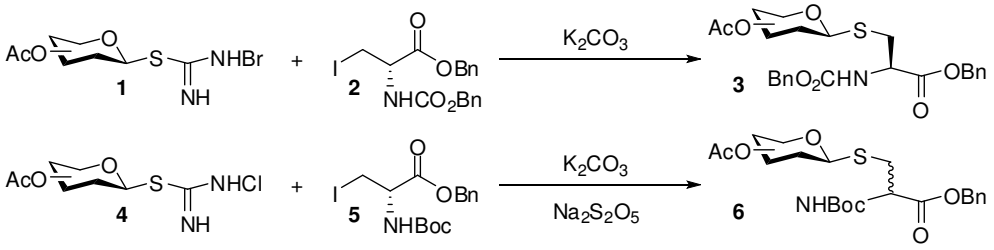
2. HYDROTHIOLATION

Hydrothiolation of alkenes is only one of the possible methods to obtain coupling bonds through sulphur, indeed the importance of this type of linkages has driven research to study methodologies to synthesize *S*-glycopeptides and *S*-polysaccharides.

2.1. Known methods for *S*-glycoconjugates synthesis

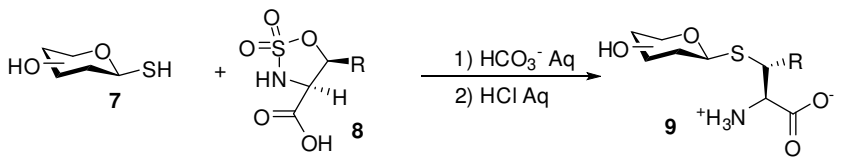
Apart from hydrothiolation of alkenes, many other synthetic methods have been developed during last decades to obtain C-S-C linkages. Herein some of the most important ones are presented and discussed with advantages and disadvantages.

Vaculik[1] and co-workers reported in 1977 a basic coupling (K_2CO_3, DMSO/H_2O) of a protected 1-thiosugar **(1)** and a β-iodoalanine **(2)** to give *S*-glycosyl aminoacid **(3)** in 72% yield. A mixture of two diasteroisomers **(6)** was found in similar conditions (K_2CO_3, $Na_2S_2O_5$/acetone/H_2O) because β-elimination is the first step to occur, followed by Michael addition on the activated α,β-unsatured ester (Scheme 5).



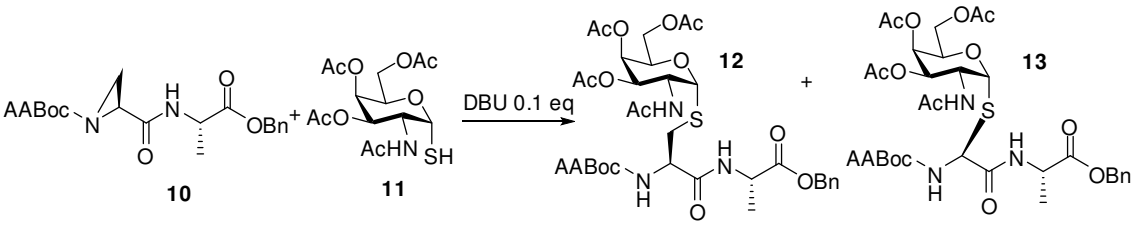
Scheme 5: *S*-glycosyl aminoacid synthesis under basic conditions.

A different approach was proposed by Halcomb[2] using aqueous buffer and non-protected thio-sugars **(7)** as starting material. The reaction was carried out with an aminoacid bearing a cyclic sulphonaminic function **(8)** like serine or threonine and was quenched with aqueous acid solutions to obtain in good yields final products as zwitterions **(9)** (Scheme 6). This method was tested also on terminal aminoacid of a peptidic chain with excellent results, however, it is effective only using monosaccharides because the acidity breaks the interglycosidic bonds; this is the biggest limit of the approach.



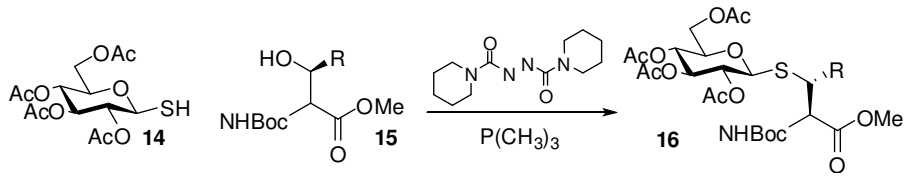
Scheme 6: Halcomb addition to cyclic suphonamic functions

Thio-sugars, like galactosamine **(11)** can also react with aziridine[3] supported on peptidic chain **(10)**, in presence of catalytic amount of a base (DBU) to give regioisomeric mixture of *S*-glycopeptides **(12, 13)** in good yield and large excess of **12**. This methodology is efficient, but, on the other hand, is not selective (Scheme 7).



Scheme 7: Coupling between thio-sugars and aziridine supported on a peptidic chain

The Mitsunobu reaction[4] has been also used for couplings of protected thio-sugars **(14)** with protected amino-acids **(15)** bearing an hydroxyl group (threonine, serine), however, only moderate yields have been observed (Scheme 8).

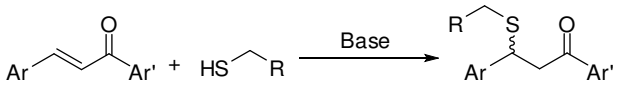


Scheme 8: Mitsunobu condensation to form glycoconjugates

Other methods to obtain these linkages were based on Lewis acids ($SnCl_4$, $BF_3{\cdot}Et_2O$) as promoters, nucleophilic substitution[5] (S_N2) of an anomeric halide, or formation of a disulphide bridge.[6–8]

2.2. Thiol-Michael Addition

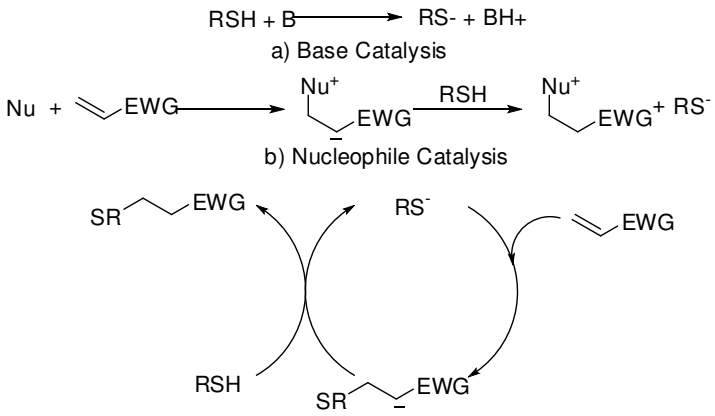
Since its discovery by Michael in 1887,[9] reactions between enolate-type nucleophile and unsaturated carbonyl have been investigated and improved in terms of yield, selectivity and efficiency. Moreover, other nucleophile have been tested as reactant to synthesize new C-C, C-S, C-O and C-X bonds within reaction products. Thiol-Michael additions,[10] namely Michael additions with thiols as nucleophiles (Scheme 9), have been studied in different conditions to obtain a wide range of products such as polymers, biological molecules or multivalent scaffolds. Since the first report by Allen,[11] the synthetic importance of this tool for organic chemists has become clear: the weakness of sulphur-hydrogen bond allows an easy formation of the nucleophile from different kinds of precursors.



Scheme 9: General scheme of thiol – Michael addition

The inherent electron density of sulphur atom ensure that thiols generate easily the thio-anion, the nucleophilic reactive species. On positive side, this easy formation permits to use this reaction as synthetic tool for a wide range of substrates and with a variety of activators, but, on the other side, the high reactivity could become a disadvantage in terms of selectivity and orthogonality, two essential criteria to fulfil to be classified as "click reaction".[12]

Two different mechanisms have been proposed as starting step of this reaction and they are both plausible, depending on pH, thiol type, solvent, temperature and catalyst. In any case is important to underline the needing to run this addition on a activated, electron poor, double bond.[13] (Scheme 10).



Scheme 10: Mechanisms for the formation of the thiol-anion and thiol-Michael reaction cycle

In the reaction cycle (Scheme 10, bottom) is shown the importance of the thio-nucleophile, that is responsible of the attack across activated alkene to generate the intermediate carbo-anion that goes to deprotonate another thiol to give Michael product as a thio-ether and a new thio-anion that keeps reaction going.[14–16] Two different mechanism (Scheme 10, top) are able to generate the thio-anion; the first is simply the deprotonation of a thiol by a base, the second obtains the same result through an intermediate with another nucleophile.

Thiol acidity is the first feature to consider, in fact easier is the anion formation, weaker is the base to use and consequently the first method is the most indicated. On the other hand, if the thiol is not incline to be deprotonated, is necessary to have a very strong base, generated *in situ* like a carbo-anion. This is the typical situation where second mechanism is favoured,

passing by a nucleophile catalysis of the enolate carbo-anion formation to obtain the first thiol-anion molecule.[17–19] (Figure 3)

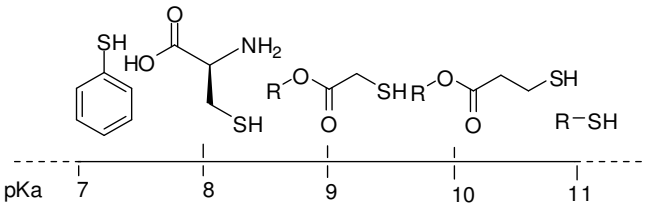


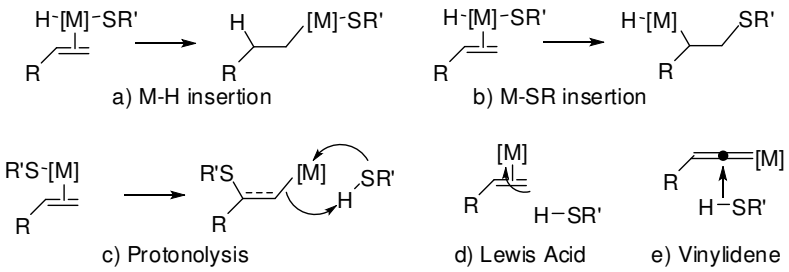
Figure 3: Range of pKa values for different thiol types that are commonly used in organic reactions (from left to right: aromatic thiols, cysteine, thioacetates, thiopropionates and aliphatic thiols).

It is very important to understand the kind of molecules used to find the best conditions for every substrate; indeed every change in a parameter can affect reaction efficiency, yield and selectivity on the basis of which mechanism is following for the first step. Electron poor double bonds are still the only substrates that can undergo this reaction, this constraining factor was the starting point for all the metal catalysis research about hydrothiolation of unsaturated carbon-carbon bonds.

2.3. Metal-Catalysed Hydrothiolation

Incorporation of sulphur into organic frameworks has been investigated in a large variety of methodologies for the importance of potential applications in pharmaceutical sciences, material chemistry or simply to obtain synthetic building blocks for larger molecules.[20–25] There is a huge number of synthetic methods to obtain these products, but they have all some drawbacks. One of the most important is to find the way to carry out hydrothiolation on electron rich alkenes, like simple terminal double bonds. The long history of sulphur addition to carbon-carbon bonds experienced an acceleration when, after more than 100 years of research, chemists understood that sulphur compounds were not potent poisons for metal catalysts they believed.[26]

Recently, several metals have been investigated as catalytic centre and most of them have been found to be effective for this addition with different levels of stereo- and regioselectivity, yield and efficiency. From the well-known palladium[27–29] to outsiders lanthanides and actinides,[30,31] passing by transition metals all the periodic table is under investigation because the control of selectivity still remains one of the most important goal in organic chemistry.[32]



Scheme 11: Various types of metal activation of thiol (a, b and c) and of the double bond (d and e).

It is evident that behaviours of thiol and of unsaturated substrate strongly determine their efficiency within the different catalytic pathways, and so the rate-determining step for the catalytic cycle may differ. It is fundamental to understand the features of the substrates in the way to choose the best catalyst and conditions to run reactions. Unsaturated compounds nature is also crucial for the synthetic approach, indeed alkenes are more reactive with radicals whereas alkynes interact more strongly with transition-metals species by π-coordination or by migratory insertion into M-X bonds.[33] In this mixture of conditions, substrates, catalysts and mechanisms it is possible that reactions follow more than one pathway to generate the final product, thus more than one by-product per time can be formed. If the catalytic way is the favoured one, the others can be non-catalytic and this cannot be controlled.

Apart from these disadvantages, metal-catalysis is a very efficient method especially to synthesize Markovnikov-type products under controlled and mild conditions. This approach is very useful as alternative to classical ionic methods, like Michael reaction, and to recent metal-free radical reactions.

2.4. Radical Hydrothiolation of Alkenes and Alkynes

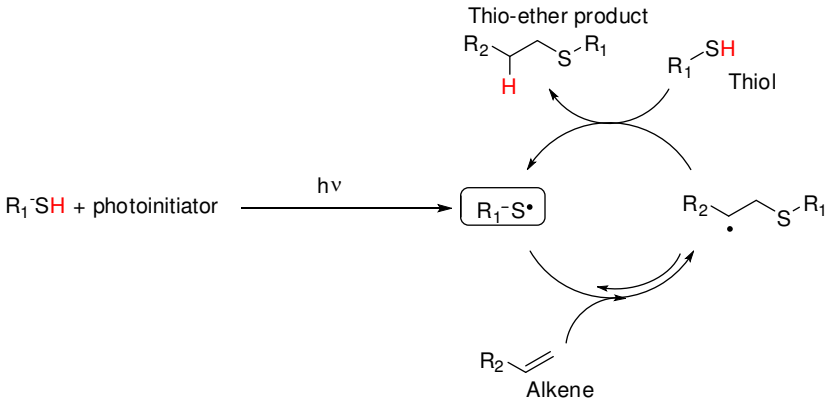
Beside ionic and metal-catalysed methods radical reactions are emerged as new interesting tools for synthetic chemists for the large potentiality in environment friendly biomolecules synthesis. In this large part of modern organic chemistry, the radical coupling generating thio-ethers linkages, namely sulphur containing bonds, is emerged as a leader technique.

The older thiol-ene coupling (TEC) was carried out the first time by Posner[34] in 1905 and the radical nature of the reaction was clarified by Kharasch and co-workers much later.[35] Radical fashion of this addition gives it some interesting characteristics like high bio-orthogonality and a very impressive regio-selectivity that make this method one of the most investigated in the last decade to build sulphur bridge in materials, large biomolecules and simple small chemical building blocks.[36] Although radical formation is known to be effective either by

thermal and photochemical way, it is obvious that thermal activation is not tolerated by biological substrates or sensible molecules, therefore, the photochemical pathway has resulted to be the best choice.[37,38] In fact thiol–ene coupling can be initiated by the greenest of all catalysts such as irradiation at a wavelength close to visible light, for example at λ_{max} 365 nm, not damaging any delicate biomolecules such as carbohydrates and peptides. Moreover the "click" status of this reaction is supported by its being highly efficient and orthogonal to a wide range of functional groups, as well as for being compatible with water and oxygen. Quite rewardingly, the reaction enables the establishment of a robust ligation motif between substrates by virtue of the stability of the thioether linkage in a wide range of chemical environments, such as strong acid and basic media as well as oxidizing and reducing conditions.

The reaction is started by a suitable photoinitiar, like 2,2-dimethoxy-2-phenylacetophenone (DMPA), to generate *in situ* the thiyl radical; that quickly reacts with the alkene and breaks the double bond to give the radical intermediate, where the new thioether linkage is already formed in anti-Markovnikov fashion and the radical centre is on the ω-1 position carbon. This carbon radical goes instantly to take a H radical from another thiol to regenerate the thiyl radical and closing the cycle giving the desired anti-Markovnikov product (Scheme 12).

TEC reaction has been extensively studied in polymer chemistry, the UV-induced cross-linking of unsaturated polymers with multifunctional thiols is currently employed in surface coating for the large advantages in respect to all other method, especially metal-catalysed ones.[39–41]

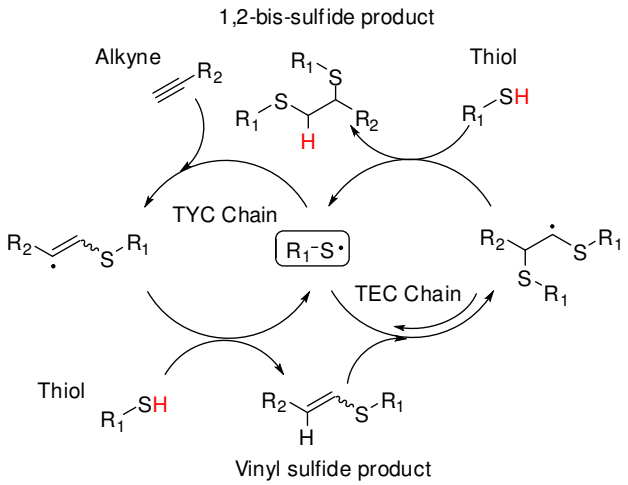


Scheme 12: Mechanism of photoinduced free-radical thiol-ene coupling

In a 2007 paper by Gress and others[42] a new definition of "thio-click" has been coined to underline the importance of this approach for polymers synthesis and the real affinity with Sharpless' concept. The wide scope of this method, the absence of transition-metal traces and

the well-defined products obtained without by-products have been emphasized by Hawker[43] in the synthesis of poly(thioether)dendrimers.

The application of TEC to biological and therapeutic molecules synthesis has been investigated by various groups in order to obtain mixed clusters of polydendrimers and carbohydrates, to couple sugars on peptidic chains and to functionalize protein. In all cases the method has resulted to be effective and products have shown good activities, demonstrating the TEC utility in biological chemistry.[44,45] Moreover, there are papers in which TEC is compared to copper-mediated azide-alkyne cycloaddition (CuAAC) for the wide possibility to synthesize molecular hybrids, chimeras or large bio-molecular architecture without renounce click chemistry criteria.[46] Summarizing these points, the main positive aspects of radical hydrothiolation of alkenes are the variety of fields it can be used in, the bio-orthogonality, the efficiency, the regioselectivity and the complete atom economy. Indeed the only known side product is the disulphide specie, that can regenerate starting thiol simply using a reductive agent. A common drawback of TEC is the reversibility of thiyl radical addition to the alkene double bond. This reversibility may vary substantially depending on the specific structure of both reagents and can be sensitive to the reaction temperature and the concentration of the thiol. For this reason, every system has to be studied in detail to find the best conditions to reach the irreversible "locking step" wherein the thioalkyl radical captures an H radical from another thiol to give the final, stable product.



Scheme 13: Mechanism of photoinduced free-radical thiol-yne coupling (TYC).

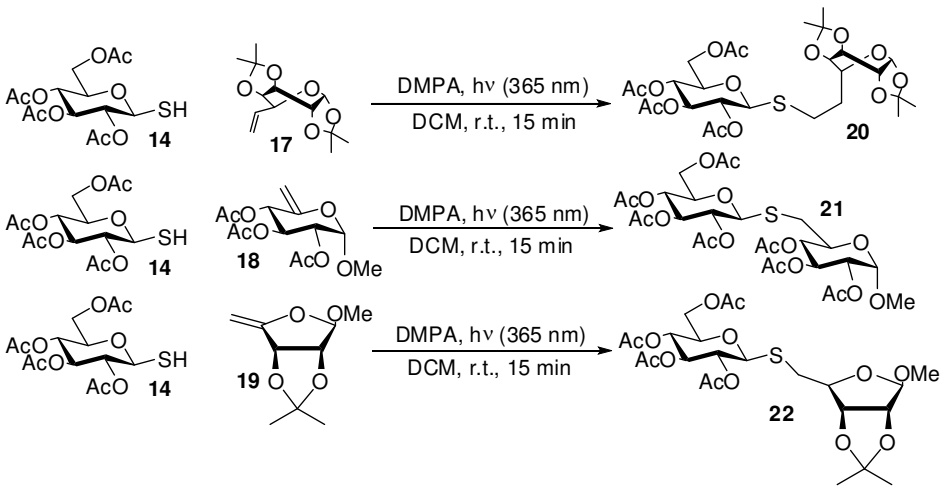
The younger sister reaction, namely thiol-yne coupling (TYC), the radical addition of one or two thiols to an alkyne has been reported the first time by Finzi and Kohler in the '30s without deeper investigation in mechanistic aspects.[47,48] In the following decades several research groups have dedicated efforts to this new method. [49–51] The mechanism of the TYC is

outlined in Scheme 13. The first step of the cycle is again the addition of a sulfanyl radical to the terminal carbon-carbon multiple bond to generate a β-sulfanyl substituted vinyl radical, hydrogen transfer from another thiol affords a vinyl sulphide product and a new sulfanyl radical to substain the chain (TYC chain). This addition is totally regioselective but scarcely stereoselective; the vinyl sulphide product is only anti-Markovnikov, but it comes out as a mixture of *E* and *Z* isomers. Therefore, one of the fundamental parameters of the "click chemistry" list is lost. The second step, the right cycle in the scheme, is the addition of an another thiyl radical to the double bond. In this case the attack should be faster than the one across triple bonds and it is reversible, when the first is usually not, especially with linear alkanesulfanyl radicals (TEC chain).[52–54] The final step is the radical protonation of the radical intermediate to close the cycle, giving the final bis-sulfide product, and regenerating the starting sulfanyl radical.

It is important to note that the reactant relative concentration plays a crucial role during the radical cycles; actually it is possible to obtain only the first addition product playing with thiol quantity. If addition is carried out in excess of thiol, it will afford only the bis-adduct, when if it is run in stoichiometric amount it will afford only the mono-addition product. [55–57] This opportunity can be used to synthesize mixed double addition products using, for example, two different thiols. The high reactivity of the vinyl sulphides is the positive side, on the other hand, the lack of stereoselectivity in TYC cycle leads to a mixture of enantiomers (or diastereomers) as final product of the second addition. Combination of thiol-ene and thiol-yne couplings (TYC-TEC homo- or etero-sequence) have been used for many studies concerning very different chemistry fields: from polymers to biological chemistry, passing through surface modification and cluster synthesis the simplicity and the relative inexpensive conditions have driven researchers to a large use of these techniques.

Thiol-yne coupling can also be catalysed by other radical activators (*i.e.* peroxides, azo-compounds, triethylborane or single electron transfer), or by different pathways like ionic or metal-catalysis. However, nowadays TYC is usually referred to the photoinduced radical reaction shown in Scheme 13.[58]

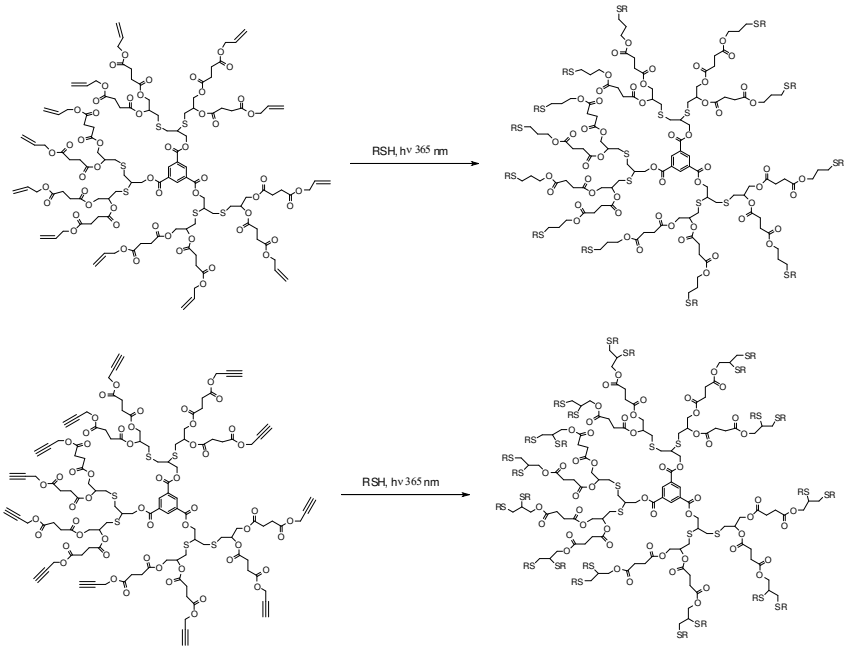
Very important results have been obtained in the field of biological chemistry using both TEC and TYC to functionalize clusters or to link molecules together. In this direction the *S*-disaccharides synthesis published by Dondoni's group in 2009 is a exemplifying model for TEC application in glycochemistry.[59]



Scheme 14: Disaccharides synthesis reported by Dondoni's group in 2009

The synthetic conditions have been optimized with a thio-glucose per-acetylated **(14)** and a di-isopropylidene-galactose-6,7-ene **(17)** to obtain the disaccharide **20** in 80% yield. The same reaction has been tested on a large library of sugar alkenes like **18** and **19** to synthesize different disaccharides with high yields and efficiency (Scheme 14).

Thiol-ene and thiol-yne have been used also for the synthesis of glycoclusters and glycdendrimers (Scheme 15). In this approach the single addition is more difficult to obtain starting from the propargylated cluster, so the TYC is used only to synthesize a multivalent molecule with a number of active points double than the one generated with TEC. This allows to study the importance of the steric hindrance respect to the number of active molecules supported onto the scaffold.



Scheme 15: synthesis of glycodendrimers by TEC (above) and TYC (below)

These are only two examples of the application of the photoinduced free-radical hydrothiolations. In this section only the study about thiol-ene across internal double bonds will be illustrated as a methodological investigation. More applications, i.e. clusters synthesis, will be illustrated in section II of this thesis.

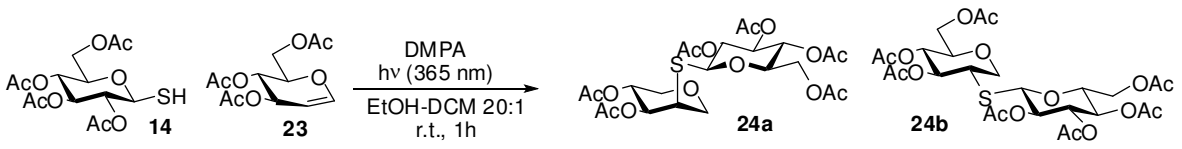
3. HYDROTHIOLATION OF GLYCALS

3.1. Aim of the study

Glycals are particular sugars with an internal double bond in a very reactive position, namely an enol-ether, that gives them a relevant role in carbohydrate chemistry as precursors or intermediates in a large variety of synthesis.[60] Probably epoxidation is the most important reaction for this functional group because epoxides are precursors of glycoconjugates and *O*- and *C*- glycosides.[61,62] Azidation of glycals is another important reaction since it gives a precursor of the amino-sugars.[63] On the other hand, only one example of radical hydrothiolation of glycals has been reported. Thermal addition of thioacetic acid activated by cumene hydroperoxide to D-glucal triacetate resulted in a mixture of diastereomers in 70:30 ratio with the major product featuring the axial SAc group.[64] Small number of investigations regarding this kind of addition has pushed us to try the equivalent photoinduced coupling on different glycals with more complex thiol such as a thiosugar. Reactivity, efficiency, stereo and regioselectivity have been posed under our focus to add a new synthetic tool in glycochemistry. Indeed, the final products are mimics of natural disaccharides, replacing *O*-glycosidic bond with a *S*-linkage.

3.2. Results and Discussion

A model reaction under standard TEC conditions between D-glucal triacetate **(23)** and 1-thioglucose peracetylated **(14)** in presence of DMPA has been investigated as first (Scheme 16).



Scheme 16: Model addition of thiosugar to glycals

After some trials conditions have been optimized in 6 eq of **14** for 1h at room temperature in a glass vial without any caution to exclude air and moisture. Under these conditions glucal was completely consumed giving the mixture of **24a** and **24b** in 80% yield and in a 50:50 ratio by NMR. Lower excess of thiol (4 eq) or shorter irradiation time (30 min) have resulted to be ineffective in order to better the stereoselectivity and have comported a big loss in terms of yield (53% and 33% respectively). It results clear that the coupling is totally regioselective but not stereoselective, it gives only the attack in position 2, but in both, axial and equatioral,

fashions. This first result is in good agreement with the other paper[64] about hydrothiolation of glycals; formation of two intermediates anomeric glycosyl radicals (AGR) is confirmed by the isolated products (Figure 3). These two radicals, stabilized by adiacent thioether groups in position 2, probably have a comparable energy that drives the two intermediates to final products in equal amount and ratio.

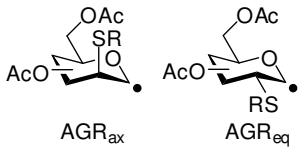


Figure 3: Intermediate anomeric glycosyl radical (axial and equatorial)

As following step we have investigated same coupling with different glycals like D-galactal **(25)**, D-allal **(27)** and D-gulal **(29)** (Table 1).

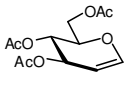
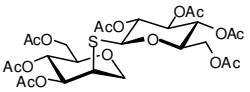
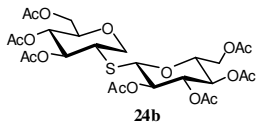
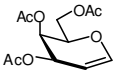
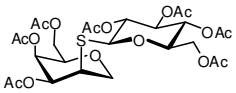
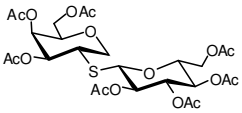
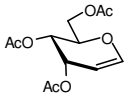
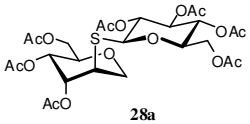
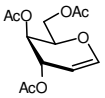
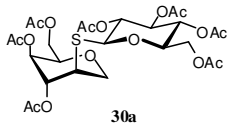
Glycal	Conversion (%)	Product(s)		Yield (%)	ax:eq ratio
 **23**	100	 **24a**	 **24b**	80	57:43
 **25**	100	 **26a**	 **26b**	59	37:63
 **27**	40	 **28a**	—	38	100:0
 **29**	35	 **30a**	—	20	100:0

Table 1: addition of thio-glucose to different glycals

The reaction is not stereoselective and glycals substituent geometry plays a crucial role for the stabilization of the radical intermediates and consequently for the ratio of the final products. Different data resulting from additions are shown in the table; on those experimental data is possible to speculate hypothesis: Going from glucal **23** to galactal **25** the conversion has remained stable at 100%, but the yields has fallen down to 60% and ratio between axial and equatorial products has passed to 37:63 in favour of the equatorial one, probably for the influence of the axial substituent in C-4 of galactal. On the other hand the addition on allal **27** and gulal **29**, that have C-3 substituent in axial position, goes exclusively to the axial final product for the larger stabilization of the intermediate radical guaranteed by 2,3-trans-diaxial

substitution. Also reaction kinetics has been affected and the result is actualized in the incomplete conversion of both the last two entries and in the low yield of isolated S-disaccharides.

In the second part of the work the role of thiol in the reaction has been posed under investigation carrying out more couplings with two different sugar-thiols, in particular peracetylated D-1-thio-galactose **31** an 2-acetamido-D-1-thio-glucose **32**, on D-glucal **23** and D-galactal **25** (Table 2).

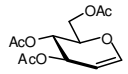
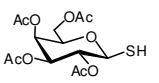
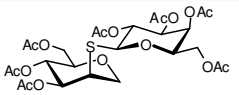
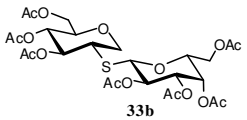
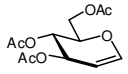
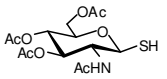
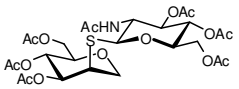
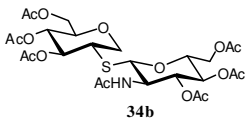
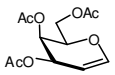
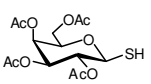
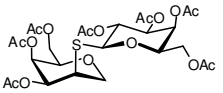
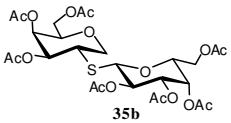
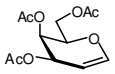
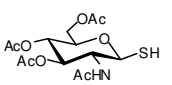
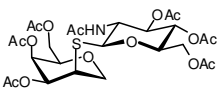
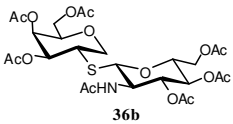
Glycal	Thiol	Products		Conversion (%)	Yield (%)	ax:eq ratio
				100	100	54:46
23	**31**	**33a**	**33b**			
				100	82	74:26
23	**31**	**34a**	**34b**			
				100	100	38:62
25	**32**	**35a**	**35b**			
				100	100	50:50
25	**32**	**36a**	**36b**			

Table 2: Coupling of galactosyl and glucosaminyl thiol to glucal and galactal

It was important to validate the methods using different glycosyl thiols, especially the one bearing the acetamido group that can differ in terms of reactivity from the others. As shown in the table, all couplings occurred with quantitative conversion and very high yields, but with lack of stereoselectivity. As it was expected from previous studies, the ratio between axial and equatorial products varies in function of the substituent on the glycals more than for thiol nature.

3.3. Conclusions

This investigation demonstrated the potential application of thiol-ene coupling to glycal internal double bonds with a total regioselectivity but a poor stereo-control. The combinations of different thiols and glycals have shown reaction stereoselectivity to be influenced by glycals substituents more than by thiol nature or geometry. Mechanistic hypotheses were postulated and validated by experimental data, and it was demonstrated to be in good agreement with the results obtained in the only other study about radical hydrothiolation of

glycals[64]. Products yield and stereo-control were the key factor to understand the mechanism, the energies of the two radical intermediates are the crucial points to investigate in order to obtain stereoselectivity. Actually, the latter is controlled mainly by the substituents on glycals, in particular at C-3. On the other hand, additions with total stereoselectivity (starting from D-allal and D-gulal) gave very low conversions and yield because increasing in equatorial radical intermediate energy affects also the whole process kinetic and consequently the reaction efficiency. By the way it is fundamental to underline the importance of the possible application of this method in carbohydrate chemistry field and the crucial role TEC can play for disaccharides mimics synthesis.

3.4. Experimental Part

See published papers, page 78: Free-radical hydrothiolation of glycals: a thiol-ene-based synthesis of S-disaccharides

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4. HYDROPHOSPHONYLATION

4.1. Introduction

Phosphorus-containing compounds play a crucial role in living organism as carriers of genetic informations and important signalling, regulatory, energy transfer and structural compounds.[1] Due to this pivotal role, biologically important phosphorus molecules have become therapeutic targets in various modern medicinal techniques, such as antisense[2] and antigene[3] approaches to the modulation of gene expression. The importance of phosphate group mimics has increased the interest in methodologies to insert phosphonates motif into larger molecules like protein, sugars and nucleic acids in the aim of synthesizing biologically active yet hydrolytically stable compounds. In fact oxygen bond in phosphate groups is the weaker point of the motif, thus replacing it with a more stable carbon linker is a good solution in terms of stability, pharmacokinetics and biological action. Moreover, since free hydroxyl groups can give absorption problems, protecting these functions with alkyl chain that can be removed by enzymatic or chemical way in cells has proved to be very effective.[4]

4.2. *H*-phosphonates

H-phosphonates have been deeply studied because they can satisfied all these criteria to give carbon linked phosphate mimics and P-H bond present in these compounds has been found to be very reactive in different reaction conditions.

Important features of *H*-phosphonates to be considered:

- Tautomeric equilibrium between phosphite and phosphonate forms
- High electrophilicity of the phosphorus centre in dialkyl esters or activated monoalkyl ester
- Conformational stability of phosphorus
- A facile oxidation to phosphorus(V) compounds

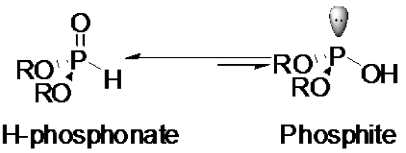


Figure 4: Tautomeric equilibrium between tetra-coordinated phosphonates (left) and tri-coordinated phosphite (right)

The equilibrium between two forms with the same phosphorus oxidation state (III) (Figure 4) is crucial because both are present in solution but they are completely different in terms of chemical reactivity. The equilibrium is completely shifted to the tetra-coordinated form and this permit to use this compound almost without caring about phosphite forms. On the other hand is important to note that another chemistry branch is born to find the condition to move the equilibrium to the trivalent form and to use this as substrate for other modifications. In terms of stability, the tetravalent form is more resistant due to the presence of the phosphoric group (P=O) that replaces the reactive pair of lone electrons. This form is less easy to oxidize and to use for electrophilic attacks, but it is a good reactant for other types of reaction. The only weak point of this form is the high susceptibility to hydrolysis of ester alkyl chains under basic conditions. It has been estimated that phosphonate dialkyl esters are 10^5 more responsive than corresponding phosphate trialkyl esters[5]. Presence of P-H bonds in tetra-coordinated derivatives is the key point of all the chemistry behind these compounds, indeed all experimental evidences have driven theorist and synthetic chemists to underline the importance of this function for the reactivity of these species. Central phosphorus electrophilicity, that is the key of reactivity, is due to linked oxygen atoms that are more electronegative, but when reactivity and electrophilicity are compared in different phosphorus species (as phosphate esters, *H*-phosphonate esters and phosphinate esters) an inverse correlation is found between these two characteristics (Figure 5).

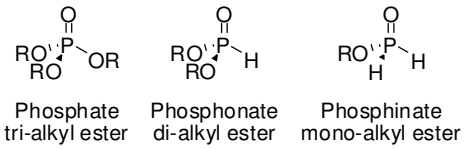
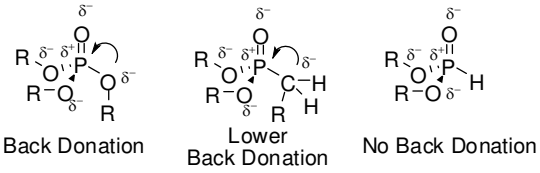


Figure 5: Different types of phosphorus compound esters

It results quite strange that less electrophilic phosphorus centres are more reactive than the more electrophilic ones, this effect cannot be attributed only to less steric hindrance, but must be driven by other electronic effect of P-H bond. The origin of this mechanism is unknown but it can be understood on the basis of chemical bonding at phosphorus that is dominated by back-donation from its substituents.[6] In phosphate triesters ($O=P(OR)_3$) the polarization of OR substituents is balanced by an efficient back-donation by lone electron pair of P-O-C bonds, in *C*-phosphonates one OR chain is replaced by a carbon directly linked to phosphorus centre that is affected by a lower electron withdrawing effect. The difference in electronegativity is close to zero when *H*-phosphonates are considered; in this case the phosphorus centre electrophilicity has to be the lowest without back-donation effect. *C*-phosphonates are supposed to have back-donation from alkyl chain to increase electron

density in the centre and to result less electronpoor than *H*-phosphonates that do not have any possibility to obtain electron back-donation from hydrogen (Scheme 17).



Scheme 17: Difference in electronic system for different phosphorus species.
(NB: electronegativity values P=2.1; O=3.5; C=2.5 and H=2.1)

For this reason *H*-phosphonates remain very electrophilic species and have been demonstrated to be excellent reactant for many additions, couplings and condensations to give, for example, *C*-phosphonates, that are more stable and less electrophilic[7,8]. One of the experimental evidences supporting this hypothesis is that phosphinates mono-alkyl esters are even more reactive species than *H*-phosphonate for the presence of two hydrogen atoms bounded to phosphorus which are able to remove more back-donation and increase electrophilicity of the centre and of the compound.[9]

4.3. Synthetic uses of *H*-phosphonates

Simple phosphonates like di-methyl or di-isopropyl are commercially available or easy to prepare, so a large part of investigations around them is focused on the synthetic importance as reactants. Literature about mono- or di-alkyl *H*-phosphonate esters use as biological compounds precursors is extremely wide; apart from oxygen containing phosphonates, sulphur, selenium and nitrogen have been studied as phosphorus substituent to synthesize large libraries of biological mimics. Moreover, hydrogen has been replaced with boron, fluorine, nitrogen, carbon, oxygen, sulphur and selenium giving several compounds with different chemical and biological features (Figure 6).

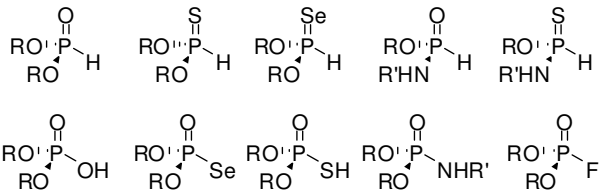


Figure 6: Different modification on *H*-phosphonate motif

Phosphorus group mimics are fundamental especially in two fields; carbohydrate and nucleotide chemistry. In the first one is crucial to find methods to insert phosphate mimics featuring higher stability with high regio- and stereoselectivity; in the other branch the best

solution has demonstrated to be the removal of the free hydroxyl group in phosphate chain. Also substitutions of O-linkages between phosphate and sugars have been tested in order to increase stability of these oligonucleotide mimics.[10] (Figure 7).

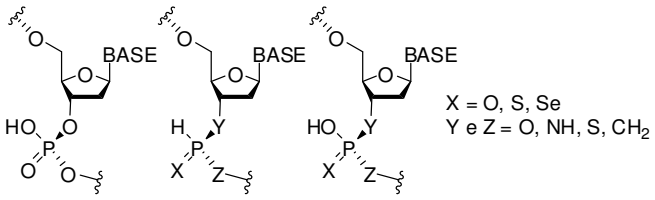
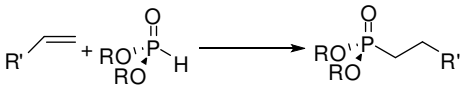


Figure 7: Natural oligonucleotide and synthetic mimics

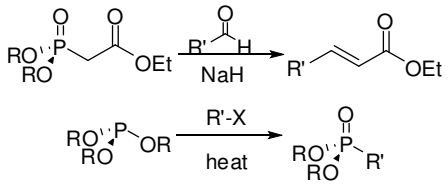
In carbohydrate chemistry it is more important the reactivity of P-H bond than its preservation in final product, because the free hydroxyl groups of phosphonates are the main anchors (together with sugar hydroxyls) for enzymatic recognition and action. For this reason it is mandatory to find reactions to synthesize carbohydrate-*C*-phosphonates from *H*-phosphonates with very high orthogonality between these functions.

One possibility is to transfer the knowledge about radical hydrothiolation to this substrate, since the general hydrofunctionalization mechanism is still valid if the sulphur alkyl chain is supposed to be replaced by phosphonate di-alkyl chains. In other words phosphonate addition to alkenes and alkynes as is shown in scheme 18 has been posed under investigation.



Scheme 18: general scheme of hydrophosphonylation of alkenes

C-phosphonates are molecule of particular biological interest since the presence of the P–C bond makes these compounds resistant to enzymatic hydrolysis and introduces conformational preferences different from those in phosphates, which are important in interactions with other biomolecules.[11] These compounds have been studied in the past as precursors of substituted olefins via Wadsworth-Horner-Emmons reaction with aldehydes[12] and they have been synthesized mainly using the Michaelis-Arbuzov reaction (Scheme 19) that, however, can suffer from low efficiency and sometimes requires long reaction times or high temperatures that could not be compatible with biological molecules stability.[13–18]

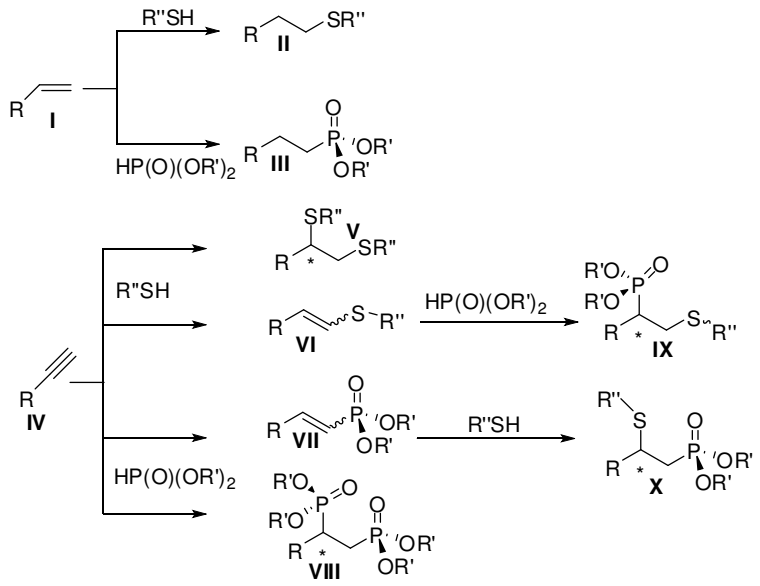


Scheme 19: Wadsworth-Horner-Emmons olefins synthesis (above) and Michaelis-Arbuzov reaction general mechanism (bottom)

Other methods have been investigated by several groups in either metal catalysis[19,20] and radical reaction field.[21–23] All these techniques are very attractive for the simplicity, because they involved common or easy-to-prepare reactants and, first of all, because all these reactions produce only linear anti-Markovnikov products with high efficiency and orthogonality. On the other hand the use of metals can lead to purification problems or to biological incompatibility due to cytotoxicity. Furthermore, radical reactions have been tested mainly with initiators, like organic peroxides, $Mn(OAc)_2$, AIBN or titanocene-epoxide system, that need high reaction temperature or long reaction time. In addition, only few complex or sensible substrates have been tested with these methods, so an extended application in biological chemistry is not possible without a safe technique developed in detail.

For all these reasons a new opportunity window was clearly opened in front of us to obtain *C*-phosphonates starting from *H*-phosphonates under the photoinduced radical conditions used for hydrothiolation. Thiol-ene and thiol-yne couplings are carried out at room temperature, with a wavelength close to visible light and using a catalytic amount of initiator. They are known to be highly efficient and to fulfil most of click-chemistry criteria, to be regioselective and orthogonal to a large variety of protecting groups or biological functions. In conclusion these methods have largely demonstrated to be tolerated by biological substrates, also by very sensible ones like *exo*-glycals.

For this new approach addition to double bonds is the first step to study, but it is very important to consider also the coupling with triple bonds, as crucial for the possibility to obtain a double phosphonylation of a carbohydrates, a protein, a nucleic acid, or a mixed addition of phosphorus and thio-compounds to a biological core to give mimic products useful for therapeutic or diagnostic purposes (Scheme 20).



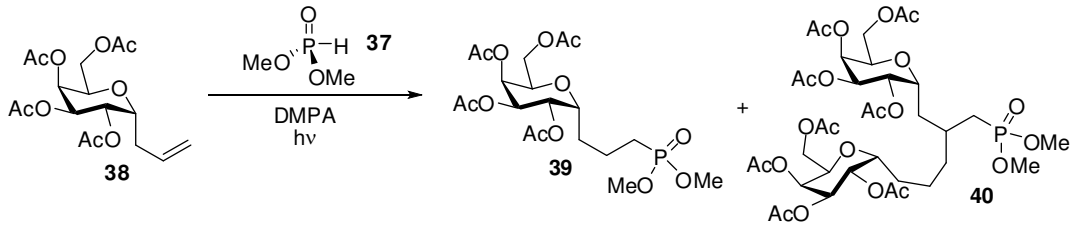
Scheme 20: theoretical addition possibilities of phosphonates and thiols to terminal alkenes and alkynes

Addition of a phosphonate to a terminal alkene **I** can result in a single product **III** if the reaction is completely regioselective and gives only linear anti-Markovnikov product like thiol-ene coupling, that affords compound **II**. A more detailed speculation about combinations of these techniques across terminal alkynes **IV** shows the importance of this investigation. Indeed, if the thiol-yne coupling in excess of thiol produces the mixture of enantiomers **V** (if R is achiral), it is possible that phosphonate addition to alkynes produces the mixture of di-substituted products **VIII**. More interesting can be the use of excess of alkyne to obtain the mono-substituted vinyl-thio-ethers **VI** or the vinyl-phosphonate analogues **VII** that are known to be very important compounds for therapeutics and for polymer synthesis. Moreover, these two internal alkenes can be used as intermediates for a second addition to obtain mixed products like **IX** or **X** or to add different thiols or phosphonates in a second step.

Another interesting point is the regio- and stereo-control of additions; the thiol-ene coupling is known to be regioselective and to afford a single product, thiol-yne instead is regioselective but not stereoselective and produces a mixture of intermediate *E/Z* alkenes that, after a second addition, lead to a stereocenter. If the rest of the molecule (R) is not chiral a mixture of enantiomers is formed, if R contains other stereocenters then the product will be a mixture of diasteroisomers with inevitable purification problems.

4.4. *H*-phosphonate addition to sugar alkenes

Reactions between dimethyl-phosphonate **37** and some enopyranoses have been examined as first approach, in particular the coupling with peracetylated allyl *C*-galactoside **38** has been chosen to optimize the reaction conditions. (Scheme 21)



Scheme 21: Optimization of photoinduced (λ_{max} = 365 nm, or sunlight) hydrophosphonylation of allyl *C*-galactoside

At the beginning conditions close to thiol-ene coupling have been adopted, i.e. a small excess of phosphonate in methanol as the solvent with a catalytic amount of DMPA as photoinitiator. Quite surprisingly, 2 equivalents of dimethyl phosphonates have led to the expected product **39** in less than 6% yield whereas 5 equivalents to less than 20% for a reaction time of 1 h (Table 3). By the way the adduct was obtained only in anti-Markovnikov form and this confirms the regioselectivity of the radical mechanism. In addition, first experiments have demonstrated the good solvent properties of dimethyl phosphonate and thus the possibility to carry out the coupling in neat conditions. Moving to solvent-free reaction and decreasing the irradiation time to 30 min with 5 or 20 equivalents of phosphonates (run 3 and 4; Table 3) the yield was higher (≈40%), but the by-product **40** was found in the crude mixture in low yields (from 12% to 25%).

Run	Equiv of 2	Time	Solvent	Yield (39)	By-Product (40)
1	2.0	60 min	MeOH	<6%	------
2	5.0	60 min	MeOH	<20%	------
3	5.0	30 min	neat	40%	12%
4	20	30 min	neat	46%	25%
5	40	30 min	neat	43%	traces
6	100	30 min	neat	91%	------
7	100	9h	neat	77%	------

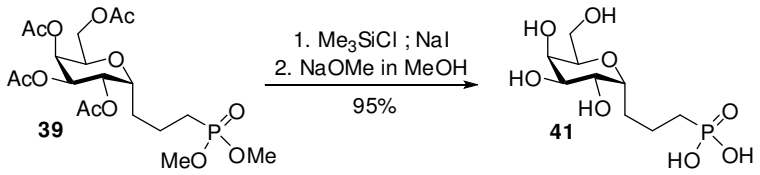
Table 3: Optimization of photoinduced radical addition of *H*-phosphonate to allyl-C-galactoside

Adduct **40** (mixture of diastereomers) structure, constituted by the sugar **38** and the *H*-phosphonate **37** in 2:1 ratio, was determined by NMR and MS analysis data. Traces of this product were also detected by NMR analysis of the crude reaction mixture using a larger excess (40 equiv.) of **37** while the yield of isolated **39** remained modest (run 5, Table 3). Only

a very large excess of phosphonate led to a quantitative yield (run 6) without the presence of the by-product. In these optimized conditions the pure product **39** was isolated simply by vacuum distillation of excess of *H*-phosphonate **37** and filtration of the resulting residue through a short column of silica to remove residual radical initiator.

The first six reactions were carried out under UV-A irradiation (λ_{max} 365 nm), that is known to leave unaltered sensible molecules as protein and carbohydrates.[24] The seventh entry shows that radical cycle can be started also by unfocussed sunlight with only a minimal loss in term of yield. Moreover, it is important to underline that as this procedure allows the recovery of the phosphonylating agent without additional purification, the process appears to be highly sustainable and very likely scalable to multigram quantities.

The second issue to complete was the total deprotection of product **39** to obtain the corresponding phosphonic acid **41** (Scheme 22). Phosphonate alkyl groups cleavage with trimethylsilyl iodine generated *in situ* followed by a deacetylation with sodium metoxide in methanol has resulted a very efficient and easy procedure to obtain the desired product in almost quantitative yield.



Scheme 22: Deprotection of phosphonate **39**

After optimization stage, the study has been widened to other peracetylated allyl-*C*-glycosides as mannose **42**, glucose **43** and *N*-acetyl-glucosamine **44** derivatives in order to obtain the corresponding glycosylalkyl phosphonates **45, 46** and **47** (Table 4). The couplings were highly effective and totally regioselective giving the desired products with excellent yield without by-products or protecting group loss.

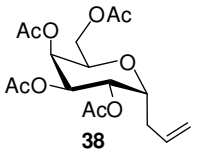
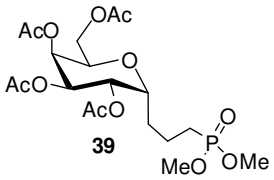
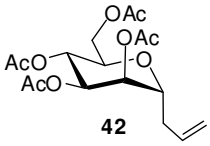
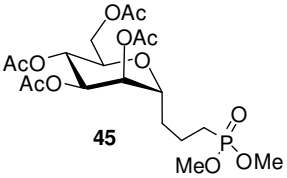
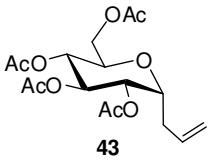
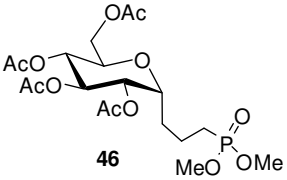
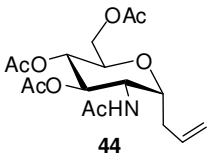
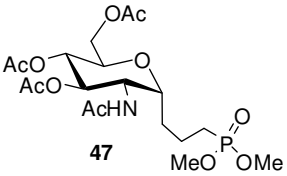
Alkene	Product	Yield
 **38**	 **39**	91%
 **42**	 **45**	91%
 **43**	 **46**	90%
 **44**	 **47**	94%

Table 4: Hydrophosphonylation of allyl-*C*-glycosides

In a second part of the study other modifications in sugar motifs have been considered; for example a diacetonide galactose alkene derivative (**48**) has been used for the coupling under standard conditions to give the product in 92% yield without decomposition of the isopropylidene protecting groups. Finally also couplings with glycals have been studied. Two *exo*-glycals, in position 6 (**49**) and in position 1 (**50**), and an *endo*-glycal (1,2-*endo*-glucal **51**) have been tested in the optimized conditions to validate the methodology (Table 5). Although the first coupling was completely effective and gave the product **52**, the additions across glycals double bonds were affected by the sugar structure and geometry. Indeed the 6-*exo*-glucal **49** underwent the coupling without problems with results very similar to those observed for allyl glycosides and yields always around 90%, but 1-*exo*-glucal generated the product **54** in less than 50% yield and one or more by-products were found in the crude reaction mixture. The latter coupling was investigated to improve the yield; the main by-product was the result of a coupling between the radical intermediate of glycoside and the DPMA fragment. However, upon decreasing photoinitiator equivalents the yield did not increase. In other words, in terms of conversion, these were still the best reaction conditions also for this substrate.

We found that the *endo*-glycal **51** was totally unreactive and no traces of product **55** were detected in the reaction mixture.[25] As mentioned before, radical intermediate stability and energy are crucial to understand this behaviour and reactivity, so it is possible to speculate about the higher stability of the radical centred in position 5 than the one in anomeric position and, of course, the complete non-reactivity of the other compound that cannot form a radical specie at all. It is also important to underline the fact that *exo*-glycal **49** has no stereocenter at position 5, therefore it can generate a D- or a L-sugar after the addition, but only the natural D-glucoside was found as product.

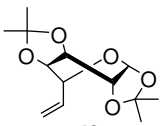
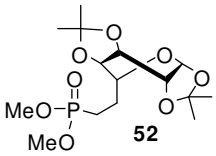
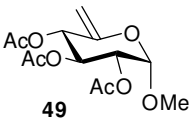
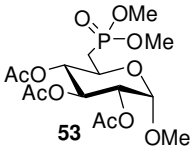
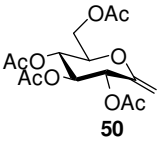
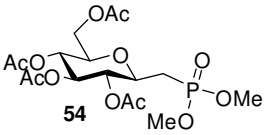
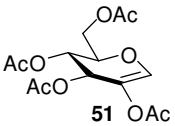
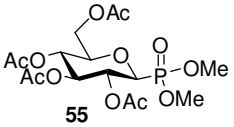
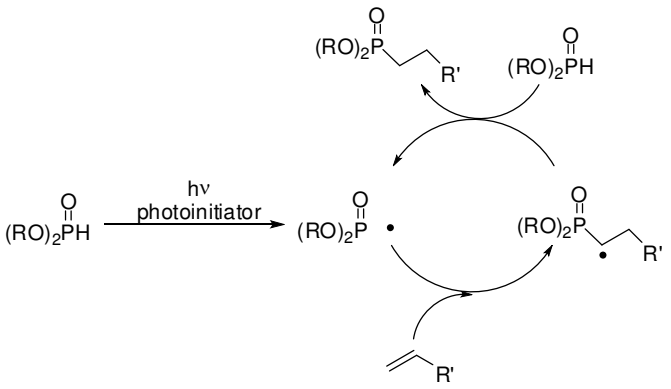
Alkene	Product	Yield
 **48**	 **52**	92%
 **49**	 **53**	88-98%
 **50**	 **54**	45%
 **51**	 **55**	No Reaction

Table 5: Hydrophosphonylation of other sugar partner and glycals

This first investigation has been useful also to determine some mechanistic aspects of phosphoryl radical cycle, in fact the addition can be compared to thiol-ene for the involved intermediates, although P-H bond has demonstrate to be more resistant than S-H. Phosphonyl-ene coupling (PEC) starts with P-H breaking by photoinitiator to generate the first radical centred on phosphorus that attacks the carbon-carbon double bond. The following intermediate has already the new P-C bond formed and the radical is now on the adjacent carbon; the latter species must react quickly with another phosphonate to close the cycle and to regenerate another phosphonyl radical, otherwise it can react with an alkene to form a sort of polymer with a single phosphorus centre with a number of other molecules linked to it.

That is the reason why it is important to carry out reactions in extremely high phosphonate concentration, in this way is statistically more probable to go in the right direction than in the wrong one. Working in neat conditions is perfect because the concentration of phosphonate is technically infinite and this permits to have good environment for the coupling (Scheme 23).



Scheme 23: Radical cycle of phosphonyl-ene coupling (PEC)

4.5. Experimental Part

See published papers, page 81: Efficiency of the Free-Radical Hydrophosphonylation of Alkenes: The Photoinduced Reaction of Dimethyl *H*-Phosphonate with Enopyranoses as an Exemplary Case

4.6. Hydrophosphonylation of alkynes

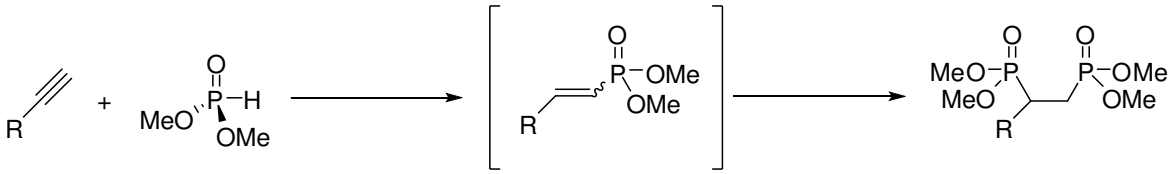
After this first study about phosphonyl-ene coupling the interest moved to the addition to triple bonds in the way to underline similitudes and differences from thiol-yne coupling. The importance of phosphorus containing compounds has already been illustrated before, but a particular interest is emerged in last years about vinyl phosphonates and double phosphonates applications.[26–28] For that reason a large variety of synthetic methods have been developed to obtain these molecules easily, with less steps as possible, without by-products and with easy purification.[29,30]

In investigation about thiol-yne coupling conditions to obtain the vinyl thioether in pure form have already been established, so the following step has been to study the phosphonate addition to find conditions for a single or double attack, to determine the geometry of the products and the regio- and stereo-selectivity of these couplings and to verify whether the radical cycle mechanism is still valid also for this case.

As already mentioned, many synthetic methods have been developed, especially for vinyl phosphonates, but most of them use metal catalysis of various type. For example organo-

copper compounds[31–37], lithium salts[38] and zirconium, palladium[39–41] or titanium complexes[42,43] have been used across activated alkenes, Arbuzov[44] or Heck[45] reactions have been used to functionalize already prepared vinyl phosphonates and some others catalytic processes[46] have been used to form the double bond on an already present skeleton. Only few methods involve radical mechanism[47] and when it happens reactions are usually thermally induced and they often need high temperatures and long reaction time. In some examples, moreover, radicals are generated by metal species, taking disadvantages from both coupling methodologies.

The initial idea was to use the know-how in metal-free photoinduced radical addition also for the addition of *H*-phosphonates to terminal alkynes of various nature and to investigate all the possibilities of this new approach. It has to be considered that this work represents the first example of a free-radical coupling in this field. As first step a model reaction has been carried out to optimize coupling conditions (Scheme 24). It was already known that a very large phosphonate excess was needed to run the coupling so it was expected to go straight to the double addition product without any way to stop the reaction isolating the vinyl phosphonate adduct.



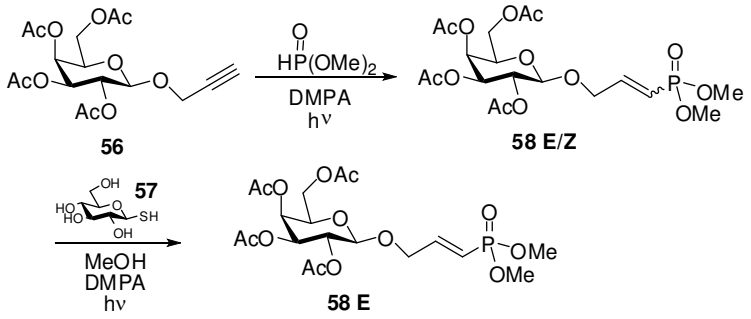
Scheme 24: Supposed mechanism of hydrophosphonylation of alkynes

Very surprisingly, with 100 equiv. of dimethyl phosphonate and 0.5 equiv. of photoinitiator (DMPA) in neat conditions, only the intermediate vinyl phosphonate was found as a mixture of isomers E/Z in 1:1 ratio. The large excess of phosphonates does not leave any margin to change conditions for a double coupling and this can be explained for the delocalization of the carbon-carbon double bond that does not allow second radical attack to work. (Scheme 25)

Scheme 25: Delocalization of vinyl phosphonate double bond

The first phosphonyl radical addition goes to deactivate the double bond and this goes to sum to the lower reactivity of *H*-phosphonate in respect to thiols. In conclusion, all factors drive the second addition to be ineffective at all.

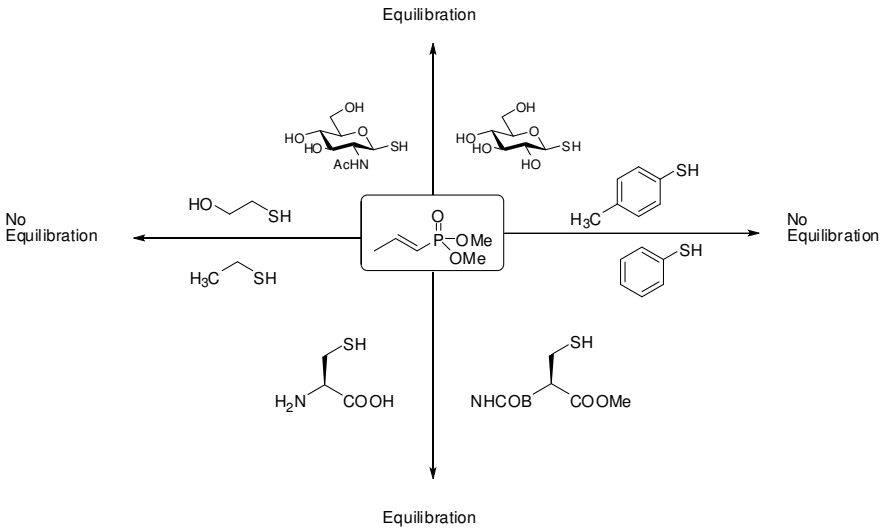
However, this addition resulted to be a good tool to synthesize vinyl phosphonates, as mixture of *Z/E* isomers, that can be used as intermediates to obtain other functionalized molecules after, for example, a thiol-ene coupling. Indeed it is known that thiols are more reactive than phosphonates and this should allow the coupling across the conjugate double bond of vinyl phosphonates. A first experiment was carried out using a peracetylated propargyl-*O*-galactose **56** as the alkyne and a free-OH glucosyl thiol **57** as second addition reactant. The hydrophosphonylation step gave a 1:1 mixture of E/Z products **58** with a 93% overall yield on the purified product, that was used as substrate for TEC with 2 equiv. of 1-thio-glucose in methanol for 30 min of irradiation to obtain only the *E* vinyl phosphonates in almost quantitative yield (Scheme 26). Surprisingly, using thiol **57** no addition products were found, instead an equilibration between *E* and *Z* forms of vinyl phosphonates occurred during the UV irradiation. Thiol was recovered without alterations and only a short filtration on silica was required to purify the *E*-vinyl phosphonates in pure form. After these first experiments our investigations have gone more in details to clarify mechanism and conditions to go straight from starting alkyne to the single *E*-isomer of vinyl phosphonate product. To do that a library of different alkynes was used.



Scheme 26: Hydrophosphonylation and equilibration of propargyl galactoside

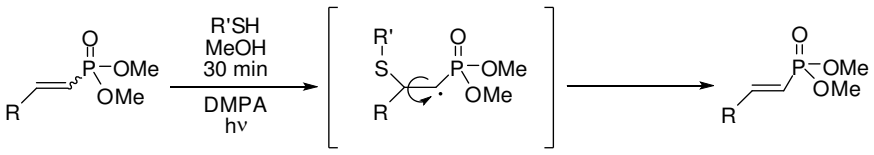
In order to clarify the radical mechanism of the equilibration, reactions in different conditions have been carried out. First the *E/Z* mixture **58** was irradiated for 30 min in methanol without photoinitiator and thiol, another reaction was performed using the photoinitiator without the thiol, finally, a reaction was carried out adding a radical inhibitor to the reaction mixture (*E/Z* mixture, DMPA, thiol). In all these cases no equilibration was observed, thus it results clear that photoinitiator is necessary to form the radical on thiol and that adding an inhibitor the entire chain is blocked from the beginning.

Changing the target of the work, it was important to use for equilibration a cheaper and simpler thiol than a sugar thiol, which has to be synthesize because is not commercially available. During this investigation different types of thiols were tried in order to test also their reactivity (Scheme 27).



Scheme 27: Different behaviour of thiols used for equilibration studies

As it is evident from the scheme above, simple aliphatic or aromatic thiols are not able to equilibrate the mixture, on the other hand, more complex thiols like sugars or aminoacids are perfectly able to do that. This effect must be correlated to mechanistic functions of radical intermediate that is formed during the equilibration. Indeed after thiyl radical formation induced by light and photoinitiator, the vinyl phosphonate must be attacked by that radical and form the radical intermediate (Scheme 28). This intermediate is more stable than the equivalent thio-ether one and the two isomers have not the same energies, so the free rotation allows to have mainly the product at lower energy, i.e. the *E* isomer.



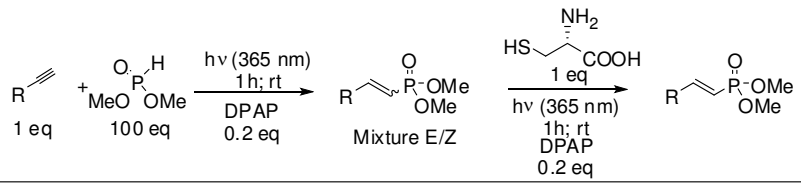
Scheme 28: proposed mechanism of the equilibration

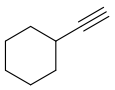
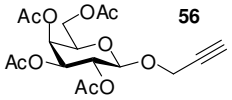
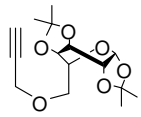
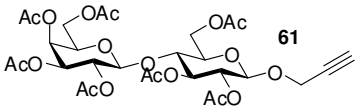
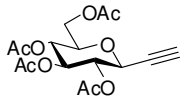
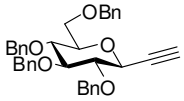
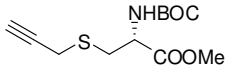
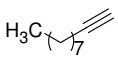
For that mechanism, the nature of the thiol (R') is important, indeed aromatic and aliphatic residues are not able to stabilize the intermediate or to differentiate energies between *E* and *Z* forms as showed by more complex molecules. It is not clear the reason of this crucial discrimination but is possible to suppose some role of chirality, spatial orientation and steric hindrance in energetic discrimination of the two forms.

In order to find the best combination of coupling and equilibration conditions we have decided to use a simple, cheap and easily available thiol like L-cysteine. The aim was to obtain the *E* vinyl phosphonate in pure form after two steps, with only a simple final purification on silica gel column. A set of various alkynes was tested under standard conditions, the *E/Z* ratio of the intermediates was measured by NMR of the crude mixture and only one purification was done to recover the final pure *E* adduct (Table 6). At the beginning cyclohexylacetylene **59** was chosen as a model: in standard conditions a quantitative yield was obtained with a final *E/Z* ratio of 95/5 starting from a 50/50 ratio of the intermediate (NMR analysis).

After that, diisopropilydene-6-propargyl-galactose **60**, peracetylated propargyl lactoside **61**, peracetylated glucosyl acetylene **62** and perbenzylated glucosyl acetylene **63** were chosen to complete the carbohydrate library. The galactose derivative **60** gave a vinyl phosphonate in 83% yield and a 95/5 *E/Z* ratio, whereas the lactose **61** afforded the product in lower yield (58%) but same E/Z ratio; perbenzylated glucosyl acetylene **63** was totally unreactive in the hydrophosphonylation. The peracetylated analogue **62** reacted only in part (80% of conversion) to give first a mixture with a *E/Z* ratio of 40/60 and then a 75/25 mixture after standard equilibration with an overall yield of 83%. It appears that this methodology cannot be applied to ethyhyl *C*-glycoside because it produces, in the best case, only mixtures of isomers in various ratios.

To complete the study the propargylated protected cysteine **64** and 1-decyne **65** were tested under standard conditions. The hydrophosphonylation step resulted in a 50/50 mixture for cysteine and 40/60 for the decyne, but for the equilibration step different methods were required. Using unprotected cysteine to equilibrate cysteine vinyl phosphonates some side-reactions between the free amino group and the methyl ester were observed. Upon replacement of the free cysteine with protected one, the equilibration afforded the *E*-vinyl phosphonates in 82% yield. The vinyl-phosphonate intermediate derived from decyne was not affected by equilibration when it was carried out on crude mixture, however, it could be transformed into an 80/20 *E/Z* mixture when the same conditions were applied to the purified intermediate. Probably the long alkyl chain is not able to drive the equilibration to a complete conversion in *E* form because is not chiral and bulky.



Alkyne	Alkene yield	Alkene E/Z ratio	Final product Yield	Final product E/Z ratio
59 	90%	50/50	<90%	95/5
 **56**	———	50/50	78%	100/0
60 	———	50/50	83%	95/5
 **61**	———	50/50	58%	95/5
62 	80% (20% Starting Material)	40/60	82%	75/25
63 	No addition			
64 	———	50/50	82% a	100/0
65 	———	40/60	57% b	80/20

a) In this case cysteine-NHBOC-COOMe has been used for isomerization to avoid byproducts formation due to nucleophylic attacks
b) Isomerization on alkenes mixture crude is not effective; purification of E/Z mix is necessary before going on

Table 6: Results of combined hydrophosphonylation and equilibration on different alkynes

In conclusion, in this study a new synthetic tool for vinyl-phosphonates has been investigated and developed to obtain only one regioisomer after an equilibration with a thiol. The data collected about diversity in terms of thiols, alkynes and conditions show the strength of this new methodology to achieve the desired product, on the other hand it has resulted clear the impossibility to synthesize double phosphonates with a double coupling on the initial triple bond. It is evident that after first addition the resulting vinyl-phosphonate is totally non-reactive toward another *H*-phosphonate. By the way, an irradiation in presence of a thiol can induce the equilibration and so the formation of a single product in very high yield and with almost quantitative conversion into *E* alkene forms.

4.7. Experimental Part

Flash column chromatography was performed on silica gel 60 (40-63 mm). ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) and ^{31}P NMR (162 MHz) spectra were recorded from $CDCl_3$ solutions at room temperature unless otherwise specified. Peak assignments were aided by ^{1}H-^{1}H COSY. In the ^{1}H NMR spectra reported below, the *n* and *m* values quoted in geminal or vicinal proton-proton coupling constants $J_{n,m}$ refer to the number of the corresponding sugar protons.

Optical rotations were measured at 20 ± 2 °C in the stated solvent; $[\alpha]_D$ values are given in $deg.mL.g^{-1}.dm^{-1}$.

The commercially available dimethyl *H*-phosphonate **(n)** and photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DMPA) were used without further purification.

The radical coupling was carried out in a glass vial (diameter: 1 cm; wall thickness: 0.65 mm), sealed with a natural rubber septum, located 2.5 cm away from the household UVA lamp apparatus equipped with four 15 W tubes (1.5 x 27 cm each).

The known compounds were prepared as reported in literature.

1-dimethyl-2-ciclohexyl-*E*-ethene 59a

(Standard procedure): The solution of cyclohexylacetylene **59** (100 mg, 0.924 mmol) in dimethyl *H*-phosphonate (8.5 mL, 92.4 mmol) with a catalytic amount of DMPA as photo-initiator (47 mg, 0.184 mmol) was irradiated at r. t. for 1h under magnetic stirring and then concentrated. The crude product (E/Z ratio 50/50 from NMR) was used for the equilibration with cysteine (111 mg, 0.924 mmol) with a catalytic amount of DMPA (47 mg, 0.184 mmol) in 500 µL di H_2O/MeOH 1:4 and then concentrated. The crude product was eluted from a column of silica gel with 3:2 AcOEt-cyclohexane + 0.5% TEA to give the product (185 mg, 92%) as a syrup with an E/Z ratio of 95/5. The E/Z ratio in the final product was determined by NMR; analytical sample of pure Z was obtained from a crude hydrophosphonylation mixture by column chromatography on silica gel with 3:2 AcOEt-cyclohexane + 0.5% TEA.

E isomer

^{1}H NMR: δ 6.76 (ddd, 1H, *J* = 6.3, 17.3, 22.8 Hz, *CH*=CH-P), 5.54 (ddd, 1H, *J* = 1.5, 17.3, 21.2 Hz, CH=*CH*-P), 3.71 (s, 3H, OMe), 3.69 (s, 3H, OMe), 2.19-2.07 (m, 1H, *CH* cyclohexyl), 1.81-1.62 (m, 5H, cyclohexyl H), 1.35-1.06 (m, 5H, *CH* cyclohexyl)

^{13}C NMR: δ 159.8 (d, CH, *J* = 4 Hz, *CH*=CH-P), 114.0 (d, CH, *J* = 188 Hz, *CH*-P), 52.2 (d, CH_3, *J* = 6 Hz, OMe), 42.2 (d, CH, *J* = 20 Hz, *CH*-CH=CH-P), 31.5 (CH_2,*CH_2* cyclohexane), 25.9 (CH_2,*CH_2* cyclohexane), 25.7 (CH_2,*CH_2* cyclohexane)

^{31}P NMR: δ 22.7

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{10}H_{20}O_3P$ $(M+H)^+$ 219.1150, found 219.1153

Z isomer

^{1}H NMR: δ 6.32 (ddd, 1H, *J* = 10.5, 13.0, 53.5 Hz, *CH*=CH-P), 5.43 (ddd, 1H, *J* = 1.0, 13.0, 20.0 Hz, CH=*CH*-P), 3.73 (s, 3H, OM*e*), 3.70 (s, 3H, OMe), 2.93 (bq, 1H, *J* = 11.0 Hz, *CH* cyclohexyl), 1.76-1.59 (m, 5H, *CH* cyclohexyl), 1.42-1.27 (m, 2H, *CH* cyclohexyl), 1.27-1.03 (m, 3H, *CH* cyclohexyl)

^{13}C NMR: δ 160.1 (d, CH, *J* = 6.0 Hz, *CH*=CH-P), 112.6 (d, CH, *J* = 185.0 Hz, *CH*-P), 52.0 (d, CH_3, *J* = 6.0 Hz, OMe), 39.5 (d, CH, *J* = 8.0 Hz, *CH*-CH=CH-P), 32.4 (CH_2,*CH_2* cyclohexane), 25.9 (CH_2,*CH_2* cyclohexane), 25.1 (CH_2,*CH_2* cyclohexane)

^{31}P NMR: δ 20.5

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{10}H_{20}O_3P$ $(M+H)^+$ 219.1150, found 219.1146

β-D-2,3,6-triacetyl-*gluco*-β(1→4)-D-2',3',4',6'-tetraacetyl-*galacto*-1-vinyl-*E*-dimethylphosphonate 61a

A solution of propargyl-O-lactoside **61** (150 mg, 0.222 mmol) in dimethyl-phosphonate (2.0 mL, 22.2 mmol) was treated as described for the preparation of **59a** to give a crude product (E/Z ratio 50/50 by NMR) used for equilibration as described for **59a** to give after column chromatography on silica gel (AcOEt + 0.5% TEA) **61a** (101 mg, 58%) as syrup with an E/Z ratio of 95/5. analytical sample of pure Z was obtained from a crude hydrophosphonylation mixture by column chromatography on silica gel with AcOEt + 0.5% TEA.

E isomer

^{1}H NMR: δ 6.75 (ddt, 1H, *J* = 3.5, 17.3, 22.8 Hz, *CH*=CH-P), 5.89 (ddt, 1H, *J* = 1.8, 17.4, 20.0 Hz, CH=*CH*-P), 5.34 (bd, 1H, $J_{3',4'}$ = 3.4 Hz, H-4'), 5.20 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, H-3), 5.10 (dd, 1H, $J_{1',2'}$ = 7.9 Hz, $J_{2',3'}$ = 10.4 Hz, H-2'), 4.95 (dd, 1H, $J_{1,2}$ = 8.0 Hz, H-2), 4.94 (dd, 1H, H-3'). 4.51 (d, 1H, H-1), 4.48 (d, 1H, H-1'), 4.45-4.38 (m, 1H, O-*CH_2*-CH), 4.19-4.10 (m, 1H, O-*CH_2*-CH), 4.16-4.04 (m, 4H, H-6a, H-6b, H-6'a and H-6'b), 3.87 (bt, 1H, *J* = 6.4 Hz, H-5'), 3.80 (t, 1H, H-4), 3.73 (d, 3H, *J* = 2.4 Hz, OMe), 3.70 (d, 3H, *J* = 2.4 Hz, OMe), 3.63-3.57 (m, 1H, H-5), 2.15, 2.12, 2.06, 2.05, 2.05, 2.04, 19.6 (7s, 21H, 7 OAc)

^{13}C NMR: δ 170.4 (C), 170.3 (C), 170.2 (C), 170.1 (C), 169.8 (C), 169.6 (C), 169.1 (C), 147.9 (d, CH, *J* = 6.0 Hz, *CH*=CH-P), 115.8 (d, CH, *J* = 176.0 Hz, *CH*-P), 101.1 (CH), 99.9 (CH), 76.1 (CH), 72.8 (CH), 72.6 (CH), 71.5 (CH), 70.8 (CH), 70.6 (CH), 69.1 (CH), 68.4 (d, CH_2, *J* = 20.0 Hz, *CH_2*CH=CH-P), 66.6 (CH), 61.8 (CH_2), 60.8 (CH_2), 52.4 (t, 2CH_3, *J* = 1.8 Hz, O*CH_3*), 20.9 (CH_3), 20.8 (CH_3), 20.7 (CH_3), 20.7 (CH_3), 20.7 (CH_3), 20.6 (CH_3), 20.5 (CH_3)

^{31}P NMR: δ 20.8

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{31}H_{46}O_{21}P$ $(M+H)^+$ 785.2269, found 785.2269

$[\alpha]_D$ = -10.3 (c = 1.0, $CHCl_3$)

Z isomer

^{1}H NMR: δ 6.58 (ddt, 1H, *J* = 5.6, 13.5, 52.0 Hz, *CH*=CH-P), 5.65 (ddt, 1H, *J* = 2.0, 13.5, 17.6 Hz, CH=*CH*-P), 5.34 (bd, 1H, $J_{3',4'}$ = 3.4 Hz, H-4'), 5.19 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, H-3), 5.10 (dd, 1H, $J_{1',2'}$ = 7.9 Hz, $J_{2',3'}$ = 9.4 Hz, H-2'), 4.94 (dd, 1H, H-3'), 4.90 (dd, 1H, $J_{1,2}$ = 8.0 Hz, H-2), 4.71-4.66 (m, 2H, O-*CH_2*-CH), 4.52 (d, 1H, H-1), 4.47 (d, 1H, H-1'), 4.16-3.04 (m, 3H, H-6a, H-6'a and H-6'b), 3.81 (bt, 1H, *J* = 7.3 Hz, H-5'), 3.80 (t, 1H, H-4), 3.78 (dd, 1H, $J_{5,6b}$ = 4.2 Hz, $J_{6a,6b}$ = 11.8 Hz, H-6b), 3.73 (d, 3H, *J* = 2.6 Hz, OMe), 3.70 (d, 3H, J = 2.6 Hz, OMe), 3.63-3.56 (m, 1H, H-5), 2.15, 2.13, 2.06, 2.05, 2.05, 2.04, 1.96 (7s, 21H, 7 OAc)

^{13}C NMR: δ 170.4 (C), 170.4 (C), 170.2 (C), 170.1 (C), 169.8 (C), 169.7 (C), 169.1 (C), 150.2 (d, CH, J = 3 Hz, CH=CH-P), 114.7 (d, CH, J = 183 Hz, CH-P), 101.1 (CH), 100.4 (CH), 77.2 (CH), 76.2 (CH), 72.7 (CH), 71.6 (CH), 71.0 (CH), 70.7 (CH), 69.0 (CH), 67.8 (d, CH_2, J = 7.5 Hz, CH_2CH=CH-P), 66.6 (CH), 61.9 (CH_2), 60.1 (CH_2), 52.3 (2d, 2CH_3, J = 0.7 Hz, OMe), 20.9 (CH_3), 20.8 (CH_3), 20.7 (CH_3), 20.7 (CH_3), 20.7 (CH_3), 20.6 (CH_3), 20.5 (CH_3)

^{31}P NMR: δ 18.3

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{31}H_{46}O_{21}P$ $(M+H)^+$ 785.2269, found 785.2260

$[\alpha]_D$ = -3.7 (c = 0.8, $CHCl_3$)

α-D-*galacto*-1,2:3,4-bis-*O*-(1-methylethylidene)-7-vinyl-E-dimethylphosponate 60a

A solution of propargyl-6-O-galactoside **60** (100 mg, 0.337 mmol) in dimethyl-phosphonate (3.1 mL, 33.7 mmol) was treated as described for the preparation of **59a** to give a crude product (E/Z ratio 50/50 by NMR) used for equilibration as described for **59a** to give after column chromatography on silica gel (AcOEt + 0.5% TEA) the product **60a** (114 mg, 83%) as syrup with an E/Z ratio of 95/5. analytical sample of pure Z was obtained from a crude

hydrophosphonylation mixture by column chromatography on silica gel with AcOEt + 0.5% TEA.

E isomer

^{1}H NMR: δ 6.74 (ddt, 1H, *J* = 3.6 Hz, 21.0, 26.0 Hz, *CH*=CH-P), 5.97 (ddt, 1H, *J* = 2.1, 19.0, 23.0 Hz, CH=*CH*-P), 5.50 (d, 1H, $J_{1,2}$ = 5.0 Hz, H-1), 4.59 (dd, 1H, $J_{2,3}$ = 2.4 Hz, $J_{3,4}$ = 8.0 Hz, H-3), 4.29 (dd, 1H, H-2), 4.23 (dd, 1H, $J_{4,5}$ = 1.9 Hz, H-4), 4.18 (ddt, 2H, *J* = 2.0 , 3.7, 14.0 Hz, O-CH_2-CH=CH), 3.99-3.93 (ddd, 1H, $J_{5,6a}$ = 5.6 Hz, $J_{5,6b}$ = 6.8 Hz, H-5), 3.71 (s, 3H, OMe), 3.68 (s, 3H, OMe), 3.68 (dd, 1H, $J_{6a,6b}$ = 10 Hz, H-6a), 3.59 (dd, 1H, H-6b), 1.52 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.31 (s, 3H, CH_3), 1.30 (s, 3H, CH_3)

^{13}C NMR: δ 149.7 (d, CH, *J* = 6.0 Hz, *CH*=CH-P), 115.1 (d, CH, *J* = 189.0 Hz, CH=*CH*-P), 109.3 (C), 108.6 (C), 96.3 (CH), 71.1 (CH), 70.6 (CH), 70.6 (d, CH_2, *J* = 20.0 Hz, CH_2-CH=CH-P), 70.5 (CH), 69.7 (CH_2), 66.8 (CH), 52.4 (d, CH_3, *J* = 1.7 Hz, P-OCH_3), 52.3 (d, CH_3, *J* = 1.7 Hz, P-OCH_3), 26.1 (CH_3), 25.9 (CH_3), 24.9 (CH_3), 24.4 (CH_3)

^{31}P NMR: δ 21.6

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{17}H_{29}NaO_9P$ $(M+Na)^+$ 431.1447, found 431.1450

$[\alpha]_D$ = -5.2 (c = 2.0, $CHCl_3$)

Z isomer

^{1}H NMR: δ 6.65 (ddt, 1H, *J* = 5.6, 13.6, 53.0 Hz, *CH*=CH-P), 5.63 (ddt, 1H, *J* = 2.0, 13.6, 18.3 Hz, CH=*CH*-P), 5.51 (d, 1H, $J_{1,2}$ = 5.0 Hz, H-1), 4.57 (dd, 1H, $J_{2,3}$ = 2.4 Hz, $J_{3,4}$ = 8 Hz, H-3), 4.47 (ddd, 2H, *J* = 2.0, 3.6, 5.6 Hz, O-CH_2-CH=CH), 4.29 (dd, 1H, H-2), 4.22 (dd, 1H, $J_{4,5}$ = 1.9 Hz, H-4), 3.97-3.92 (ddd, 1H, $J_{5,6a}$ = 4.5 Hz, $J_{5,6b}$ = 5.9 Hz, H-5), 3.71 (s, 3H, OMe), 3.68 (s, 3H, OMe), 3.64 (d, 1H, H-6a), 3.62 (d, 1H, H-6b), 1.52 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.31 (s, 3H, CH_3), 1.30 (s, 3H, CH_3)

^{13}C NMR: δ 151.7 (d, CH, *J* = 4.0 Hz, *CH*=CH-P), 114.3 (d, CH, *J* = 183.0 Hz, CH=*CH*-P), 109.3 (C), 108.6 (C), 96.3 (CH), 71.1 (CH), 70.6 (CH), 70.5 (CH), 69.5 (CH_2), 69.0 (d, CH_2, *J* = 8.0 Hz, CH_2-CH=CH-P), 66.6 (CH), 52.2 (CH_3), 52.1 (CH_3), 26.0 (CH_3), 25.9 (CH_3), 24.9 (CH_3), 24.4 (CH_3)

^{31}P NMR: δ 18.7

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{17}H_{30}O_9P$ $(M+H)^+$ 409.1627, found 409.1634

$[\alpha]_D$ = -24.3 (c = 1.5, $CHCl_3$)

β-D-2,3,4,6-tetraacetyl-*galacto*-vinyl-*E*-dimethylphosphonate 56a

A solution of propargyl-1-O-galactoside **56** (335 mg, 0.870 mmol) in dimethyl-phosphonate (6.0 mL, 65.4 mmol) was treated as described for the preparation of **59a** to give a crude product (E/Z ratio 50/50 by NMR) used for equilibration as described for **59a** to give after column chromatography on silica gel (AcOEt / cyclohexane 3:1 + 0.5% TEA) the product **56a** (110 mg, 78%) as syrup in pure E form. analytical sample of pure Z was obtained from a crude hydrophosphonylation mixture by column chromatography on silica gel with AcOEt / acetone 1:1 + 0.5% TEA.

E isomer

^{1}H NMR: δ 6.77 (ddt, 1H, *J* = 3.5, 17.0, 22.8 Hz, *CH*=CH-P), 5.91 (ddt, 1H, *J* = 2.0, 17.0, 20.0 Hz, CH=*CH*-P), 5.38 (dd, 1H, $J_{3,4}$ = 3.4 Hz, $J_{4,5}$ = 1.0 Hz, H-4), 5.25 (dd, 1H, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 10.5 Hz, H-2), 5.01 (dd, 1H, H-3), 4.52 (m, 1H, CH_2-CH=CH-P), 4.50 (d, 1H, H-1), 4.22 (m, 1H, CH_2-CH=CH-P), 4.16 (dd, 1H, $J_{5,6a}$ = 6.6 Hz, $J_{6a,6b}$ = 11.4 Hz, H-6a), 4.10 (dd, 1H, $J_{5,6b}$ = 6.6 Hz, $J_{6a,6b}$ = 11.4 Hz, H-6b), 3.90 (dt, 1H, H-5), 3.72 (d, 3H, *J* = 2 Hz, OMe), 3.70 (d, 3H, J = 2 Hz, P-OMe), 2.14, 2.06, 2.04, 1.98 (4s, 12H, 4 OAc)

^{13}C NMR: δ 170.4 (C), 170.2 (C), 170.1 (C), 169.4 (C), 147.9 (d, CH, *J* = 6.0 Hz, *CH*=CH-P), 115.8 (d, CH, *J* = 189.0 Hz, CH=*CH*-P), 100.7 (CH), 70.8 (CH), 70.7 (CH), 68.6 (CH), 68.4 (d, CH_2, *J* = 22.0 Hz, CH_2-CH=CH-P), 66.9 (CH), 61.2 (CH_2), 52.5 (d, CH_3, *J* = 5.6 Hz, OMe), 52.4 (d, CH_3, J = 5.6 Hz, P-OMe), 20.8 (CH_3), 20.7 (CH_3), 20.7 (CH_3), 20.6 (CH_3)

^{31}P NMR: δ 20.9

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{19}H_{30}O_{13}P$ $(M+H)^+$ 497.1424, found 497.1427

$[\alpha]_D$ = +10.9 (c = 1.2, $CHCl_3$)

Z isomer

^{1}H NMR: δ 6.60 (ddt, 1H, *J* = 5.9, 13.6, 52.3 Hz, *CH*=CH-P), 5.63 (ddt, 1H, *J* = 2.0, 13.6, 17.5 Hz, CH=*CH*-P), 5.36 (dd, 1H, $J_{3,4}$ = 3.4 Hz, $J_{4,5}$ = 1.0 Hz, H-4), 5.19 (dd, 1H, $J_{1,2}$ = 7.9 Hz, $J_{2,3}$ = 10.5 Hz, H-2), 4.99 (dd, 1H, H-3), 4.73-4.68 (m, 2H, CH_2-CH=CH-P), 4.51 (d, 1H, H-1), 4.15 (dd, 1H, $J_{5,6a}$ = 6.7 Hz, $J_{6a,6b}$ = 11.3 Hz, H-6a), 4.10 (dd, 1H, $J_{5,6b}$ = 6.7 Hz, H-6b), 3.89 (dt, 1H, H-5), 3.71 (d, 3H, *J* = 2.3 Hz, OMe), 3.69 (d, 3H, J = 2.3 Hz, OMe), 2.13, 2.04, 2.03, 1.96 (4s, 12H, 4 OAc)

^{13}C NMR: δ 170.4 (C), 170.3 (C), 170.1 (C), 169.5 (C), 150.3 (d, CH, *J* = 3.3 Hz, *CH*=CH-P), 116.1 (d, CH, *J* = 183.3 Hz, CH=*CH*-P), 101.0 (CH), 70.9 (CH), 70.7 (CH), 68.8 (CH), 67.9

(d, CH_2, *J* = 7.8 Hz, *CH_2*-CH=CH-P), 66.9 (CH), 61.6 (CH_2), 52.3 (d, CH_3, *J* = 2.3 Hz, OMe), 52.2 (d, CH_3, J = 2.3 Hz, OMe), 20.8 (CH_3), 20.7 (CH_3), 20.7 (CH_3), 20.6 (CH_3)

^{31}P NMR: δ 18.3

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{19}H_{29}NaO_{13}P$ $(M+Na)^+$ 519.1243, found 519.1249

$[\alpha]_D$ =+36.6 (c = 1.0, $CHCl_3$)

L-Cysteine, *N*-[(1,1-dimethylethoxy) carbonyl]-, methyl ester, S – vinyl- *E*-dimethylphosphonate 64a

A solution of cysteine-OMe-N-BOC **64** (115 mg, 0.420 mmol) in dimethyl-phosphonate (4.0 mL, 43.6 mmol) was treated as described for the preparation of **59a** to give a crude product (E/Z ratio 50/50 by NMR) used for equilibration with cysteine-OMe-N-BOC-S-propargyl (99 mg, 0.420 mmol) and DMPA (21 mg, 0.084 mmol) to give after column chromatography on silica gel (AcOEt + 0.5% TEA) the product **64a** (132 mg, 82%) as syrup in E pure form. Analytical sample of pure Z was obtained from a crude hydrophosphonylation mixture by column chromatography on silica gel with AcOEt + 0.5% TEA.

E isomer

^{1}H NMR: δ 6.69 (ddt, 1H, *J* = 6.9, 16.9, 20.8 Hz, *CH*=CH-P), 5.75 (dd, 1H, *J* = 19.5, 16.9 Hz, CH=*CH*-P), 5.32 (bd, 1H, *J* = 7.5 Hz, *NH*), 4.52 (bdt, 1H, *J* = 5.5, 7.3 Hz, *CH_α*), 3.78 (s, 3H, COOMe), 3.75 (d, 3H, *J* = 1.8 Hz, P-OMe), 3.72 (d, 3H, *J* = 1.8 Hz, P-OMe), 3.30-3.25 (m, 2H, S-*CH_2*-CH=CH-P), 2.93 (dd, 1H, *J* = 4.8, 13.9 Hz, *$CH_{\beta 1}$*), 2.82 (dd, 1H, *J* = 5.5, 13.9 Hz, *$CH_{\beta 2}$*), 1.45 (s, 9H, OtBu).

^{13}C NMR: δ 171.3 (C), 155.1 (C), 147.8 (d, CH, *J* = 5.8 Hz, *CH*=CH-P), 118.4 (d, CH, *J* = 187.0 Hz, CH=*CH*-P), 80.3 (CH), 52.7 (CH_3, COOMe), 52.5 (d, CH_3, *J* = 0.8 Hz, P-OMe), 52.4 (d, CH_3, *J* = 0.8 Hz, P-OMe), 34.7 (d, CH_2, *J* = 25.0 Hz, *CH_2*-CH=CH-P), 33.3 (CH_2), 28.3 (3 CH_3)

^{31}P NMR: δ 19.9

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{14}H_{26}NaNO_7PS$ $(M+Na)^+$ 406.1065, found 406.1071

$[\alpha]_D$ =-6.8 (c =1.0 ; $CHCl_3$)

Z isomer

^{1}H NMR: δ 6.50 (ddq, 1H, *J* = 7.6, 8.6, 12.8, 51.6 Hz, *CH*=CH-P), 5.70 (bs, 1H, *NH*), 5.65 (ddt, 1H, *J* = 1.0, 12.8, 17.3 Hz, CH=*CH*-P), 4.54 (bdt, 1H, *J* = 7.5, 4.7 Hz, *CH_α*), 3.75 (s, 3H, COOMe), 3.82-3.70 (m, 1H, S-*CH_2*-CH=CH-P), 3.75 (d, 3H, J = 1.3 Hz, P-OMe), 3.72 (d,

3H, J = 1.3 Hz, P-OMe), 3.52 (dddd, 1H, *J* = 1.2, 2.8, 7.5, 13.9 Hz, S-CH_2-CH=CH-P), 3.01 (dd, 1H, *J* = 4.7, 13.9 Hz, $CH_{\beta 1}$), 2.89 (dd, 1H, *J* = 7.0, 13.9 Hz, $CH_{\beta 2}$), 1.45 (s, 9H, tBu).

^{13}C NMR: δ 171.3 (C), 155.1 (C), 147.8 (d, CH, *J* = 5.8 Hz, *CH*=CH-P), 118.4 (d, CH, *J* = 187.0 Hz, CH=*CH*-P), 80.3 (CH), 52.7 (CH_3, COOMe), 52.5 (d, CH_3, *J* = 0.8 Hz, P-OMe), 52.4 (d, CH_3, *J* = 0.8 Hz, P-OMe), 34.7 (d, CH_2, *J* = 25.0 Hz, CH_2-CH=CH-P), 33.3 (CH_2), 28.3 (3 CH_3)

^{31}P NMR: δ 18.6

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{14}H_{26}NaNO_7PS$ $(M+Na)^+$ 406.1065, found 406.1067

$[\alpha]_D$ = -45.5 (c =1.0 ; $CHCl_3$)

1-decen-*E*-dimethylphosphonate 65a

A solution of 1-decyne **65** (100 mg, 0.723 mmol) in dimethyl-phosphonate (6.5 mL, 70.9 mmol) was treated as described for the preparation of **59a** to give a crude product (E/Z ratio 40/60 by NMR) used for equilibration as described for **59a** to give after column chromatography on silica gel (AcOEt / cyclohexane 3:2 + 0.5% TEA) the product **65a** (102 mg, 57%) as syrup in pure E form and a mixture of E and Z forms (30 mg E/Z 1:3). Analytical sample of pure Z was obtained from a crude hydrophosphonylation mixture by column chromatography on silica gel with AcOEt / cyclohexane 1:1 + 0.5% TEA.

E isomer

^{1}H NMR: δ 6.81 (ddt, 1H, *J* = 6.6, 17.1, 22.0 Hz, *CH*=CH-P), 5.61 (ddt, 1H, *J* = 1.5, 17.1, 21.4 Hz, CH=*CH*-P), 3.73 (s, 3H, OMe), 3.70 (s, 3H, OMe), 2.27-2.18 (m, 2H), 1.49-1.39 (m, 2H), 1.36-1.19 (m, 10H), 0.88 (t, 3H, *J* = 7.1 Hz)

^{13}C NMR: δ 155.2 (d, CH, *J* = 4.4 Hz, *CH*=CH-P), 115.2 (d, *J* = 188.1 Hz, CH=*CH*-P), 55.3 (CH_3), 55.2 (CH_3), 31.8 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 27.4 (CH_2), 27.4 (CH_2), 22.7 (CH_2), 14.1 (CH_3)

^{31}P NMR: δ 21.9

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{12}H_{26}O_3P$ $(M+H)^+$ 249.1620, found 249.1623

Z isomer

^{1}H NMR δ 6.51 (ddt, 1H, *J* = 7.7, 13.0, 53.5 Hz, *CH*=CH-P), 5.54 (dd, 1H, *J* = 13.0, 20.0 Hz, CH=*CH*-P), 3.71 (s, 3H, OMe), 3.70 (s, 3H, OMe), 2.55-2.45 (m, 2H), 1.46-1.36 (m, 2H), 1.36-1.16 (m, 10H), 0.86 (t, 3H, *J* = 7.0 Hz)

^{13}C NMR: δ 155.4 (d, *J* = 4.8 Hz, *CH*=CH-P), 114.7 (d, *J* = 184.6 Hz, CH=*CH*-P), 52.1 (CH_3), 52.0 (CH_3), 31.8 (CH_2), 30.8 (d, CH_2, *J* = 8.0 Hz, *CH_2*-CH=CH-P), 29.4 (CH_2), 29.2 (CH_2), 28.9 (CH_2), 28.9 (CH_2), 22.7 (CH_2), 14.1 (CH_3)

^{31}P NMR: δ 20.4

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{12}H_{26}O_3P$ $(M+H)^+$ 249.1620, found 249.1623

2,3,4,6-acetyl-1-C-vinylphosphonate-glucose 62a

A solution of 1-C-acetylen-glucose **62** (100 mg, 0.281 mmol) in dimethyl-phosphonate (2.6 mL, 28.1 mmol) was treated as described for the preparation of **59a** to give a crude product (E/Z ratio 40/60 by NMR) containing 20% of starting material. The mixture of alkenes is purified by column chromatography on silica gel with AcOEt / acetone 8:1 + 0.5% TEA and the mixed fraction is equilibrate with cysteine (51 mg, 0.421 mmol) and DMPA (14 mg, 0.054 mmol) to give after column chromatography on silica gel with AcOEt / acetone 8:1 the product **62a** (87 mg, 66%) in pure E form as a syrup and residual Z form syrup (29 mg, 22%). Analytical sample of pure Z was obtained from a crude hydrophosphonylation mixture by column chromatography on silica gel with AcOEt / acetone 8:1 + 0.5% TEA.

E isomer

^{1}H NMR: δ 6.61 (ddd, 1H, *J* = 4.6, 17.2, 22.0 Hz, *CH*=CH-P), 6.00 (ddd, 1H, *J* = 1.6, 17.2, 19.2, CH=*CH*-P), 5.24 (t, 1H, *J* = 9.4 Hz, H-3), 5.09 (t, 1H, *J* = 9.8 Hz, H-4), 4.90 (t, 1H, *J* = 9.8 Hz, H-2), 4.25 (dd, 1H, $J_{5,6a}$ = 4.8 Hz, $J_{6a,6b}$ = 12.4 Hz, H-6a), 4.14 (dd, 1H, $J_{5,6b}$ = 2.2 Hz), 4.09-4.03 (m, 1H, H-1), 3.77-3.69 (m, 1H, H-5), 3.74 (d, 3H, *J* = 2.2 Hz, OMe), 3.71 (d, 3H, *J* = 2.2 Hz, OMe), 2.10, 2.05, 2.04, 2.01 (4s, 12H, 4 OAc)

^{13}C NMR: δ 170.7 (C), 170.4 (C), 169.4 (C), 169.4 (C), 145.6 (d, *J* = 6.5 Hz, *CH*=CH-P), 118.9 (d, *J* = 188.0 Hz, CH=*CH*-P), 77.2 (CH), 75.7 (CH), 73.9 (CH), 71.1 (d, *J* = 1.7 Hz, C-1), 68.1 (CH), 62.1 (CH_2), 52.6 (d, *J* = 5.5 Hz, OMe), 52.5 (d, *J* = 5.5 Hz, OMe), 20.8, 20.7, 20.7, 20.6 (4 OAc)

^{31}P NMR: δ 19.5

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{18}H_{28}O_{12}P$ $(M+H)^+$ 467.1318, found 467.1322

$[\alpha]_D$ = +13.4 (c =0.8 ; $CHCl_3$)

Z isomer

^{1}H NMR δ 6.36 (ddd, 1H, *J* = 8.9, 13.2, 51.0 Hz, *CH*=CH-P), 5.81 (ddd, 1H, *J* = 0.6, 13.2, 15.8, CH=*CH*-P), 5.31 (t, 1H, *J* = 9.4 Hz, H-3), 5.09 (t, 1H, *J* = 9.5 Hz, H-4), 5.06 (bt, 1H, J = 9.9Hz, H-1), 4.99 (t, 1H, *J* = 9.5 Hz, H-2), 4.26 (dd, 1H, $J_{5,6a}$ = 4.5 Hz, $J_{6a,6b}$ = 12.5 Hz, H-6a), 4.12 (dd, 1H, $J_{5,6b}$ = 2.1 Hz), 3.83-3.78 (m, 1H, H-5), 3.77 (d, 3H, *J* = 2.6 Hz, OMe), 3.75 (d, 3H, *J* = 2.6 Hz, OMe), 2.07, 2.02, 2.01, 1.99 (4s, 12H, 4 OAc)

^{13}C NMR: δ 170.7 (C), 170.1 (C), 169.9 (C), 169.6 (C), 146.4 (d, *J* = 3.0 Hz, *CH*=CH-P), 121.5 (d, *J* = 184.1 Hz, CH=*CH*-P), 77.3 (CH), 75.6 (CH), 74.0 (d, *J* = 7.8 Hz, C-1), 73.6 (CH), 68.4 (CH), 62.0 (CH_2), 52.6 (d, *J* = 5.6 Hz, OMe), 52.1 (d, *J* = 5.6 Hz, OMe), 20.8, 20.7, 20.6, 20.6 (4 OAc)

^{31}P NMR: δ 16.9

HRMS (ESI/Q-TOF) *m/z* calcd for $C_{18}H_{28}O_{12}P$ $(M+H)^+$ 467.1318, found 467.1331

$[\alpha]_D$ = -22.6 (c =0.5 ; $CHCl_3$)

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SECTION II

Applications of Hydrotiolation

5. APPLICATION FOR MULTIVALENT CLUSTERS

5.1. Multivalency. A brief introduction

The definition of multivalency regards the simultaneous interaction between multiple functionalities on one entity and complementary functions on another. Multivalency is a self-assembly pathway that combines advantages like reversibility, self-sorting and self-correction with the possibility to achieve thermodynamic and/or kinetic stability at very low concentrations[1] (Figure 8).

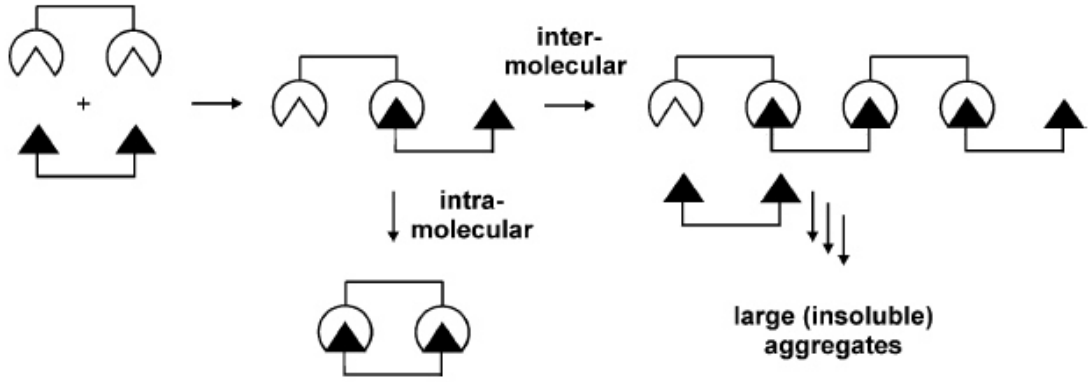


Figure 8: Multivalent interactions

Multivalency has become a central topic in scientific fields since it became clear that it governs many biological interactions[2] as, for example, interactions between proteins and carbohydrates. Binding of viruses, bacteria or other pathogens to cell membranes happens through recognition of a saccharidic portion by carbohydrate-binding proteins like lectins.[3–19] From these first observations multivalency concept has been successfully applied to the development of inhibitors with extremely low dissociation constants.[20,21]

Multivalency describes the binding of two or more entities that involves the simultaneous interactions between multiple, complementary functionalities on these entities.[22] It is important to define the concept of valency: the valency of an entity is the number of separate connections of the same kind that it can form through host-guest interactions with complementary functions. When two complementary entities form a complex, the valency of the complex is the number of shared interactions between two single compounds. All host-guest interaction with more than one connection are considered multivalent.[23]

A second crucial point to underline is the formation process of multivalent complexes. In fact after first connection is established, the second binding does not necessarily occur in a

multivalent fashion (intra-molecular), but it can also go to form an inter-molecular interaction giving a sort of polymer chain core (Figure 8).[24] It is clear that complex architecture, single entity geometry and process thermodynamic are the fundamental aspects of this issue.

When one part is a biological rigid compound like a protein, with a low density of three-dimensionally arranged host sites, it is more probable to have an inter-molecular binding with multivalent molecules in solution; on the other hand, if the biological target is linear along the same axis, this permits the easy formation of the intra-molecular complex.[25–27]

After spatial and architectural consideration, thermodynamic drives the multivalent complex formation according to enthalpy and entropy. There are a large number of papers dealing with calculations of binding free energy in multivalent complex, but it is possible to assume the global free energies (ΔG^0) affected from both enthalpy (ΔH^0) and entropy (ΔS^0) in every case.

A traditional view of the multivalent complex formation is that it is governed mainly by entropy, with binding enthalpy assumed to be proportional to the number of interactions. In this model entropy drives also the mode of binding.[28,29] A binding is always a loss in terms of entropy, but if the intra-molecular binding enthalpy is able to balance that loss, the entire process goes to intra-molecular binding formation. In this philosophy the implication is that multivalent bindings are associated with favourable entropies compared to corresponding number of multiple monovalent interactions. This is in sharp contrast with the large negative entropy terms that are typically found for multivalent systems.

Effective concentration

Effective concentration represents the probability of interaction between two counterparts already interlinked in one site. It symbolize the "real" concentration of one of the entities as experienced by its counterpart[30]. (Figure 9)

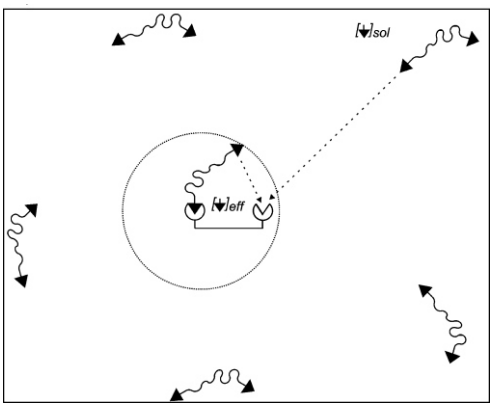


Figure 9: A schematic representation of "effective concentration" concept

As shown in Figure 9, after the first binding is established there is an alteration in the concentration experienced by the second subunit in case of an intra- or inter-molecular second binding. The circle line around the first bond is a region where the effective concentration of ▲must be compared to the [▲] in solution; if $[▲]_{eff}$ is higher than $[▲]_{sol}$ an intra-molecular binding will take place, the other way around an inter-molecular one will be more probable.

Despite problems associated with the exact determination of C_{eff} it is predicted that it depends on the inverse cube of the linker length, which is explained by the increase of the probing volume accessible by an uncomplexed guest site upon increase of the linker length.[31–33] In conclusion, this explains logically that with low concentration intra-molecular binding is more probable and inter-molecular become more probable linearly with concentration.

Multivalency and cooperativity

Assessment of cooperativity in multivalent systems is notoriously difficult and there are numerous examples in literature where multivalent binding is declared negatively or positively cooperative in a wrong way simply because this assessment was based on mono- or multivalent association constants.

Cooperativity has been defined for consecutive monovalent interactions at a multivalent platform, but it is still not completely clear how to establish if a multivalent interaction is also positive or negative cooperative.[34] Ercolani and coworkers[35] have clearly exemplified that cooperativity in multivalent interactions can only be assessed by considering the inter- and intra-molecular processes separately and independently (*i.e.* only if equilibrium constants have same dimensions). In conclusion, it appears that cooperativity in multivalent system is often extremely scarce; one example of positive cooperativity is the self-assembly of DNA double helix, however the majority of synthetic multivalent systems reported in literature presents a negative cooperativty.[36]

Kinetics

It results quite clear that kinetics of multivalent interactions are fundamentally different from monovalent ones. The overall dissociation rate is determined by the dissociation rate of the monovalently bound species of the multivalent complex. (Figure 10), this rate can be assumed equal to the intrinsic dissociation rate constant of the corresponding monovalent interaction. In the end the dissociation rate constant is dependent on the effective concentration, because the monovalent specie exists only in function of the formation of the divalent one and in

function of the complete dissociation. This means that complex, made by multivalent interactions, can be made to combine high affinity, kinetic control and reversibility.[37]

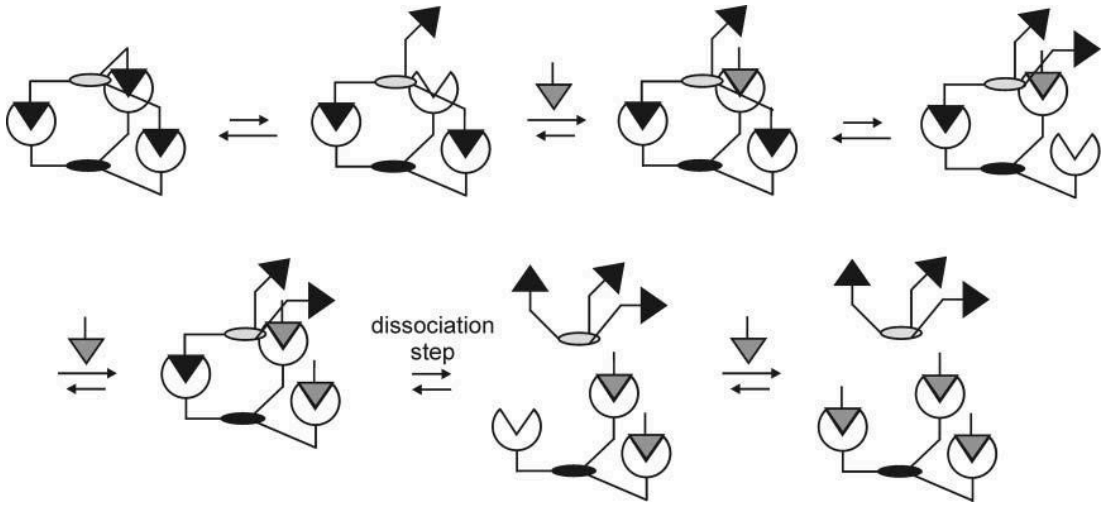


Figure 10: Stepwise dissociation of a multivalent complex by competition with a monovalent guest functionality.

5.2. Multivalency in Biological Systems

Some relevant biological examples of multivalent interaction will be illustrated in order to clarify the huge importance of this concept both for therapeutic and diagnostic studies. In biological systems interactions between different cells are driven by multivalent recognition of sugars, proteins or glyco-proteins; cell adhesion is attributed to multiple linking of glyco-proteins present on cell membrane, cell infection is due to a first recognition of pathogen by cell surface lectins or carbohydrates and so on.

Influenza virus adhesion and infection is a perfect example of this mechanism[38,39] (Figure 11). In the first step the virus attacks the cell membrane through a multiple interaction of a lectin present on virus surface and the final unit of saccharidic chains present on target cell membrane, that usually is represented by a sialic acid unit. Other connections take place after the first one to link strongly the virus and the cell before endocytosis and consequently the infection. Many viruses act in this way, using sugars present on target cells as a first step for infection.[40,41]

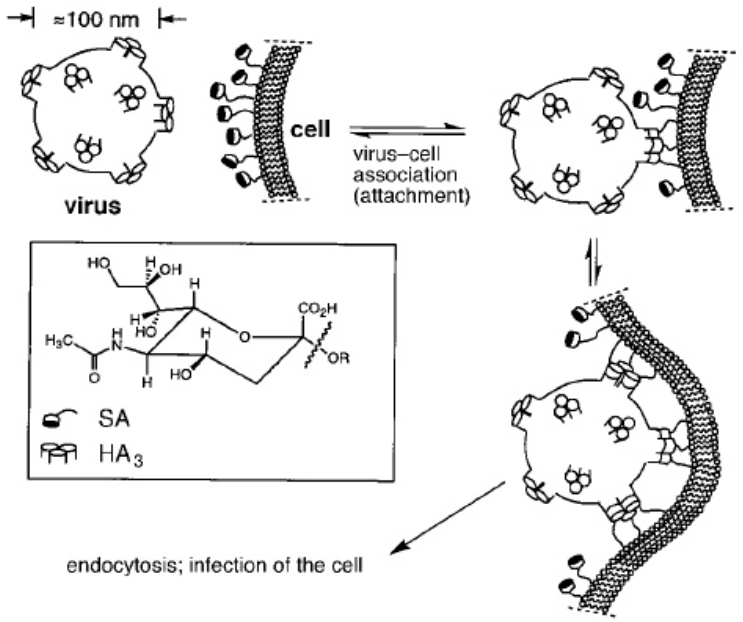


Figure 11: Virus typical way of adhesion and infection

Bacteria usually follow the same adhesion way of virus, however, sometime a mediator can be present to interact with both target cell and pathogen, in order to hide the risk of the bacterium or virus to the cell.[42,43]

Another application of multivalency is the adhesion of neutrophils to arterial endothelial cells.[44,45] Neutrophils are initially suspended in the flowing blood and they are moving faster,

then the interaction with particular selectins expressed on epithelial cell only during inflammation makes them go slower and closer to target cells. After that neutrophils start to roll over the cell and to migrate in extra-vessel space passing from the cell-cell lateral space (Figure 12). Interactions between selectins and glyco-protein containing the tetrasaccharide sialyl Lewis X (sLe^x) is one of the most important and investigated in modern pharmaceutical chemistry.[46,47]

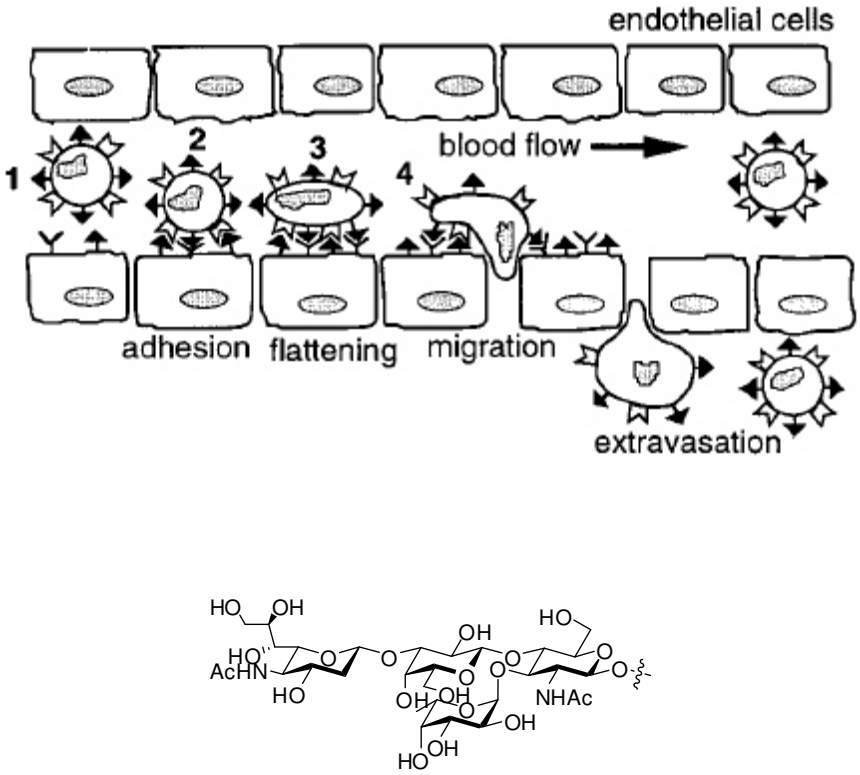


Figure 12: Adhesion and migration of neutrophils in inflammation situation (above) and structure of sialyl Lewis X (sLe^x)

Last, but not least, example of natural multivalent system is the antibodies-macrophages system.[48,49] The single, uncomplexed, antibody is not able to interact with macrophages, but when a virus, a bacterium, a drug or a "non-self" cell is surrounded by several antibodies linked through mannose-mannose interaction, the multiple antibodies system become able to interact with the macrophage and to drive to degradation the pathogen[50,51] (Figure 13).

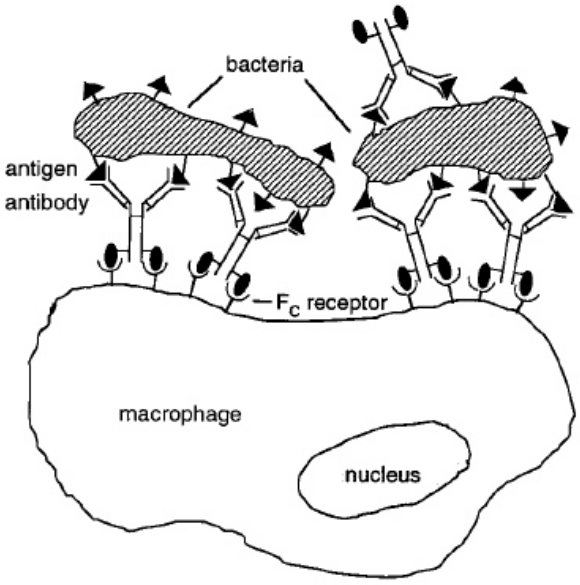


Figure 13: Antibodies-macrophage system

In conclusion it results that investigations about carbohydrates multivalency are very attractive for chemists for the potential applications of synthetic clusters. To this aim, many research groups have developed new techniques to obtain different multivalent molecules to test for disparate purposes.

5.3. Octasilsequioxane: an useful scaffold for multivalent compounds

Moving from all these considerations and evidences our interest focused on potential applications of radical couplings to multivalent systems synthesis. The main principles of multivalent chemistry are simple: a good cluster is able to support a fixed number of molecules, is highly reactive when it must couple a large number of molecules in order not to leave any intermediates with an unknown number of functionalities, is typically inactive if not functionalized and it confers good pharmacological and toxicological properties to the final compound. Also the synthetic tool, usually a coupling, must be efficient, regio- and stereo-selective and must not leave any toxic traces in the compound after purification. The right combination of a good scaffold, a good synthetic method and a good epitope gives extraordinary properties to multivalent synthetic compounds.

Polyhedral oligomeric silsesquioxanes[52,53] (POSS – Figure 14) are receiving a lot of attention for the rigid globular architecture and the precise clustering of eight ligands in space. Thus, POSS can serve as nanobuilding blocks for constructing functional materials,[54–60] as supports for organometallic catalysts,[61] and as biocompatible drug carriers.[62,63] POSS-derived materials exhibited no significant cell toxicity demonstrating their potential as biomaterials.

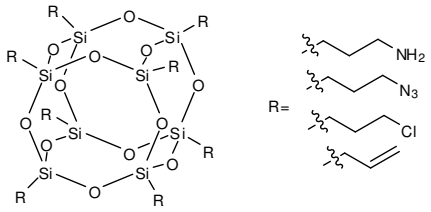


Figure 14: Polyhedral oligomeric silsesquioxanes (POSS)

Starting materials for construction of more complex POSS derivatives are based on reactive functional groups like amino, azido, chloro or vinyl at the periphery that are commercially available or can be obtained from simple organosilicon precursors.

Historically, the first approach was proposed by Feher,[64,65] who used an octa-amino POSS to obtain series of peptidyl and glycosyl POSS by standard amine coupling of *N*-protected peptides or sugar lactones. Unfortunately this method presented serious disadvantages like the scarce availability of the starting octa-amine POSS, low yields for the amine coupling and the difficulties to achieve a complete conjugation to each apex of the cluster.

An efficient coupling, needed to avoid difficult separation of partially functionalized products, has driven research to novel approaches. For instance, Fessner[66] and Chiara[67] proposed the octa-azido POSS as starting material for CuAAC.[68] Unfortunately octa-azido POSS is known to be a very hazardous compound due to the formation of azidomethane as by-product during its preparation, moreover, the copper traces are not easily removable from the product leading to molecules not compatible with bio-organic purpose.[69]

To overcome these drawbacks, metal-free couplings have been widely investigated and different solutions[70–78] have been found for every product or situation. In our studies the metal-free photoinduced radical addition of thiol to alkenes and alkynes[79,80] have been used as ligation tools. TEC and TYC are known to be induced by a small amount of photoinitiator and an irradiation at wavelength close to visible light (365 nm) that is known to be compatible with organic molecules like proteins and carbohydrates.[81–84] In addition, regio- and stereoselectivity of these couplings are very important features, and the only by-product is a disulphide if the corresponding thiol is used in excess. The orthogonality respect to several protecting groups is another crucial characteristic that make these additions very interesting tools for multivalent cluster synthesis.

Thiol-ene functionalization of POSS derivatives

The first work about thiol-ene on POSS was published by Lee and coworkers[85] in 2004 and reported on the conjugation of *N*-mannosyl and *N*-lactosyl γ-thiobutyramides with octa-vinyl POSS. In another study[86] was reported the addition of a glucose unit to a hepta-vinyl POSS-polylactide conjugate (VPOSS-PLLA) *via* thiol-ene coupling, but no validation or extension of this methodology were done before the publication of our investigation.

An extension of the potential application of TEC was reported on two different types of POSS (Figure 15), the vinyl derivative and the PEGylated one that bears the alkene moieties farer from the core. Couplings were carried out with either peptides and sugars and selected products were evaluated for their inhibition properties toward lectins. This study permitted to validate efficiency of thiol-ene conditions, to optimize purification processes, to speculate about importance of spatial configuration of multivalent clusters and to find products with significant inhibition properties.

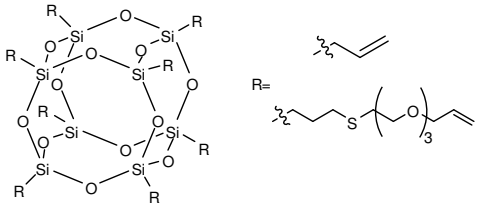
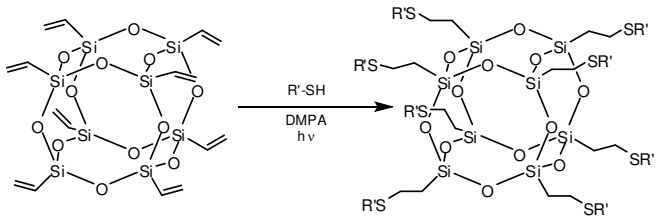


Figure 15: Two POSS derivatives used for thiol-ene couplings

First, an optimization study was carried out using octavinyl POSS and 1-thio-β-D-glucopyranose[87] **7** as a model, but only partially substituted products were found in reaction mixture despite of the thiol excess used (1.5-4 eq. per -ene). Very likely, the steric congestion prevented the complete conjugation on every apex of the clusters, thus the first solution was to insert a small spacer using *C*-glucosylpropyl thiol[88] **66**. This second attempt gave the product in quantitative yield (by ^{1}H-NMR) after 1 h of irradiation. The desired product was separated from the disulphide by column chromatography on Sephadex LH-20. Another sugar thiol (the *C*-mannosylpropyl **67**) was coupled with octavinyl POSS to obtain in the same conditions quite identical results; i.e. complete conjugation of each apex of the silsequoxane core. Single signal in ^{29}Si NMR spectroscopy shows without doubts that integrity of the central core of the cluster is maintained unaltered during coupling and purification process, so the method has demonstrated to be solid and efficient in all aspects of the issue.

Other groups used POSS derivatives as clusters for peptidic conjugation, employing techniques, like CuAAC,[89] not fully compatible with those compounds. In order to use TEC

as ligation tool, cysteine containing molecules were, evidently, our targets, so we carried out coupling with commercially available cysteine hydrochloride ethyl ester **68**, glutathione **69** (Glu-Cys-Gly) and RGDC peptide **70** (Arg-Gly-Asp-Cys) (Table 7).



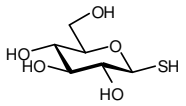
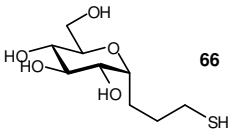
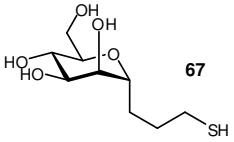
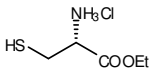
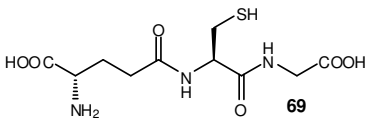
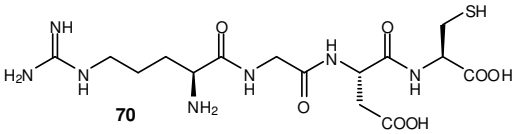
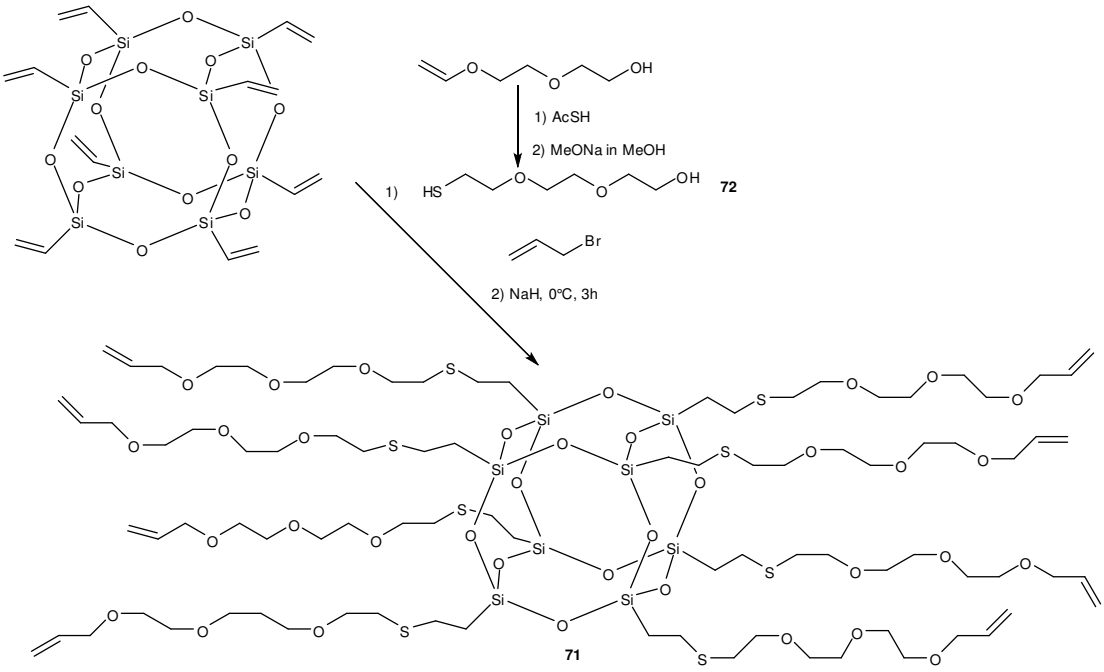
Thiol	Thiol eq./ene	Solvent	Time	Yield
 **7**	1.5-4.0	DMF	1h	———
 **66**	2	DMF-THF	1h	94%
 **67**	2	DMF-THF	1h	93%
 **68**	1.5	DMF	45min	84%
 **69**	1.5	DMF	45min	75%
 **70**	3	DMF-H_2O	2h	———

Table 7: Thiol-ene coupling based conjugations on octavinyl POSS.

Cysteine afforded good results even using smaller excess and shorter reaction time, glutathione in same condition was less reactive but still gave satisfactory results. On the other hand, the tetra-peptide RGDC did not afford any product also increasing both irradiation time and thiol equivalents per alkene unit.

All products structures were verified by MS analysis in order to be sure about the complete hydrothiolation of every apex of the cluster and to verify the purification of the target molecules before the inhibition tests.

The problems due to steric hindrance pushed us to introduce a spacer in the sugar thiols and also a spacer onto the POSS derivative to improve some features like water solubility and biocompatibility. For that reason a PEGylated octaallyl derivative **71** was prepared from octavinyl POSS simply by thiol-ene coupling with a PEG-thiol **72** (synthesized from commercial vinyl-PEG and thioacetic acid), followed by a nucleophilic attack of free hydroxyl POSS to allyl bromide[90,91] (Scheme 29). The new scaffold was used to couple 1-thio-β-D-glucopyranose **7** in order to prove the difference from the first case, when it was not effective for steric hindrance problems. This time the addition gave the corresponding product in high yield and, more important, without incomplete sustitution or purification problems.



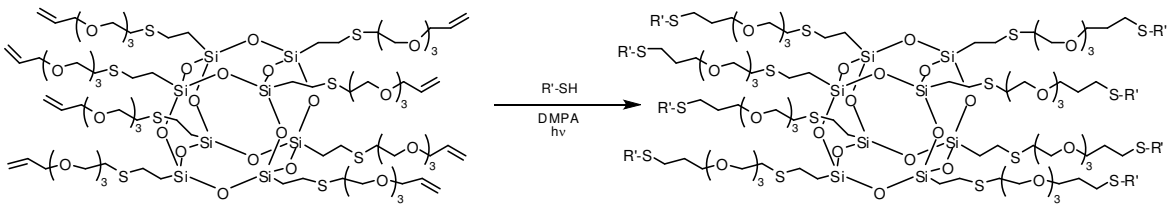
Scheme 29: Syntesis of PEGylated octaallyl POSS.

Then, another sugar thiol, namely 1-thio-β-D-glucosamine **73**, was used to validate the methodology. It gave almost the same yield of the corresponding octavalent glyco-conjugate. Lactosyl thiol **74** was also tested to extend the study to more bulky carbohydrates; in this case the total yield is lower than others sugars, but only the complete conjugation product was found as confirmation of the validity of TEC as ligation tool. The lower yield is probably due to purification problem by Sephadex column chromatography, which is based on polysaccharides that can interact with highly functionalized products. The purification step can likely be improved, but in our work no more efforts were made in this direction.

To complete PEGylated POSS library, couplings with glutathione **69** and with tetrapeptide RGDC **70** were carried out under the same conditions as above apart from the use of the hydrochloride salt of glutathione to avoid solubility problems. Results of all these couplings are shown in Table 8.

All couplings were effective and gave corresponding clusters with satisfactory to high yield in pure isolated form. Following the same trend of couplings on octavinyl POSS, larger are the substituents and lower is the global yield, particularly in the purification process. Hydrogen interactions between saccharidic or peptidic units and dextran matrix of Sephadex column are stronger with lactose than with glucose and with RGDC than with glutathione. All pure products were characterized by NMR spectroscopy, elemental analysis and MALDI-TOF MS before submission to the enzymatic inhibition test with specific lectins for each sugar.

Two lectins were chosen to test glycoconjugates: the concanavalin A (ConA), from *Canavalia ensiformis*, which is specific for the α-D-mannopyranosides and, to a lesser extent, the α-D-glucopyranosides, and the wheat germ agglutinin (WGA), from *Triticum vulgaris*, which is specific for N-acetyl-D-glucosamine (D-GlcNAc).



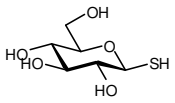
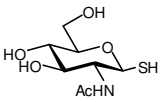
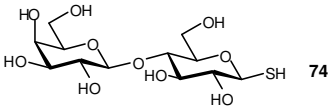
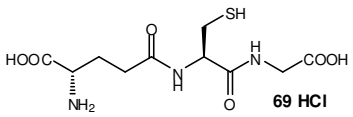
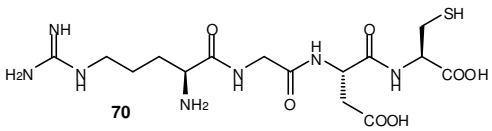
Thiol	Thiol eq./ene	Solvent	Time	Yield
 **7**	3	MeOH-DMF-H_2O	1h	79%
 **73**	3	DMF-H_2O	1h	82%
 **74**	3	DMF-H_2O	1h30min	50%
 **69 HCl**	3	MeOH	1h	78%
 **70**	3	MeOH	1h	61%

Table 8: TEC based conjugations on octa-allyl PEGylated POSS

First, the ability of glucosylated and mannosylated glycoclusters (from octavinyl POSS) to inhibit the binding of horseradish peroxidase-labelled ConA (ConA-HRP) to an α-D-mannose-polyacrylamide conjugate (α-D-Man-PAA) was measured by an Enzyme-Linked Lectin Assay (ELLA)[92] following a previously reported procedure. Methyl α-D-mannopyranoside (Me α-D-Man) and methyl α-D-glucopyranoside (Me α-D-Glc) were used as monovalent references.

A similar assay was performed with WGA and PEGylated POSS-based GlcNAc cluster as the inhibitor while 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and glucosylated glycocluster were used as the monovalent reference and the negative control, respectively. The results are shown in Tables 9 and 10 and in Figure 16.

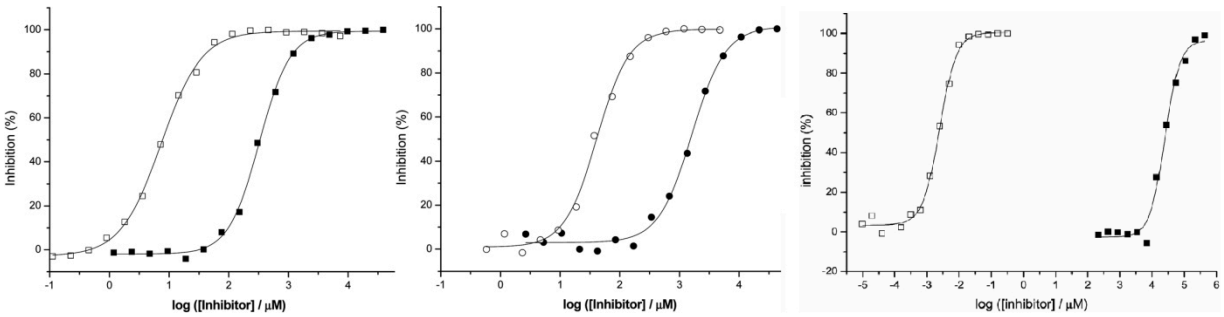


Figure 16: Inhibition curves of methyl α-D-mannopyranoside (■) and mannosylated glycocluster 8(□) (Left) Inhibition curves of methyl α-D-glucopyranoside (●) and glucosylated glycocluster (◯) (Centre) Inhibition curves for the binding of WGA-HRP to D-GlcNAc-PAA by GlcNAc (■) and glycocluster (□). (Right)

Product	**n[b]**	**IC_{50} (µM)[a]**	**rp[c]**	**rp/n[d]**
Me α-D-Glc	1	1422±129	1	1
Glucosylated glycocluster	8	40.4±0.7	35.2	4.4
Me α-D-Man	1	328±27	1	1
Mannosylated glycocluster	8	6.8±0.9	48.2	6

Table 9: ELLA data for the inhibition of the binding of ConA-HRP to α-D-Man-PAA with glucosylated or mannosylated glycoclusters. **Notes.** a) Each experiment was carried out in triplicate b) Number of sugar units in the molecule c) Relative potency = IC_{50} (mono)/ IC_{50} (cluster) d) Relative potency/number of sugars.

Product	**n[b]**	**IC_{50} (µM)[a]**	**rp[c]**	**rp/n[d]**
D-GlcNAc	1	28000±2500	1	1
Glucosamine glycocluster (PEG)	8	0.003±0.0006	9.6×10^6	10^6
Glucosylated glycocluster (PEG)	8	No inhibition	---	---

Table 10: ELLA data for the inhibition of the binding of WGA-HRP to D-GlcNAc-PAA with PEGylated POSS-based glycoclusters **Notes:** a,b,c,d as table 9. e) no inhibition detected at 100 µM.

In the first case both glycoclusters show modest inhibitory properties with absolute IC_{50} values of 40 and 7 µM for the glucosylated and mannosylated derivatives, respectively. In terms of relative potency compared to corresponding monosaccharides the cluster effect is around 40 and, if compared to sugar units, it decrease to 4.4 and 6.

On the other hand the second test gave very important results: glucosamine-PEGylated glycocluster had an IC_{50} of 3 nM and relative potency 10^6 time higher than monovalent reference. The role of sugar is underlined by the negative control with a glucosylated cluster that did not show inhibition at 100 µM. Since it differs from the other only for a hydroxyl

function that means that the lectin binding depends exclusively on the sugar units while the POSS core is not affecting the interaction.

Spatial proximity of linking sites of Concanavalin A (ca. 65 Å) is well-known in literature[93,94] and the steric hindrance of glucosyl and mannosyl clusters apexes could drive multivalent interactions to be efficient only in part. On the opposite, WGA is a dimeric protein with a total of eight binding sites separated by 14 Å, but insertion of PEG spacer gives to glucosamine cluster more flexibility and allows it to reach every sites of the lectin.[95–98]

In conclusion, it appears clear the importance of an efficient ligation tool to synthesize libraries of multivalent clusters, because only binding tests can discriminate between active and inactive compounds. The ELLA test is only a preliminary experiment but gives very precious informations about inhibition properties of new compounds, studies on promising molecules can go further with other assays like isothermal titration calorimetry (ITC) or surface plasmon resonance (SPR).

Thiol-yne functionalization of POSS derivatives

The exciting results of thiol-ene coupling based clusterization of POSS derivatives with carbohydrates have stimulated us to go further into more detailed investigations. In a second study a library of sugars have been supported on PEGylated POSS propargyl derivative **75** obtained following the same procedure used for synthesis of **71** but changing the allyl bromide with propargyl bromide in the final step (Figure 17).

The use of thiol-yne coupling as ligation tool allows to obtain in efficient way very densely glycosylated clusters starting from simple sugars thiols and easily available alkyne platforms. The PEG spacer was used also for this POSS derivative to maintain positive effects in terms of water solubility, biocompatibility and space congestion. In fact, as resulted evident from previous work, a spacer that separates core and binding apexes of the cluster is crucial to allow interactions in a complete multivalent fashion.

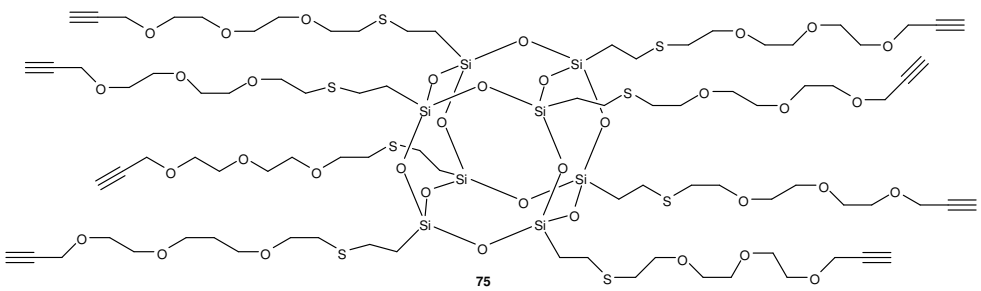


Figure 17: PEGylated POSS propargyl derivative

The well-known TYC radical mechanism[99–106] (Scheme 13) drives, in excess of thiol reactant, to the formation of 1,2-bis adduct across starting alkyne triple bond; in comparison to the TEC coupling on POSS derivative high density of sugars in final product can be predicted to be balanced by the steric congestion in every apex that will be inevitable using this methodology (Figure 18).

In order to be sure about total conversion of starting octaalkynyl POSS derivatives into the corresponding double substituted products some ^{1}H-NMR diagnostic signals were identified in each species. The high symmetry T_8 system of POSS gave very simple spectra in every case; propargyl and alkenyl protons (4.2 ppm and 5.5-6.5 ppm respectively) are very easy to identify and to detect if present in the spectra. The disappearance of these signals demonstrated that this simple analytical method was useful to determine the completeness of the coupling.

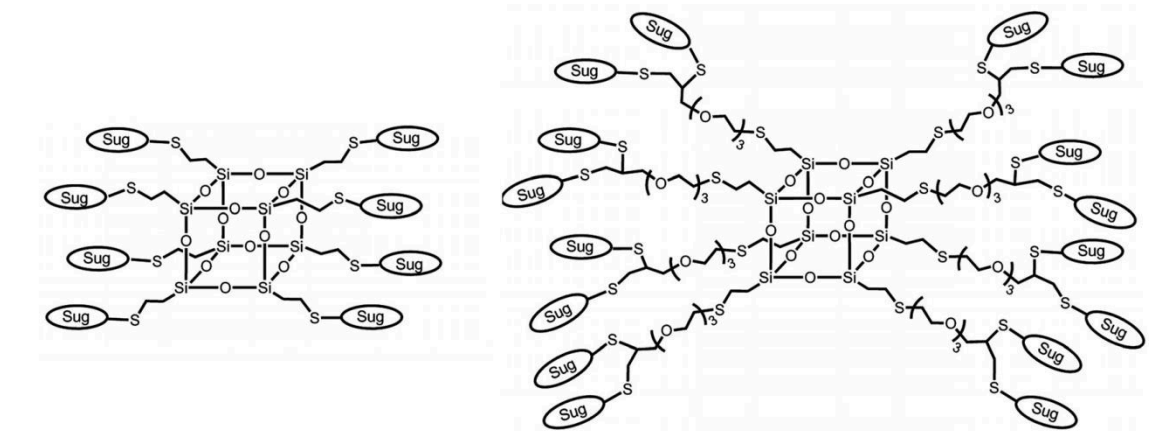


Figure 18: POSS-glycoconjugates obtained with TEC (left) and with TYC (right)

The first sugar used was the 2-acetamido-2-deoxy-1-thio-β-D-glucopyranose **73** that gave the best inhibition results in previous study. A complete conjugation was obtained with 4 equivalents per alkyne after 1 h of irradiation at standard wavelength (365 nm), structural characterization demonstrated the effective addition of sugar units and only a gel filtration chromatography was necessary to purify the product from disulphide, the only by-product of the reaction.

Encouraged by this first positive experiment galactosyl thiol **76** was used to obtain the corresponding hexadecavalent glycocluster in satisfactory yield. In this case, however, 4 equivalents per alkyne were not sufficient to complete the addition and mixture of alkene intermediates was detected by ^{1}H-NMR analysis. By the way, increasing thiol excess to 8 eq./yne the efficiency of the coupling was re-established. The low reactivity of **76** can be

ascribed to some steric interaction between sulfhydryl group of free sugar and the C-4 axial hydroxyl of the first sugar linked with the cluster. For this reason the use of the galactosyl-propyl-thiol **77** (4 eq. per alkyne) allowed to obtain the double addition product in good yield. This is another important evidence of the essential role of spatial distribution of sugars around cluster terminus (Figure 19).

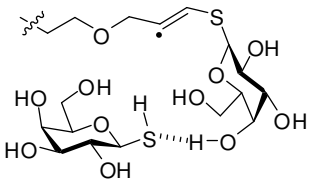
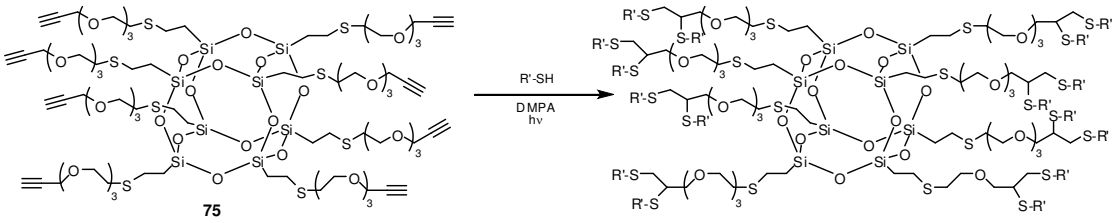


Figure 19: interaction between 1-SH and C-4 axial OH of galactosyl-thiol during the second step of thiol-yne coupling.

Bis-adducts were obtained in high yield also with glucosyl-propyl-thiol **66** and mannosyl-propyl-thiol **67** as reported in Table 11.



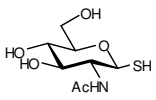
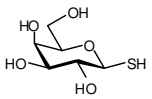
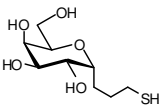
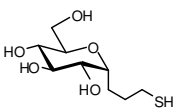
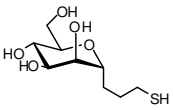
	Thiol	Thiol eq./ene	Solvent	Yield
73		4	DMF	60%
76		8	MeOH-DMF 1:1	50%
77		4	MeOH-DMF 1:1	70%
66		4	MeOH-DMF 1:1	82%
67		4	MeOH-DMF 1:1	80%

Table 11: Photoinduce hydrothiolation of POSS derivative **75**

Binding tests on these compounds were carried out using the same ELLA protocol as the previous study in order to evaluate absolute and relative inhibition properties of the new multivalent products in comparison to thiol-ene based ones.

In a paper reported in the literature[107] a problem emerged regarding the instability of some POSS based triazole-linked glycoconjugates in aqueous media, therefore a stability test was carried out on mannosyl glycoconjugate obtained from **67**. Under ELLA test conditions (pH 7.4, T = 37 °C, 2 h) but for longer time (16 h) in deuterated water, the ^{1}H-NMR analysis demonstrated the stability of these compounds.

ELLA tests were performed using the glucosylated conjugate obtained from **66** and the mannosylated conjugate obtained from **67** with Concanavalin A and glucosaminylated conjugate obtained from **73** with wheat germ agglutinin (WGA). Results shown in Tables 12 and 13 were quite good for all compounds, but it is necessary to underline some crucial points in order to better understand the essential structural features of multivalent interactions for these family of clusters. In general the inhibition properties are increasing doubling sugar units in the cluster, the tendency is still the same with mannose more effective than glucose, and with a notable relative potency in the case of mannosyl cluster.

On the other hand the difference between first and second generation of these cluster leaves some doubts about the role of the PEG linker in the final effect. However, it remains one of the most important result as inhibition values present in literature for ConA substrates.[108] The higher values of hexadecavalent clusters can be due to the higher density of sugars on apexes, to the higher flexibility of the PEGylated clusters or to the best distribution of sugars respect to lectin binding sites.

Product	**n[b]**	**IC_{50} (μM)[a]**	**rp[c]**	**rp/n[d]**
Me α-D-Glc	1	2108±75	1	1
Glucosylated glycocluster (PEG)	16	4.4±0.6	479	30
Glucosylated glycocluster	8	40.4±0.7	35.2	4.4
Me α-D-Man	1	459±26	1	1
Mannosylated glycocluster (PEG)	16	0.179±0.011	2564	160
Mannosylated glycocluster	8	6.8±0.9	48.2	6

Table 12: ELLA data for the inhibition of the binding of the lectin ConA-HRP to α-d-Man-PAA by glucosylated and mannosylated clusters. **Notes:** a) Each experiment was carried out in triplicate b) Number of sugar units in the molecule c) Relative potency = IC_{50} (mono)/ IC_{50} (cluster) d) Relative potency/number of sugars

Moving to inhibition test of the binding of WGA by glucosamine clusters more precise consideration can be made because in this case the core structure of the multivalent products is the same. In both case a PEGylated POSS was used and only the number of sugar units was changed. It resulted a very impressive IC_{50}, very close to that showed by the octavalent cluster one, that, compared to monovalent reference, was just a little bit higher but that became lower if related to the number of sugar units present on the molecule (Table 13). This means that interactions are still effective in multivalent fashion, but that probably only one of two sugar molecules is really involved in binding protein site and the other is partially disturbing this interaction.

Product	**n[b]**	**IC_{50} (μM)[a]**	**rp[c]**	**rp/n[d]**
D-GlcNAc	1	28000±2500	1	1
Glucosamine glycocluster	8	0.003±0.0006	9.6×10^6	10^6
Glucosylated glycocluster	16	$0.002 \pm 5 \times 10^{-5}$	14.4×10^6	9.0×10^5

Table 13: ELLA data for the inhibition of the binding of WGA-HRP to D-GlcNAc-PAA with PEGylated POSS-based octavalent and hexadecavalent glycoclusters[a] **Notes:** a,b,c,d as Table 12.

In conclusion, it is possible to generalize that inhibition properties must be evaluated case by case in order to find the best fitting solution for each target; adding huge number of active molecules is not always the good way to establish the stronger or the better interaction on biomolecules, especially when a very good result (inhibition in nM range) has been already achieved.

Finally, it must also be pointed out that all of the isolated products were mixtures of diastereoisomers, very likely in 1:1 ratios, due to the lack of stereoselectivity of the thiyl radical addition to the vinyl thioether intermediate. No attempts were made to separate individual stereoisomers, as this matter was beyond the scope of the present work. Therefore, it cannot ruled out that separation or stereoselective synthesis of pure stereoisomers could allow the identification of even stronger and more selective lectin ligands.

5.4. Experimental Part

See published papers. Page 109: Glycoside and peptide clustering around the octasilsesquioxane scaffold via photoinduced free-radical thiol–ene coupling. The observation of a striking glycoside cluster effect. Page 118: Thiyl Glycosylation of Propargylated Octasilsesquioxane: Synthesis and Lectin-Binding Properties of Densely Glycosylated Clusters on a Cubic Platform

5.5. Dendrimers: Flexible scaffolds for glycoclustering

Investigations regarding carbohydrate-protein interactions and synthesis of inhibitors of these bindings are at the forefront of glycobiology today; the importance of these interactions in biological and pathological processes has been explained in the introduction and includes fertilization, intercellular communication, viral or bacterial infection, inflammation, tumor cell metastasis, and immune response.[109,110]

As shown before, the classical biological interaction is formed by multiple linkages that in total become stronger than the sum of the single interactions. This particular feature, namely *avidity* drives to cluster effect, which is the base of multivalency. Libraries of different glycoclusters have been prepared and evaluated by several research groups, but definition of structure-effect relations are impossible to establish *a priori* because interactions with different targets are dependent on several structural features of both the interacting compounds.[111] Spacing, orientation, density, flexibility, and overall architecture of molecules are influencing the multivalent interaction, as much as the nature and the structure of the target; for that reason several platform have been tested during decades (cyclopeptides,[112,113] glycoproteins,[114–116] dendrimers and dendrons,[117–119] polymers,[120,121] polymeric and gold nanoparticles,[122–124] fullerenes,[125–127] calixarenes,[128,129] cyclodextrins,[130–132] DNA,[133–135] and silsesquioxanes).

Dendrimers are macromolecular compounds that comprise a series of branches around an inner core, they can be synthesized from the centre to the periphery (divergent synthesis) or in top-down approach starting from the outermost residues (convergent synthesis). Dendrimers are built starting from monomers with general formula AB_n (usually n = 2 or 3) and every layer added (generation) makes peripheral functional group number doubles or triples.[136,137] Some dendrimers are commercially available (PAMAM, Astramol), but universe of these molecules is potentially infinite. They are obviously very dynamic but is possible to generalize that, especially for high generation ones, spherical structure is preferred.[138]

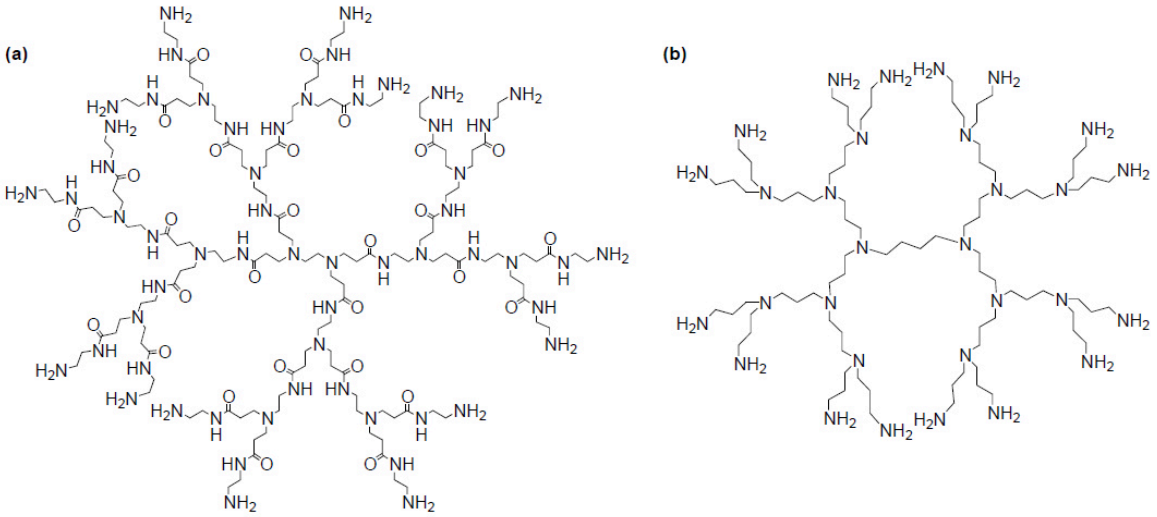


Figure 20: Two commercially available dendrimers: second generation PAMAM **(a)** and third generation poly(propylene imine) (Atramol) **(b).**

Glycoconjugation of dendrimers has become one of the major application for these molecules and specific platforms have been developed in order to improve the coupling conditions and the biological characteristics.[139–147] Other examples of applications include also dendrimers clustering peptides,[148–150] the use as carrier for gene therapy[151,152] and for *in situ* drug delivery.[153] In each branch very impressive progresses have been achieved in last years, as demonstration of the importance of these platforms for supramolecular chemistry and biochemistry.[154]

For our investigation a polyester-based dendrimer was chosen and 4 different generations of multivalent products were synthesized. The commercial 1,1,1-tris(hydroxymethyl) propane (trimethylolpropane, TMP) dendrimers TMP-G1-OH_6 **78**, TMP-G2-OH_{12} **79**, TMP-G3-OH_{24} **80**, and TMP-G4-OH_{48} **81** (Figure 21) were separately treated with excess (1.3 eq./OH group) of 4-(2-(allyloxy)ethoxy)-4-oxobutanoic anhydride in the presence of pyridine and 4-dimethylaminopyridine (DMAP) to give the corresponding allylated dendrimers TMP-G1-ene_6 **82**, TMP-G2-ene_{12} **83**, TMP-G3-ene_{24} **84**, TMP-G4-ene_{48} **85** in 41-68% isolated yields after column chromatography on silica gel.

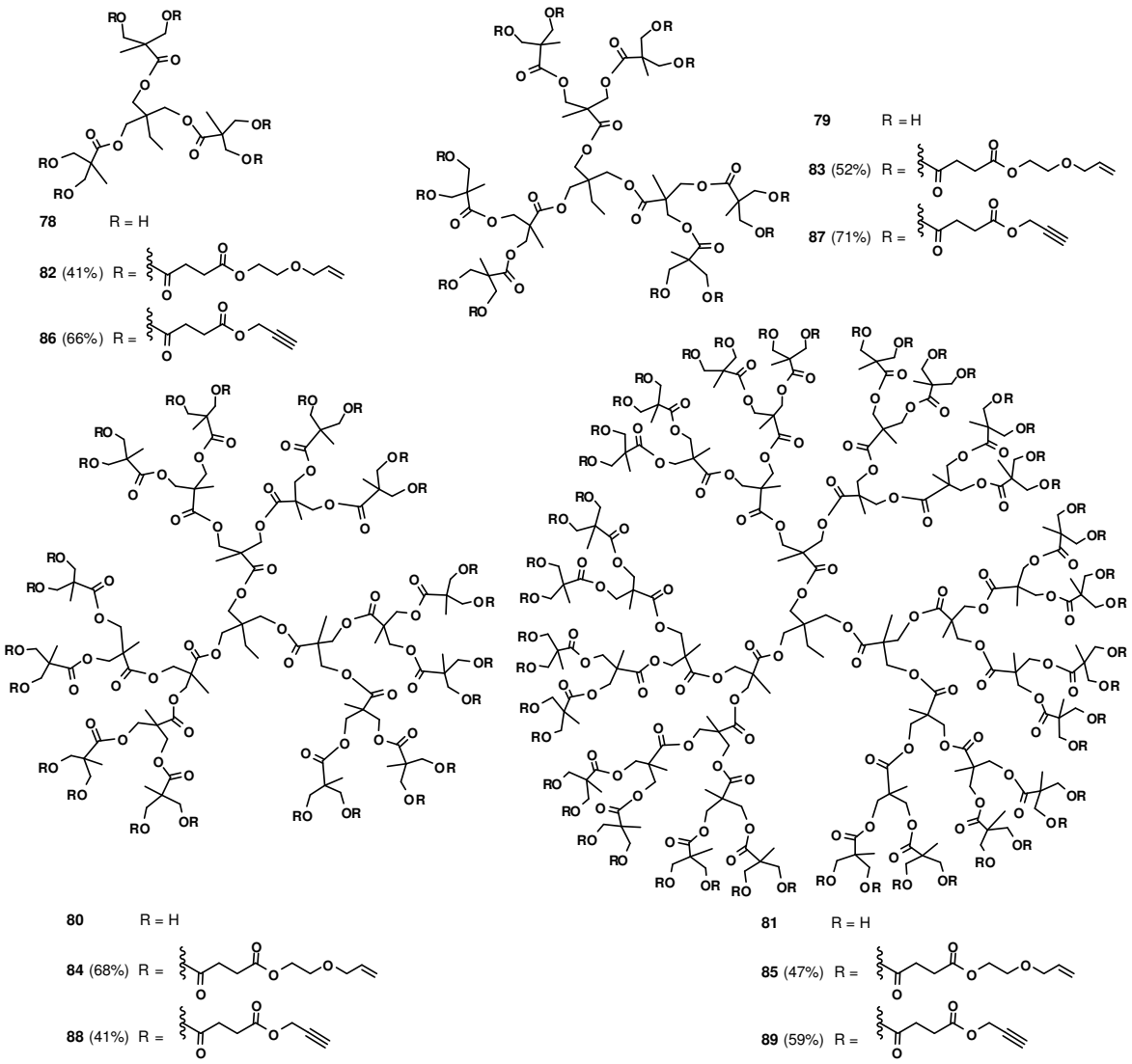


Figure 21: Alkene (**82**-**85**) and alkyne (**86**-**89**) functional dendrimers prepared from TMP-GX-OH_n **78**-**81**

In a similar way, treatment of the hydroxylated dendrimers **78**-**81** with 4-oxo-4-(prop-2-yn-1-yloxy)butanoic anhydride (1.3 eq./OH group) afforded the propargylated dendrimers TMP-G1-yne_6 **86**, TMP-G2-yne_{12} **87**, TMP-G3-yne_{24} **88**, TMP-G4-yne_{48} **89** that were recovered by column chromatography on silica gel in 41-71% yields. The TMP-GX-ene_n **82**-**85** and TMP-GX-yne_n **86**-**89** dendrimers were characterized by ^1H and ^{13}C NMR spectroscopy and MALDI-TOF mass spectrometry. The latter analysis clearly demonstrated that for each dendrimer all the hydroxyl functions were converted into the corresponding allyl- or propargyl-armed ester.

The eight dendrimers represent a small library to investigate, but the differences between generations and from allylated to propargylated ones give a wide overview of the different glycoconjugation possibilities. In order to study the inhibition properties 2-acetamido-2-

deoxy-1-thio-β-D-glucose **73** was chosen as functional group for the striking cluster effect showed in POSS derivatives toward WGA lectin.

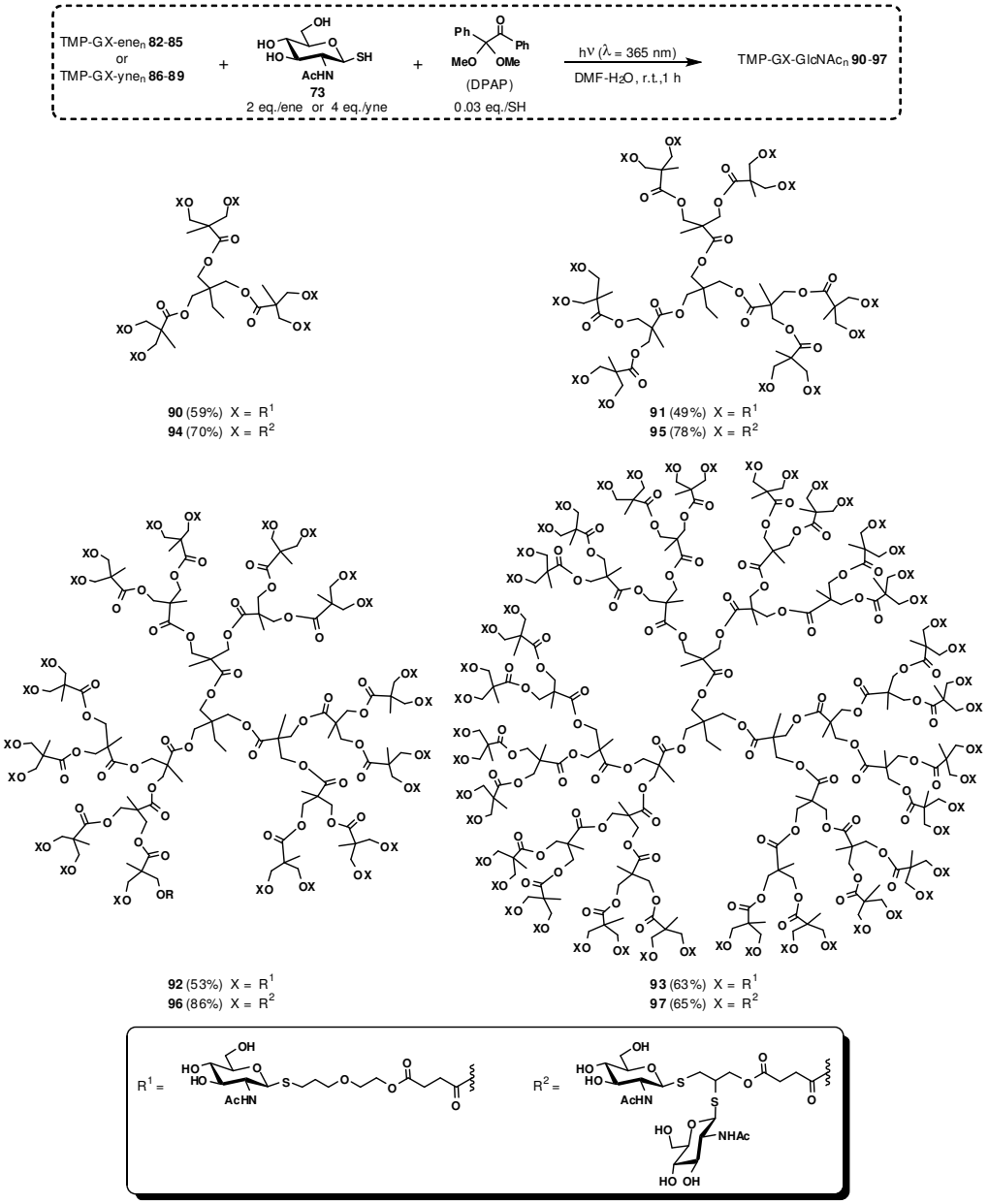
Allylated compounds **82**-**85** were used for thiol-ene coupling and propargylated compounds **86**-**89** for thiol-yne coupling to obtain as products mono-substituted glycodendrimers **90**-**93** and di-substituted ones **94**-**97** with isolated yield between 49-63% and 65-86%, respectively (Scheme 30). Purification of the crude mixtures was achieved by gel filtration chromatography (Sephadex LH20) that wass able to separate high MW compounds, like glycodendrimers, from low MW molecules like unreacted thiol or the corresponding disulphide. Control through ^{1}H-NMR analysis demonstrated complete consumption of alkene (5-6 ppm) and alkyne (ca. 2.5 ppm) species and the presence of only completely conjugated products in final reaction mixture. MALDI-TOF MS analysis was used to have confirmations about glycodendrimers **90**-**93** structures, on the other hand gel permeation chromatography (GPC) analysis did not allow to establish their molecular weight and dispersity because values significantly above the expected ones were observed. This is probably due to a massive presence of hydrogen bonds that permit aggregation of more molecules and avoid good results for this technique. Di-substituted glycodendrimers **94**-**97** were characterized by MALDI-TOF MS analysis in good general agreement with predicted values. Generation 3 and 4 glycodendrimers (**96** and **97**) were also characterized by consistent elemental analysis of hydrated forms (experimental values for carbon and hydrogen always within 0.3% from calculated values).

This library of synthesized glycodendrimers (Scheme 30) features carbohydrates density ranging from 6 units per molecule of **90** up to an impressive 96 sugars present on **97**. Intermediate compounds exhibit 12, 24 and 48 units per molecule, but with substantial differences in terms of geometry and spatial disposition of them. For example the G-2 glycodendrimer **91** and the G-1 **94** have both 12 sugar units but their exposition and freedom are very different from the first to the second. Increasing the MW of the central core and, consequently, sugar units supported very large macromolecules are synthesized and the last, the bigger glycodendrimer **97**, can be compared to an "artificial protein"[155,156] also in terms of globular structure.

The lectin inhibition tests from all these compounds are very important to better understand the relation between structural features of the platform, the size of the branched part, its geometry and the spatial distribution of the sugar units around the core. With substantial amounts of those glycodendrimers inhibition tests were done using the well-known WGA

lectin as target, in order to compare also results obtained with POSS conjugates. In fact only few studies reported glycodendrimers inhibition properties toward WGA, while more papers report results with lower size clusters as, for instance, silsesquioxane.[157] Our POSS glycoconjugates showed a relative potency related to sugar units around 10^6 for both the octavalent and the hexadecavalent one, that is one of the best result toward WGA reported in the literature.

For both series (allylated and propargylated) it was possible to notice very low values of IC_{50}, always in the nanomolar range; the first series **90-93** showed a stronger diminution of IC_{50} passing from 6-sugars glycodendrimer to the 48-sugars one than the second series **94-97** that shows a marked reduction only from first generation to the second (Table 14).



Scheme 30: Glycodendrimers **90-93** and **94-97** prepared via thiol-ene and thiol-yne couplings, respectively.

In multivalent compounds the most important feature is the relative potency and its values related to one sugar unit. In the present case, only the glycodendrimer **93** shows higher values than average, but still lower than the octa-substituted POSS derivative.

In conclusion, it is undeniable that all glycodendrimers studied are exposing peripheral sugar units in a favorable direction to exhibit excellent inhibition properties towards WGA and that adding sugar units to the cluster is not always leading to stronger effect. Geometry is another crucial factor for multivalent interactions, indeed glycodendrimers with same sugars units, but obtained by thiol-ene or thiol-yne show different values.

Product	**n**[b]	**IC_{50} (μM)**[a]	**rp**[c]	**rp/n**[d]
D-GlcNAc	1	$2.8x10^{4}\pm2.5x10^{3}$	1	1
POSS-Glucosamine glycocluster	8	$3x10^{-3}\pm6x10^{-4}$	9.6×10^{6}	10^{6}
POSS-Glucosylated glycocluster	16	$2x10^{-3}\pm5x10^{-5}$	$14.4x10^{6}$	$9.0x10^{5}$
TMP-G1-$GlcNAc_6$ **90**	6	$2.7x10^{-2} \pm 1x10^{-2}$	$1.04x10^{6}$	$1.7x10^{5}$
TMP-G2-$GlcNAc_{12}$ **91**	12	$2.7x10^{-3} \pm 4x10^{-5}$	$10.3x10^{6}$	$8.6x10^{5}$
TMP-G3-$GlcNAc_{24}$ **92**	24	$4.5x10^{-3} \pm 4x10^{-3}$	$6.4x10^{6}$	$2.6x10^{5}$
TMP-G4-$GlcNAc_{48}$ **93**	48	$2.6x10^{-4} \pm 4x10^{-3}$	$109x10^{6}$	$22.7x10^{5}$
TMP-G1-$GlcNAc_{12}$ **94**	12	$8.3x10^{-3} \pm 4x10^{-4}$	$3.37x10^{6}$	$2.8x10^{5}$
TMP-G2-$GlcNAc_{24}$ **95**	24	$1.39x10^{-3} \pm 2x10^{-4}$	$2.01x10^{6}$	$8.4x10^{5}$
TMP-G3-$GlcNAc_{48}$ **96**	48	$1.46x10^{-3} \pm 3x10^{-4}$	$19.2x10^{6}$	$4.0x10^{5}$
TMP-G4-$GlcNAc_{96}$ **97**	96	$1.06x10^{-3} \pm 2.0x10^{-4}$	$26.4x10^{6}$	$2.7x10^{5}$

Table 14: ELLA data for the inhibition of the binding of WGA-HRP to PAA-GlcNAc with glycoclusters **90**-**97**.[a]

Notes: [a] Each experiment was realized in triplicate. [b] Number of sugar unit in the glycodendrimer.

[c] Relative potency = IC_{50}(monosaccharide)/IC_{50}(glycodendrimer). [d] Relative potency/number of sugar units.

The results achieved in this work are very important because strong multivalent interaction were tested and verified between WGA and different types of glycoclusters and for the first time some biological assays were carried out on dendrimers. Moreover photo-induced free-radical thiol-ene and thiol-yne couplings were used as ligation tool also on these molecules and they have demonstrated to be completely effective, selective and orthogonal.

5.6. Experimental Part

Flash column chromatography was performed on silica gel 60 (40-63 μm). Optical rotations were measured at 20 ± 2 °C in the stated solvent; $[\alpha]_D$ values are given in $deg \cdot mL \cdot g^{-1} \cdot dm^{-1}$. ^{1}H NMR (400 MHz) and ^{13}C NMR spectra (100 MHz) were recorded in the stated solvent at room temperature unless otherwise specified. In the ^{1}H NMR spectra reported below, the *n*

and *m* values quoted in geminal or vicinal proton-proton coupling constants $J_{n,m}$ refer to the number of the corresponding sugar protons. MALDI-TOF mass spectroscopy was conducted on a Bruker UltraFlex with a SCOUT-MTP Ion Source (Bruker Daltonics) equipped with a N_2-laser (337 nm), a gridless ion source and a reflector. The laser intensity was set to the lowest value possible to acquire high-resolution spectra. The spectra were acquired using reflector mode with an acceleration of 25 kV to the extent possible; however for compounds over 20 kDa a linear mode was required. The instrument was calibrated using SpheriCalTM calibrants purchased from Polymer Factory Sweden AB. A THF solution of either 9-nitroanthracene or 2,5-dihydroxybenzoic acid (DHB) (10 mg/mL) doped with sodium trifluoroacetate was used as the matrix. The obtained spectra were analyzed with FlexAnalysis Bruker Daltonics version 2.2. Size exclusion chromatography (SEC) measurements were performed on a TOSOH EcoSEC HLC-8320GPC system equipped with an EcoSEC RI detector and three columns (PSS PFG 5µm; Microguard, 100 Å, and 300 Å) (MW resolving range: 300-100,000 Da) from PSS GmbH, using DMF (0.2 mL/min) with 0.01 M LiBr as the mobile phase at 50 °C. A conventional calibration method was created using narrow linear poly(methyl methacrylate) standards. Corrections for flow rate fluctuations were made using toluene as an internal standard. PSS WinGPC Unity software version 7.2 was used to process data. The photoinduced thiol-ene and thiol-yne reactions were carried out in a glass vial located 2.5 cm away from the household UVA lamp apparatus equipped with four 15 W tubes (1.5 x 27 cm each). The commercially available photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DPAP) was used without further purification. The dendrimers **78-81** were purchased from Polymer Factory Sweden AB, Stockholm, Sweden. Horseradish peroxidase-labelled *Triticum vulgaris* lectin (wheat germ agglutinin) (WGA-HRP), Bovine Serum Albumin (BSA), and SIGMA*FAST O*-phenylenediamine dihydrochloride (OPD) were purchased from Sigma-Aldrich. The 2-acetamido-2-deoxy-D-glucose-polyacrylamide (D-GlcNAc-PAA) was obtained from Lectinity Holding, Inc., Moscow.

TMP-G1-ene$_6$ (82). To a solution of TMP-G1-OH$_6$ **78** (1.23 g, 2.55 mmol) in pyridine (3.7 mL) and anhydrous CH_2Cl_2 (10 mL) were added DMAP (375 mg, 3.07 mmol) and 4-(2-(allyloxy)ethoxy)-4-oxobutanoic anhydride (7.71 g, 19.95 mmol). The mixture was stirred at room temperature for 16 h, then diluted with H_2O (4 mL) and stirred for 16 h to destroy the excess of anhydride. The mixture was extracted with CH_2Cl_2 (100 mL) and the organic phase was washed with 10% aqueous $NaHSO_4$ (5 x 20 mL) and 10% aqueous Na_2CO_3 (2 x 20 mL), then dried ($MgSO_4$) and concentrated. The residue was eluted from a column of silica gel

column with *n*-heptane-AcOEt (from 4:1 to 1:4) to give **82** (1.66 g, 41%) as a syrup. MALDI-TOF MS *m/z* calcd for $C_{75}H_{110}NaO_{36}$ $(M+Na)^+$ 1609.667, found 1609.978.

TMP-G2-ene$_{12}$ (83). The TMP-G2-OH_{12} **79** (1.00 g, 0.85 mmol) was functionalized as described for the preparation of **82** to give **83** (1.49 g, 52%) as a syrup. MALDI-TOF MS *m/z* calcd for $C_{159}H_{230}NaO_{78}$ $(M+Na)^+$ 3412.50, found 3411.79.

TMP-G3-ene$_{24}$ (84). The TMP-G3-OH_{24} **80** (1.00 g, 0.39 mmol) was functionalized as described for the preparation of **82** to give **84** (1.85 g, 68%) as a syrup. MALDI-TOF MS *m/z* calcd for $C_{327}H_{470}NaO_{162}$ $(M+Na)^+$ 7016.18, found 7013.98.

TMP-G4-ene$_{48}$ (85). The TMP-G4-OH_{48} **81** (1.00 g, 0.19 mmol) was functionalized as described for the preparation of **82** to give **85 (**1.24 g, 47%) as a syrup. MALDI-TOF MS *m/z* calcd for $C_{663}H_{950}NaO_{330}$ $(M+Na)^+$ 14223.55, found 14233.78.

TMP-G1-yne$_6$ (86). To a solution of TMP-G1-OH_6 **78** (1.31 g, 2.71 mmol) in pyridine (4 mL) and anhydrous CH_2Cl_2 (12 mL) were added DMAP (400 mg, 3.27 mmol) and 4-oxo-4-(prop-2-yn-1-yloxy)butanoic anhydride (6.25 g, 21.24 mmol). The mixture was stirred at room temperature for 16 h, then diluted with H_2O (4 mL) and stirred for 16 h to destroy the excess of anhydride. The mixture was extracted with CH_2Cl_2 (100 mL) and the organic phase was washed with 10% aqueous $NaHSO_4$ (5 x 20 mL) and 10% aqueous Na_2CO_3 (2 x 20 mL), then dried ($MgSO_4$) and concentrated. The residue was eluted from a column of silica gel column with *n*-heptane-AcOEt (from 4:1 to 1:4) to give **9** (2.35 g, 66%) as a syrup. MALDI-TOF MS (matrix: DHB) *m/z* calcd for $C_{63}H_{74}NaO_{30}$ $(M+Na)^+$ 1333.416, found 1333.394.

TMP-G2-yne$_{12}$ (87). The TMP-G2-OH_{12} **79** (1.00 g, 0.85 mmol) was functionalized as described for the preparation of **86** to give **87** (1.71 g, 71%) as a syrup. MALDI-TOF MS (matrix: DHB) *m/z* calcd for $C_{135}H_{158}NaO_{66}$ $(M+Na)^+$ 2857.890, found 2857.906.

TMP-G3-yne$_{24}$ (88). The TMP-G3-OH_{24} **80** (1.00 g, 0.39 mmol) was functionalized as described for the preparation of **86** to give **88** (0.94 g, 41%) as a syrup. MALDI-TOF MS (matrix: DHB) *m/z* calcd for $C_{279}H_{326}NaO_{138}$ $(M+Na)^+$ 5910.53, found 5911.00.

TMP-G4-yne$_{48}$ (89). The TMP-G4-OH$_{48}$ **81** (1.00 g, 0.19 mmol) was functionalized as described for the preparation of **86** to give **89** (1.32 g, 59%) as a syrup. MALDI-TOF MS (matrix: DHB) *m/z* calcd for $C_{567}H_{662}NaO_{282}$ $(M+Na)^+$ 12012.24, found 12017.85.

TMP-G1-GlcNAc$_6$ (90). To a solution of TMP-G1-ene$_6$ **82** (50 mg, 31.5 μmol), thiol **73** (90 mg, 0.38 mmol), and 2,2-dimethoxy-2-phenylacetophenone (DPAP, 2.9 mg, 11.3 μmol) in DMF (300 μL), partially concentrated under vacuum (ca. 0.1 mbar) to remove the traces of Me_2NH, was slowly added H_2O (200 μL). The mixture was irradiated (λ_{max} 365 nm) under vigorous stirring at room temperature for 1 h and then concentrated. The residue was eluted from a column of Sephadex LH-20 (2 x 50 cm) with 1:1 MeOH-H_2O to give **90** (56 mg, 59%) as a white powder. MALDI-TOF MS *m/z* calcd for $C_{123}H_{200}N_6NaO_{66}S_6$ $(M+Na)^+$ 3032.079, found 3032.226.

TMP-G2-GlcNAc$_{12}$ (91). The dendrimer TMP-G2-ene$_{12}$ **83** (51 mg, 15.0 μmol) was allowed to react with the thiol **73** as described for the preparation of **90** to give, after column chromatography on Sephadex LH-20 (1:1 MeOH-H_2O), **91** (46 mg, 49%) as a white powder. MALDI-TOF MS *m/z* calcd for $C_{255}H_{410}N_{12}NaO_{138}S_{12}$ $(M+Na)^+$ 6255.198, found 6255.467.

TMP-G3-GlcNAc$_{24}$ (92). The dendrimer TMP-G3-ene$_{24}$ **84** (56 mg, 8.0 μmol) was allowed to react with the thiol **73** as described for the preparation of **90** to give, after column chromatography on Sephadex LH-20 (1:1 MeOH-H_2O), **92** (54 mg, 53%) as a white powder. MALDI-TOF MS *m/z* calcd for $C_{519}H_{830}N_{24}NaO_{282}S_{24}$ $(M+Na)^+$ 12710.75, found 12712.49.

TMP-G4-GlcNAc$_{48}$ (93). The dendrimer TMP-G4-ene$_{48}$ **85** (58 mg, 4.1 μmol) was allowed to react with the thiol **73** as described for the preparation of **90** to give, after column chromatography on Sephadex LH-20 (1:1 MeOH-H_2O), **93** (66 mg, 63%) as a white powder. MALDI-TOF MS *m/z* calcd for $C_{1047}H_{1670}N_{48}NaO_{570}S_{48}$ $(M+Na)^+$ 25612.67, found 25776.81.

TMP-G1-GlcNAc$_{12}$ (94). A solution of the dendrimer TMP-G1-yne$_6$ **86** (16 mg, 12.2 μmol), thiol **73** (69 mg, 0.29 mmol), and DPAP (2.2 mg, 8.8 μmol) in AcOEt (50 μL), DMF (300 μL), partially concentrated under vacuum (ca. 0.1 mbar) to remove the traces of Me_2NH, and H_2O (50 μL) was irradiated (λ_{max} 365 nm) under vigorous stirring at room temperature for 1 h and then concentrated. The residue was eluted from a column of Sephadex LH-20 (2 x 50 cm)

with 1:1 MeOH-H_2O to give **94** (35.5 mg, 70%) as a syrup. MALDI-TOF MS (matrix: THAB) *m/z* calcd for $C_{159}H_{254}N_{12}NaO_{90}S_{12}$ $(M+Na)^+$ 4181.53, found 4182.30.

TMP-G2-GlcNAc$_{24}$ (95). The dendrimer TMP-G2-yne$_{12}$ **87** (17 mg, 6.0 μmol) was allowed to react with the thiol **73** (68 mg, 0.29 mmol) in 4:1 DMF-H_2O (500 μL) as described for the preparation of **94** to give, after column chromatography on Sephadex LH-20 (1:1 MeOH-H_2O), **95** (40 mg, 78%) as a syrup. MALDI-TOF MS (matrix: THAB) *m/z* calcd for $C_{327}H_{518}N_{24}NaO_{186}S_{24}$ $(M+Na)^+$ 8554.23, found 8555.10.

TMP-G3-GlcNAc$_{48}$ (96). The dendrimer TMP-G3-yne$_{24}$ **88** (17 mg, 2.9 μmol) was allowed to react with the thiol **73** (66 mg, 0.28 mmol) in 4:1 DMF-H_2O (500 μL) as described for the preparation of **94** to give, after column chromatography on Sephadex LH-20 (1:1 MeOH-H_2O), **96** (43 mg, 86%) as a syrup. Anal. Calcd. for $C_{663}H_{1046}N_{48}O_{378}S_{48}\cdot 100H_2O$: C, 41.74; H, 6.58; N, 3.52; S, 8.07. Found: C, 41.48; H, 6.46; N, 3.22; S, 7.27.

TMP-G4-GlcNAc$_{96}$ (97). The dendrimer TMP-G4-yne$_{48}$ **89** (10 mg, 0.83 μmol) was allowed to react with the thiol **73** (38 mg, 0.16 mmol) in 2:1:1 DMF-H_2O-MeOH (400 μL) as described for the preparation of **94** to give, after column chromatography on Sephadex LH-20 (1:1 MeOH-H_2O), **97** (19 mg, 65%) as a syrup. Anal. Calcd. for $C_{1335}H_{2102}N_{96}O_{762}S_{96}\cdot 80H_2O$: C, 44.28; H, 6.30; N, 3.71; S, 8.50. Found: C, 44.38; H, 6.38; N, 3.26; S, 9.00.

Enzyme-Linked Lectin Assay (ELLA). 96-well microtiter Nunc-Immuno plates (Maxi-Sorp) were coated with PAA-GlcNAc (100 μL per well, diluted from a stock solution of 5 $\mu g\cdot mL^{-1}$ in 50 mM carbonate buffer pH 9.6) for 1 h at 37 °C. The wells were then washed with T-PBS (3 x 100 $\mu L\cdot well^{-1}$, PBS pH 7.4 containing 0.05% (v/v) Tween 20). This washing procedure was repeated after each incubation step. The coated microtiter plates were then blocked with BSA in PBS (3% w/v, 1 h at 37 °C, 100 μL per well). Serial two-fold dilutions of each inhibitor was pre-incubated 1 h at 37 °C in PBS (60 μL per well) in the presence of WGA-HRP (60 μL) at the desired concentration. The above solutions (100 μL) were then transferred to the blocked microtiter plates which were incubated for 1 h at 37 °C. After incubation, the plates were washed with T-PBS (3 x 100 μL per well) then the color was developed using OPD (100 μL per well, 0.4 $mg\cdot mL^{-1}$ in 0.05 M phosphate-citrate buffer) and urea hydrogen peroxide (0.4 $mg\cdot mL^{-1}$). The reaction was stopped after 10 min by adding H_2SO_4 (30% v/v, 50 μL per well) and the absorbance was measured at 490 nm. The

percentage of inhibition was plotted against the logarithm of the concentration of the sugar derivatives. The sigmoidal curves were fitted and the concentrations at 50% inhibition of binding of WGA-HRP to PAA-GlcNAc coated plates were determined (IC_{50}). The percentages of inhibition were calculated as given in the equation below, where A = absorbance. The IC_{50} values were systematically performed in triplicate.

$$\% \text{ inhibition} = [(A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})}) / A_{(\text{no inhibitor})}] \times 100$$

6. Application for macromolecules functionalization

Another important application of thiol-ene and thiol-yne couplings is the functionalization of large macromolecules, like proteins, in order to glycosylate or to mark them for biological, therapeutic or diagnostic purposes. Several methodologies and reactions have been developed to achieve very precise modifications in last decades;[158–161] molecular and positional control of these alteration[162] have been studied for active site modification, complexation, protein surface alteration, cell membrane glycoprotein variation[163] and *in vivo* conjugation.[164] The utility of these investigations is all about mechanism and functionality of natural protein post-translational modifications. It is well-known that this process, which affects protein after biosynthesis and folding, incorporates a variety of small active groups (phosphates, sugars, lipids, alkyl and acyl groups).[165] Glycosylation, by the way, is the most common and complex of these modifications and it can affect deeply both protein structure and function. The carbohydrates supported on trans-membrane proteins are responsible for cell-cell interaction and, consequently, for pathogen infection, cell adhesion, communication, growth and differentiation. Native glycoproteins are known to have a large microheterogeneity that makes very difficult the determination of structural essential features and the artificial modification of expressed protein. Methods to obtain directly proteins with post-translational modifications are still very attractive, as much as synthetic techniques to insert functional tags to a modified protein by site-directed mutagenesis are.[166–168]

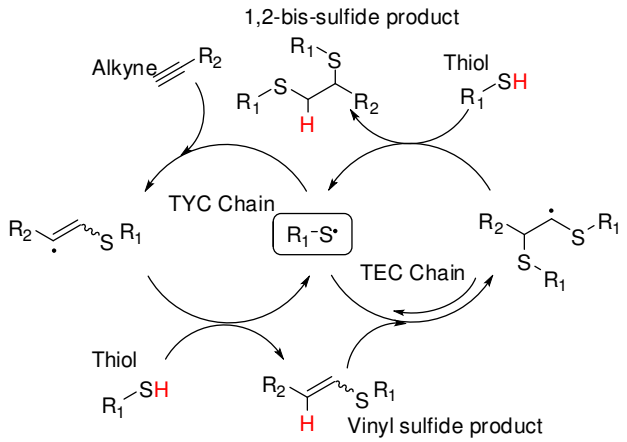
In this direction several examples are reviewed in literature,[169] including the disulphide-linked glycoproteins[170] formation from cysteine containing proteins, followed by desulfurization that rapidly gives the corresponding thioether linked glycoproteins.[171] The same final conjugate has been demonstrated to be also obtained by radical addition of an allyl sugar to the cysteine residue of the protein.[172,173] The irradiation wavelength used for thiol-ene or thiol-yne couplings is in the UV part of the spectrum, but very close to the visible part and, more important, has been demonstrated to not interact or to be incompatible with biomolecules. Moreover, the metal-free nature of the addition makes these radical coupling very attractive when large complex biomolecules are used as target.

Although thiol-ene coupling has been used successfully for protein modifications, thiol-yne coupling has not been used as multiple ligation tool. Indeed in other works the reaction potentiality to undergo a double different addition has been already explored, therefore this chance can be used for protein modification in order to insert a double

functional tag on the same cysteine residue of the native (or mutagen) protein. Only one drawback was noticed in a previous work; if present in native form, cystine disulfide bridges can be cut and the corresponding cysteine residues can undergo rapidly radical coupling to form other thioether conjugates. This side-reaction can affect structurally the protein, but can also be used to form multi-site-selective protein modifications.

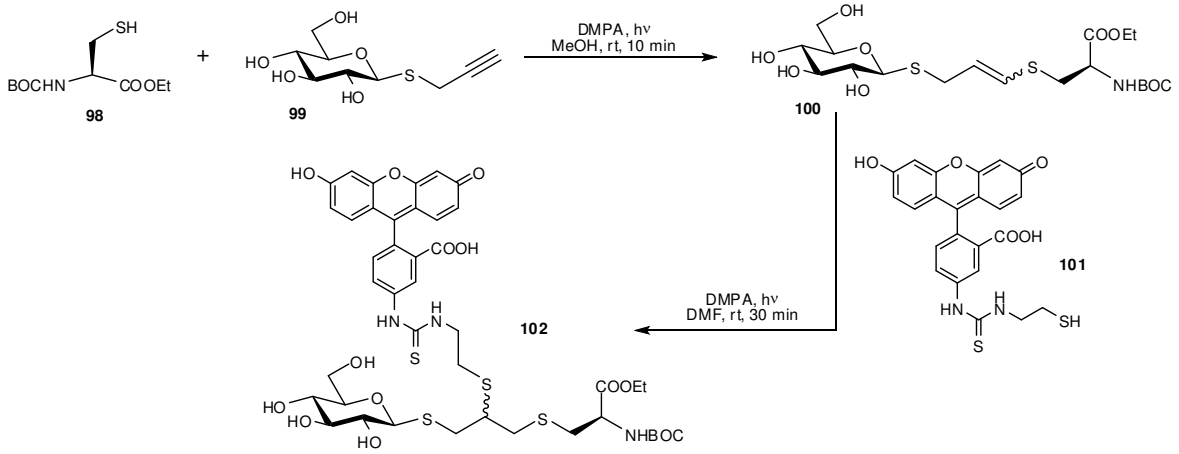
The double addition of two different thiol residues across an alkyne has been already exposed, but is important to notice that only working on alkyne excess respect to the first thiol is possible to obtain the vinyl thioether necessary for the second thiol addition (Scheme 31).

The aim of our work is to obtain a double addition product supported on cysteine residues of native BSA (bovine serum albumin), but before scaling up to natural protein we have tested more simplex substrates in order to verify the efficiency of the coupling and the compatibility of reactants.



Scheme 31: Mechanism of photoinduced free-radical thiol-yne coupling

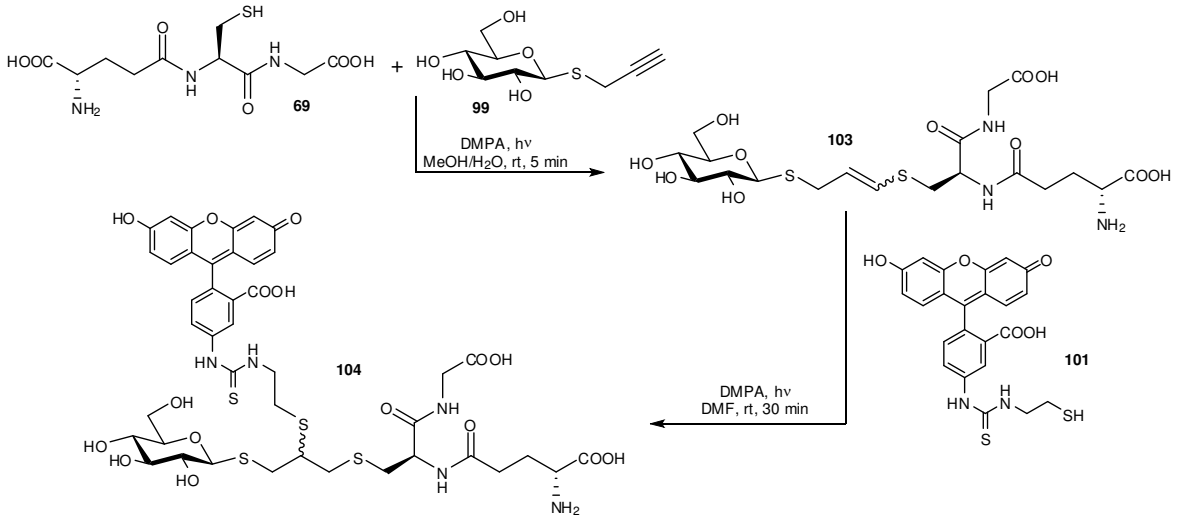
First the simple cysteine derivative **98** was used as model to optimize the reaction with 4 eq. of propargyl 1-thio-D-glucopiranoside **99** under standard thiol-yne condition in presence of catalytic amount of photoinitiator (DMPA) in MeOH. After 10 min of irradiation the NMR spectrum of the reaction mixture revealed the presence of olefinic protons signals in the 5-6.5 ppm region. Sugar alkyne excess was fully recovered by silica gel chromatography and the vinyl thioether intermediate **100** was obtained in pure form but in quite low yield (ca. 30%). This key initial experiment confirmed the feasibility of step one of our intended two-step process (Scheme 32).



Scheme 32: Thiol-yne couplings on cysteine derivative **98**

With the alkene intermediate in our hands a second coupling was tested using the fluorescein derivative **101** (4 eq.) in DMF for 30 min of irradiation to give the diasteroisomeric mixture **102** in 35% yield after gel filtration chromatography (Sephadex LH20) purification. The NMR spectrum of the mixture showed the complete conversion of the alkene.

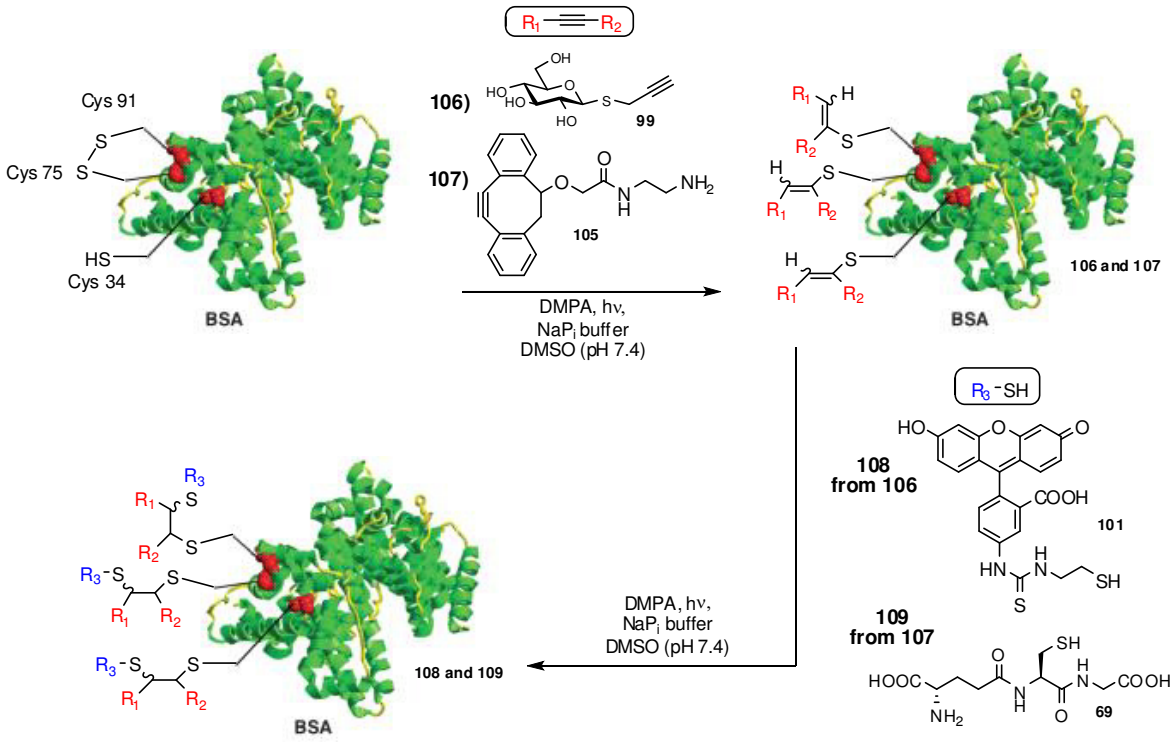
Another model experiment (Scheme 33) was carried out using glutathione **69** as cysteine containing tripeptide. After 5 min of irradiation under the same conditions used for cysteine, alkene intermediate **103** was obtained in good isolated yield (64%). The second coupling with fluorescein afforded the final double adduct **104** with almost complete conversion (>95% by NMR) but very modest isolated yield (ca. 15%).



Scheme 33: Thiol-yne couplings on glutathione **69**.

Differences between conversion value and isolated yield for both these compounds are related to purification problems due to the amphiphilicity of the products. However the key point of

these two model couplings is the possibility to develop a strategy for a dual modification of same cysteinyl residue with a biologically relevant molecule (sugar) and a label (fluorescein).



Scheme 34: Thiol-yne couplings on BSA with different reactants and methodologies

After the preliminary studies reported above, the BSA was considered as cysteine containing substrate for the same glycoconjugation followed by fluoro-labelling strategy. Experimentally, a large excess of sugar **99** was added (33 eq.) together with 3 eq. of the initiator DMPA in a DMSO/phosphate buffer (pH 7.4 – 5% v/v) and the mixture was irradiated at 365 nm for 5 min at room temperature without any caution to exclude air or moisture. The intermediate mixture was purified by size-exclusion centrifugation to remove small size reagent such as the excess of sugar alkyne **99**, the resulting solution, containing the alkene intermediate **106**, was mixed with phosphate buffer at pH 7.4 and with an excess of fluorescein **101** (160 eq.) and DMPA (16 eq.) dissolved in DMSO. That mixture was irradiated as described above at room temperature for 10 min. MALDI TOF MS analysis of the resulting synthetic conjugate **108** (found 68546 Da; calculated 68565 Da) indicated overall incorporation of three molecules of **99** and three molecules of **101** consistent with sequential dual modification at three cysteinyl sites (Scheme 34): the free cysteine at 34 and two thiyls 75 and 91 created by the photoinduced opening of the corresponding 75–91 cystine, as previously observed. Consistent with these observations the fluorescence spectrum (Figure 22) confirmed the incorporation of fluorescein residues. It should be noted that if

disulfides provide structural integrity critical to function then this type of cysteine cleavage and modification may therefore clearly prove detrimental to protein activity.

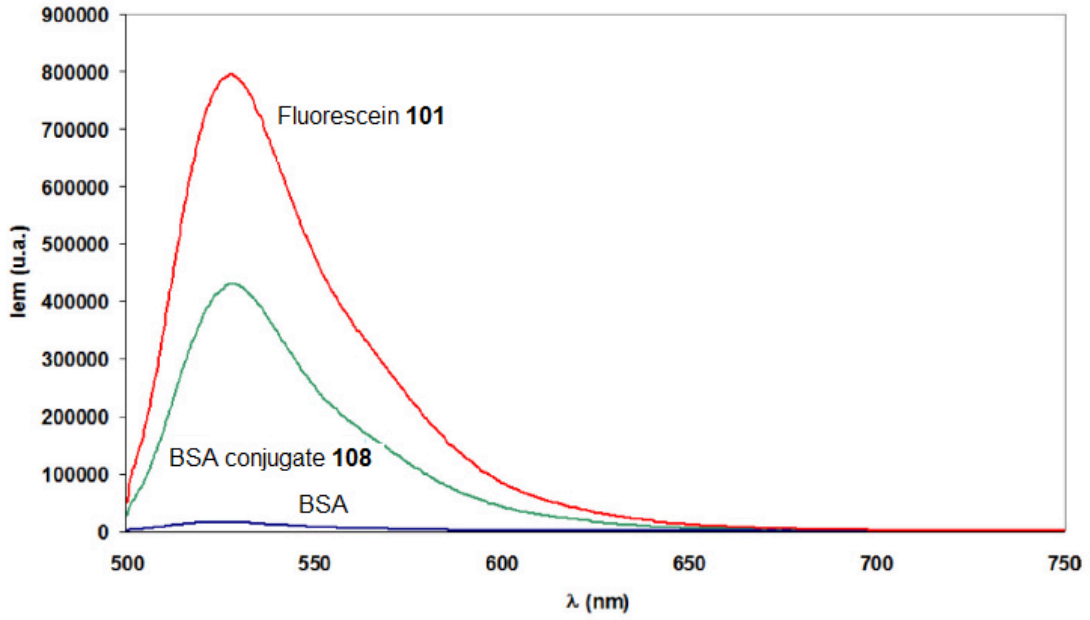


Figure 22: Fluorescence emission spectra (λ_{ex} = 490 nm) of phosphate buffer solution (pH 7.40) of BSA, BSA conjugate **108** and fluorescein thiol **101**

These crucial experiments reveals that BSA, a very common mammalian protein, reacts undergoing a dual modification (glycoconjugation plus fluoro-labeling) at up to three different sites *via* thiol-yne coupling strategy. It also open to the possibility that other different reagents containing alkynes may react in analogous way; foremost the possibility to test strained cycloalkynes, which are used in the Cu-free cycloaddition reaction with azides.[174,175]

In this logic a coupling with commercially available strained cyclo-octyne **105** was carried out under the same irradiation conditions used for the experiments described above. The first coupling product **107** was identified by MALDI-TOF MS analysis, purified by size-exclusion centrifugation and used as starting material for a second radical addition with glutathione **69** to obtain after centrifugation the final double addition product **109**. Also this compound was characterized by MALDI-TOF MS analysis in order to confirm the complete consumption of the three cysteine residues and the addition of both the reagents used.

Furthermore, the reaction of cyclo-octyne **105** with BSA in absence of thiols, light irradiation and photo-initiator was tested. The formation of the single addition product **110** was

demonstrated by mass spectroscopy analysis and tryptic digestion followed by MS/MS analysis (Figure 23).

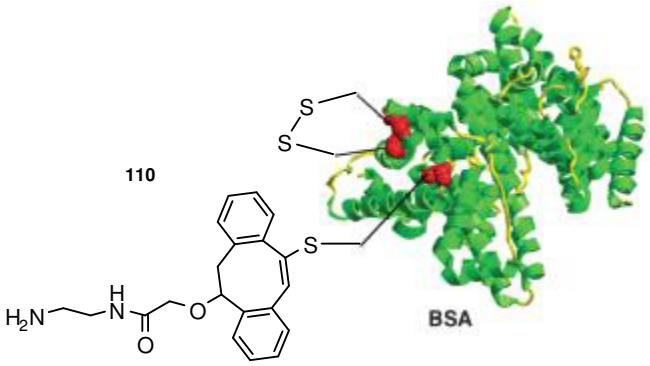


Figure 23: Single addition of cyclo-octyne **105** to native BSA

In these conditions only the free cysteine residue reacted to form the coupling product. It is crucial to consider the role of a disulphide bridge in protein structure and function; if breaking it comports a loss in term of activity it is evident that photo-induced coupling is not the most favourable method. It is worth noting that reaction between strained cyclo-alkynes and thiols are effective also without light irradiation,[176] and they are possible also on biological complex substrates like proteins.

Although conditions for *in vitro* experiments can never adequately reproduce those *in vivo* our results confirm that alternative reactive pathways exist for such strained alkyne reagents. Taken together with these results is possible to think that thiols in such albumins may act as potential unwanted reaction partners during experiments as those exposed here. It should be noted that other reactions that involve the use of excessive double-bond containing reagents (such as the so-called photoclick variants[177]) may also suffer from similar limitations.

6.1. Experimental Part

See Published Papers, page 124: Multi-molecule reaction of serum albumin can occur through thiol-yne coupling.

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Free-radical hydrothiolation of glycals: a thiol-ene-based synthesis of *S*-disaccharides

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ABSTRACT

A method for the synthesis of a new family of 1-deoxy *S*-disaccharides has been established via free-radical hydrothiolation of glycals by sugar thiols (thiol-ene coupling). The photoinduced coupling between four tri-*O*-acetyl-D-glycals and three different sugar thiols reveals that the reaction efficiency and stereoselectivity are highly dependent on the stereochemistry of the OAc groups at C3 and C4 of the glycal.

Glycans and their conjugates deeply influence many fundamental biological processes in living organisms.[1] They mediate a variety of events, including inflammation, immunological response, fertilization, cancer metastasis, and viral and bacterial infection.[2] Hence, there is an urgent need to provide usable quantities of natural carbohydrates with a well defined structure and composition to be used in studies of those phenomena at molecular levels. There is, however, substantial difficulty to obtain complex natural O-linked oligosaccharides in a pure and homogeneous form from natural sources because of the presence of mixtures of glycosylated species (glycoforms). Moreover, these compounds display an intrinsic instability toward chemical and enzymatic degradation due to the readily hydrolizable exocylic carbon–oxygen bond. Therefore, synthetic efforts have been directed toward the supply of carbon- and sulfur-linked isosteres, that is, compounds bearing a methylene group or a sulfur atom in place of the oxygen atom of the glycosidic bond that is present in natural products. Thus, simple *C*-disaccharides[3] and *S*-disaccharides[4] have been prepared because these products can be used as probes of recognition specificity and may provide important insight into the mechanism of glycoside elaboration by carbohydrate processing enzymes. In turn, these glycomimetics may become effective inhibitors of those enzymes and therefore evolve into lead compounds of pharmaceutical relevance. It has to be noted, however, that for biological studies, *S*-oligosaccharides may be more suitable than *C*-analogues because the sulfur derivatives, despite some differences in C–S and C–O bond lengths, as well as in C–S–C and C–O–C bond angles,[5] represent the smallest step away from natural *O*-glycosides in the backbone space.[6] While several chemical syntheses of *S*-disaccharides have been established over the second half of last century,[4] the free-radical thiol-ene coupling (TEC) between alkenyl sugars and sugar thiols was reported[7] for the first time by our group in 2009. Under optimized conditions (irradiation time, reagent ratio, and solvent) TEC afforded S-linked disaccharides in elevated yields. Given our ongoing research in the use of TEC in glycochemistry[8] as a very efficient metal-free click process,[9] we would like to report here on a new TEC-based approach to *S*-disaccharides by using glycals as ene partners. Protected glycals are well known precursors to functionalized carbohydrates due to the occurrence of a variety of addition reactions to the endocyclic carbon–carbon double bond.[10] Epoxidation is probably the most important of these addition reactions[11] because the so-called glycal epoxides that are formed are precursors to *O*- and *C*-glycosides and glycoconjugates. Other reactions, however, such as azidation reactions[12] bear substantial importance because of the easy transformation of the 2-azido substituted products into 2-amino derivatives. Quite surprisingly, to the best of our knowledge there is only a single example that has been reported in 1970 on the free-radical hydrothiolation of a glycal.[13] This consists of the thermal addition of thiolacetic acid to D-glucal triacetate in the presence of the free-radical initiator cumene hydroperoxide. The reaction afforded a mixture of two diastereomers in 70:30 ratio with the major product featuring the axial SAc group and the

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minor one having the same group equatorial. The product distribution was interpreted on the basis of the different stability of glycosyl radical intermediates. We were, thus, spurred to investigate in more detail the substrate scope and stereochemical outcome of the photoinduced addition of sugar thiols to isomeric glycals en route to 1-deoxy *S*-disaccharides and the relevant results are reported below.

We first investigated the model reaction between 3,4,6-tri-*O*-acetyl-D-glucal **1a** and peracetylated 1-thio-β-D-glucopyranose **2a** (Table 1) under previously established standard conditions for *S*-disaccharide synthesis via TEC,[7] that is, irradiation at wavelength close to visible light (λ_{max} 365 nm) in the presence of catalytic 2,2-dimethoxy-2-phenylacetophenone (DPAP) as radical initiator. The reaction was carried out at room temperature in a glass vial without any caution to exclude air and moisture. After some experimentation optimized reaction conditions were established. These entailed using 6 equiv of **2a**, 1 h irradiation and 20:1 EtOH–CH_2Cl_2 as the solvent. Under these conditions the glucal **1a** was totally consumed (100% conversion) to give a mixture of products **3a** and **4a** in 80% isolated overall yield and 57:43 ratio. The use of a lower excess of **2a** (4 equiv) or a shorter irradiation time (30 min) resulted in a partial conversion of glucal **1a** (73% and 33%, respectively) with consequent significant lower overall yield of **3a** and **4a** (53% and 33%, respectively). These products were formed in about 1:1 ratio also under these conditions. Hence, the reaction was essentially lacking in stereoselectivity while it appeared to be totally regioselective with the thiyl radical generated from thiol **2a** attacking exclusively the C2 carbon of the glucal **1a**. In agreement with that suggested for the addition of thiolacetic acid to **1a**,[13] these results are consistent with the initial formation of two anomeric glycosyl radical (AGR) intermediates stabilized by the adjacent oxygen atom, namely AGR_{ax} in which the RS group is axial and AGR_{eq} in which the same group is equatorial (Fig. 1). In the present case these intermediates must have comparable energies with the consequence that the products **16** and **17** are formed in essentially equal amounts.

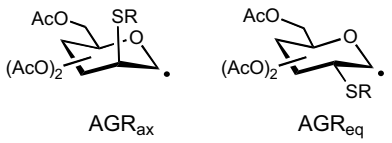
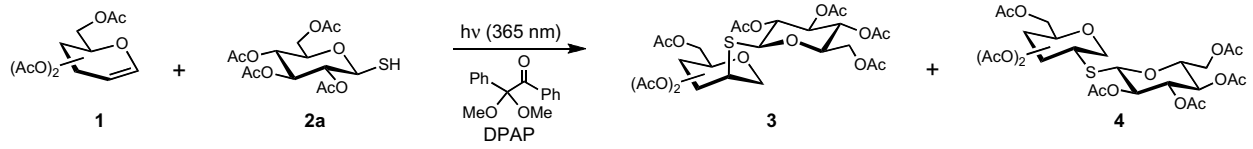
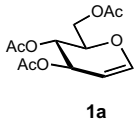
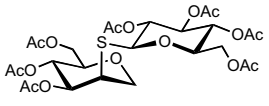
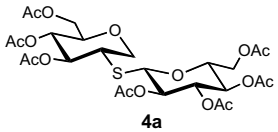
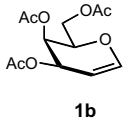
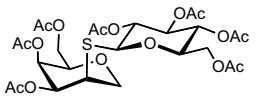
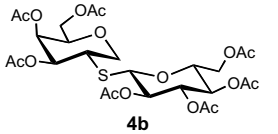
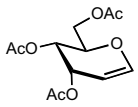
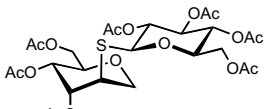
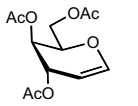
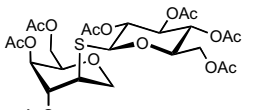


Figure 1. Anomeric glycosyl radical (AGR) intermediates formed by addition of a thiyl radical to glycals.

It was soon realized that the relative amounts of AGR_{ax} and AGR_{eq} depended considerably on the stereochemistry of the OAc groups at C3 and C4 in the starting glycal. Accordingly, the addition of the sugar thiol **2a** to the D-galactal triacetate **1b** under the above standard conditions afforded a mixture of diastereomeric sulfides **3b** and **4b** in good yield and with a net excess of the latter displaying the equatorially linked RS group (Table 1). This is consistent with the preferential formation of AGR_{eq} over AGR_{ax} due to the shielding exerted by the axial OAc group at C4 in **1b**. This stereochemical outcome was completely reversed in the hydrothiolation of D-allal triacetate **1c** by **2a** as this reaction afforded exclusively the axially substituted sulfide **3c** although in only fair yield (Table 1). Thus, it appears that in this case the axial OAc group at C3 of **1c** induces the exclusive formation of the AGR_{ax} radical intermediate via an addition of the thiyl radical to the double bond *anti* to the alkoxy group at C-3 of the glycal to give a 2,3-trans-diaxial substituted product.

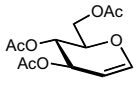
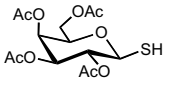
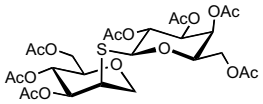
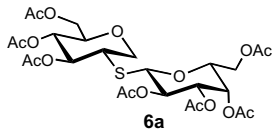
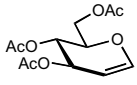
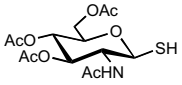
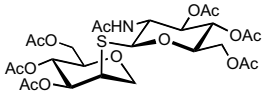
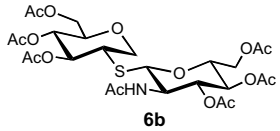
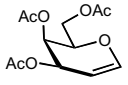
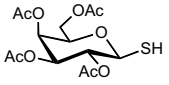
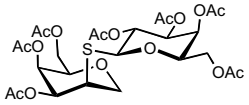
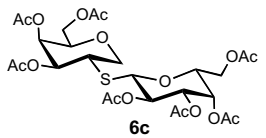
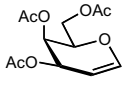
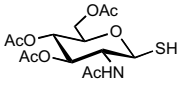
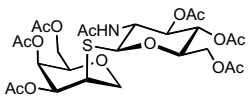
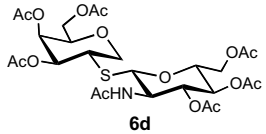
Table 1
Hydrothiolation of glycals **1**[a]



[a] Glycal	Conversion (%)	Product(s)		Yield[b] (%)	3:4 ratio[c]
 **1a**	100	 **3a**	 **4a**	80	57:43
 **1b**	100	 **3b**	 **4b**	59	37:63
 **1c**	40	 **3c**		38	100:0
 **1d**	35	 **3d**		20	100:0

[a] Reaction conditions: 6 equiv of **2a**, 0.6 equiv of DPAP, 20:1 EtOH–CH_2Cl_2, 1 h.
[b] Overall yield of products isolated by column chromatography on silica gel.
[c] Ratio determined by ^1H NMR.

Table 2
Hydrothiolation of glucal **1a** and galactal **1b** by different sugar thiols[a]

Glycal	Thiol	Products		Conversion (%)	Yield[b] (%)	5:6 ratio[c]
 **1a**	 **2b**	 **5a**	 **6a**	100	100	54:46
 **1a**	 **2c**	 **5b**	 **6b**	100	82	74:26
 **1b**	 **2b**	 **5c**	 **6c**	100	100	38:62
 **1b**	 **2c**	 **5d**	 **6d**	100	100	50:50

[a] Reaction conditions: 6 equiv of **2a**, 0.6 equiv of DPAP, 20:1 EtOH–CH_2Cl_2, 1 h.
[b] Overall yield of products isolated by column chromatography on silica gel.
[c] Ratio determined by ^1H NMR.

An identical stereoselectivity was observed in the reaction of **2a** with D-gulal triacetate **1d** to give diastereoisomer **3d** despite the presence of the axial OAc group at C4 (Table 1). This, however, affected the kinetics of the process as shown by the incomplete conversion of glycal **1d** and the low yield of isolated *S*-disaccharide **3d**.

Next, the substrate scope of the reaction was investigated by considering the addition of two more thiols **2b** and **2c** to glucal **1a** and galactal **1b** (Table 2). The choice of thiol **2c** relied on the fact that the 2-acetamidoglucosyl moiety (GlcNAc) is a key structural motif that is present in numerous biologically active carbohydrates. Moreover the presence of the NHAc group might serve to validate the stability of this important functionality under the conditions of TEC. Thus, it was with our great delight that the photoinduced hydrothiolation of **1a** and **1b** by thiols **2b** and **2c** under the above optimized conditions occurred with essentially quantitative conversion of the glycals to give the corresponding pairs of diastereoisomers **5a**–**6a**, **5b**–**6b**, **5c**–**6c** ,and **5d**–**6d** in very high isolated yields. All reactions, however, were scarcely stereoselective as the two *S*-disaccharides **5** and **6** were formed in comparable or even equal amounts. Thus, it appears that the substitution pattern in the thiol exerts scarce or no effect on the stereochemical course of the reaction.

In conclusion, it has been demonstrated for the first time that glycals can undergo a photoinduced addition of glycosylthiyl radicals with total regioselectivity to give 1-deoxy *S*-disaccharides in good to excellent yields. While the addition of three different sugar thiols to D-glucal and D-galactal leads to pairs of *S*-disaccharide diastereoisomers in comparable amounts, the additions of a sugar thiol to D-allal and D-gulal are totally stereoselective as they both afford a single product although in low yields.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.11.140.

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Efficiency of the Free-Radical Hydrophosphonylation of Alkenes: The Photoinduced Reaction of Dimethyl *H*-Phosphonate with Enopyranoses as an Exemplary Case

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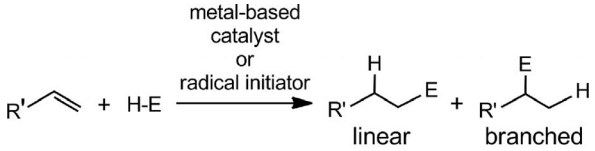
Keywords: Carbohydrates / C-glycosides / Phosphonates / Photochemistry / Radical reactions

Free-radical hydrophosphonylation of alkenes by *H*-phosphonates has been reported by other groups to occur under harsh conditions. On the other hand, we demonstrate in this paper that the hydrophosphonylation of alkene functionalized carbohydrates (enopyranoses) by dimethyl *H*-phosphonate can be carried out with high efficiency under neutral conditions at room temperature and with short reaction times using UV/Vis light irradiation in the presence of a suitable photoinitiator. Indeed, a range of structurally different allyl *C*-glycosides and *exo*-glycals were transformed into the corresponding glycosylalkyl phosphonates that were isolated in excellent yields (88–98 %), except for one case (45 %). A mechanism similar to that of the free-radical photoinduced hydrothiolation of alkenes is proposed.

Introduction

The click chemistry concept, as formulated by Sharpless and co-workers in the early 2000s,[1] highlights a set of criteria that have to be fulfilled by privileged reactions in order to serve as useful ligation tools for the facile assembly of molecular building blocks into larger constructions. One of these criteria involves atom economy, that is, the need for high conversion efficiency in terms of all atoms involved. Among the variety of reactions that comply with this requirement, the hydrofunctionalization of unsaturated carbon–carbon bonds, such as the addition of an H-E residue (E = heteroatom bearing group) across unactivated alkenes, appears to be of considerable importance. A prototypical example of this class of reactions is the hydroboration of alkenes (Scheme 1, H-E = HBR_2) discovered by H. C. Brown in the middle of the last century.[2] The enormous synthetic utility of this reaction[3] is attributable to the easy conversion of the transient organoborane into a variety of useful compounds including alcohols, amines, alkyl halides.[2,4] Other typical hydrofunctionalization reactions include hydroamination[5] (H-E = HNR_2), hydrosilylation[6] (H-E = $HSiR_3$), hydrostannylation[7] (H-E = $HSnR_3$), and hydrothiolation[5d,8] (H-E = HSR) of unactivated alkenes (Scheme 1). These reactions can give the linear and/or branched product depending on i) the catalyst, ii) the substituents in both the olefin and E residue, and iii) the reaction conditions selected. It is worth noting that, although these reactions are useful entry points to important intermediates for organic synthesis such as boranes, amines, silanes, stannanes, and sulfides respectively, the free-radical hydrothiolation has recently gained an additional value as a metal-free ligation tool for peptide and protein glycosylation.[9] This reaction is considered an exemplary case of click chemistry.[10] Another reaction of the H-E addition type to unsaturated carbon–carbon bonds is the hydrophosphonylation of alkenes[11] [H-E = $H(O)P(OR)_2$] to give alkyl phosphonates (Scheme 1), a class of phosphorus derivatives that are important as phosphate isosteres, isopolar analogues and precursors to olefins via the Wadsworth–Horner–Emmons reaction[12] with aldehydes. Indeed, this atom economical method of preparing phosphonates appears more convenient than the Michaelis–Arbusov reaction[11] because the latter suffers from low efficiency and sometimes requires high temperatures and long reaction times thus limiting its substrate scope. Moreover, the Michaelis–Arbusov reaction generates one equiv. of alkyl halide as a by-product which, in turn, can react with the phosphite thus drastically reducing reaction yield and efficiency.



Scheme 1. Some typical hydrofunctionalization reactions of alkenes.

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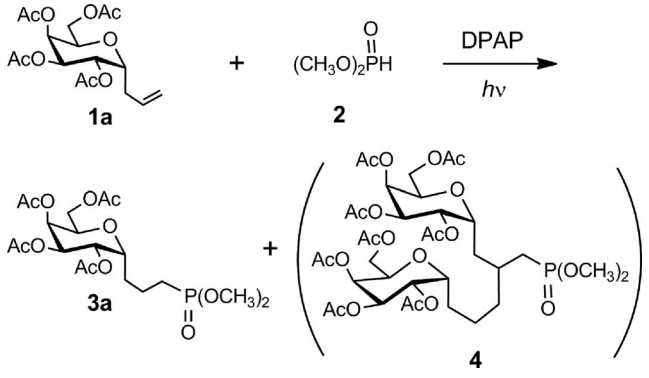
Results and Discussion

Although examples of hydrophosphonylation of alkenes using transition-metal catalysis have been reported,[11a,11b] radical reactions promoted by organic peroxides,[13–16] AIBN,[17] $Mn(OAc)_2$ in the presence of air,[18] and the titanocene/epoxide system[19] are quite attractive because they are operationally simple while using inexpensive and commercially available initiators and, most importantly, afford exclusively linear anti-Markovnikov adducts. Unfortunately, the scope of these radical reactions was established by using rather simple alkyl and aryl-substituted alkenes, thus extension to more complex substrates such as *exo*-glycals,[20] have not garnered much attention. Indeed a drawback of these reactions is the severity of the conditions employed, such as heating at 90–140 °C for several hours, which may not be tolerated by delicate bioactive substrates. Hence, we envisaged overcoming this limitation by adopting our photochemical method employed in the hydrothiolation of alkenes and alkynes.[21] This approach involves irradiation at room temperature at a wavelength close to visible light in the presence of small amounts of a suitable photoinitiator. We were aware of only one example of an alkene hydrophosphonylation by actinic radiation reported by Stiles using a G. E. AH-4 mercury lamp.[13] Thus, we considered the photoinduced hydrophosphonylation of rather delicate biomolecules such as enopyranoses. This reaction would lead to glycosyl phosphonates, a class of hydrolytically stable glycosyl phosphate mimics reported to be of considerable importance as enzyme inhibitors.[22] To determine the viability of the planned hydrophosphonylation, we first examined the reaction of the peracetylated allyl *C*-galactoside **1a** with dimethyl *H*-phosphonate **2** induced by irradiation at λ_{max} 365 nm in the presence of 2,2-dimethoxy-2-phenylacetophenone (DPAP) as the initiator. As in the case of hydrothiolation, we carried out this reaction at room temperature in glass vials without excluding air and moisture. Initial runs were performed using MeOH as the solvent and with a slight excess of **2** (Table 1, Entries 1 and 2). Gratifyingly, these experiments revealed the feasibility of the hydrophosphonylation reaction although target phosphonate **3a**, actually an anti-Markovnikov adduct, was isolated by chromatography in very low yield. Similar results were obtained in runs carried out under solvent-free conditions and with a more substantial excess of *H*-phosphonate **2** (Table 1, Entries 3 and 4). Moreover, a by-product was isolated in low yields and was elucidated on the basis of NMR and MS analyses as **4** (as a mixture of diastereomers) composed of sugar **1a** and *H*-phosphonate **2** in a 2:1 ratio. Traces of this product were also detected by NMR analysis of the crude reaction mixture using a larger excess of **2** whereas the yield of isolated **3a** remained moderate (Table 1, Entry 5). Finally, optimized conditions leading to a high yield of isolated **3a** and the total absence of by-product **4** were established using a very large excess of **2** (100 equiv., Table 1, Entry 6). Notably, the branched Markovnikov product was not observed in any of the runs carried out with **1a** and **2**. Thus, pure product **3a** was isolated simply by vacuum distillation of excess of *H*-phosphonate **2** and filtration of the resulting residue through a short column of silica to remove residual DPAP. As this procedure allows the recovery of the phosphonylating agent, the process appears to be highly sustainable and very likely scalable to multigram quantities. Finally, it has to be pointed out that irradiation of the reaction mixture requires only a simple and economical household UVA lamp equipped with four 15 W tubes. This is noteworthy since the UVA lamp furnishes low energy light (λ_{max} 365 nm) which is known to leave unaltered even sensitive biomolecules such as carbohydrates and proteins.[23] Notably, in the absence of UVA irradiation, photoactivation could be induced by exposure to unfocussed sunlight. Accordingly, reaction of **1a** with **2** carried out under these very mild conditions and in the presence of the DPAP initiator was found to render phosphonate **3a** as the sole product in very good isolated yield (Table 1, Entry 7).

Table 1. Optimization of photoinduced (λ_{max} = 365 nm, or sunlight) hydrophosphonylation of allyl *C*-galactoside **1a**.

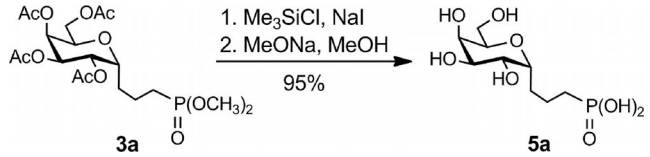


Entry[a]	Equiv. **2**	Time [h]	Solvent	Yield (**3a**)[b]	Product (**4**)[b]
1[c]	2.0	1	MeOH	<6%	–
2[c]	2.0	1	MeOH	<20%	–
3[c]	5.0	0.5	neat	40%	12%
4[c]	20	0.5	neat	46%	25%
5[c]	40	0.5	neat	43%	traces
6[c]	100	0.5	neat	91%	–
7[d]	100	9	neat	77%	–

[a] All runs were carried out in the presence of 0.5 equiv. of 2,2-dimethoxy-2-phenylacetophenone (DPAP). [b] Isolated yield after chromatography. [c] Run carried out using irradiation at λ_{max} 365 nm. [d] Run carried out using sunlight irradiation.

Another issue addressed in this initial study entailed the transformation of phosphonate **3a** into the corresponding deprotected phosphonic acid. To this end the phosphonate group was hydrolyzed using trimethylsilyl iodide and the *O*-acetyl groups of the sugar moiety were removed by treatment with sodium methoxide to give the phosphonic acid **5a** in essentially quantitative yield (Scheme 2).

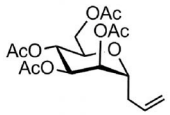
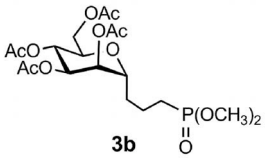
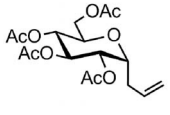
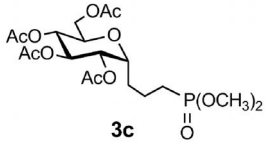
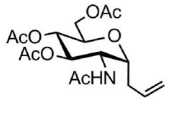
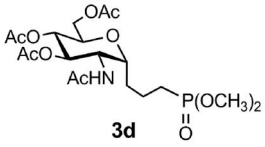
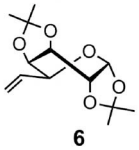
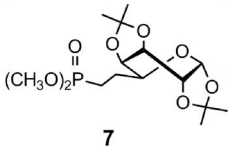
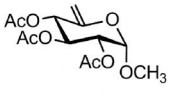
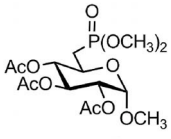
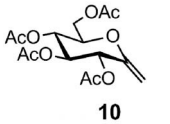
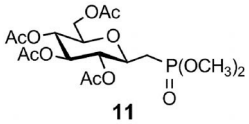
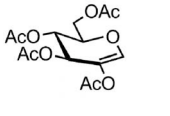
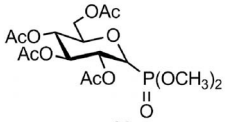
In a second instance, the substrate scope of the reaction was examined exploiting the above optimized photoinduced conditions. At first three additional allyl *C*-glycosides, (the peracetylated mannose, glucose and 2-acetamido-glucose derivatives **1b**, **1c**, and **1d** respectively), were treated with dimethyl *H*-phosphonate **2**. In all cases successful reactions



Scheme 2. Demethylation and deacetylation of phosphonate **3a**.

were registered as the corresponding pure glycosylalkyl phosphonates **3b**, **3c** and **3d** were isolated in excellent yields (Table 2). A more drastic sugar modification was introduced by using the diacetonide protected galactose-derived alkene **6**. Also in this case, successful photoinduced hydrophosphonylation of the alkene functional group by **2** was achieved. The corresponding phosphonate **7**, with the unaffected isopropylidene protecting group of the sugar moiety, was isolated in high yield (Table 2). Finally, a further structure modification was considered by reacting **2** with methylene *exo*-glycals **8** and **10**, two sugar-based enol ethers. To our great delight, hydrophosphonylation of **8** under the above standard conditions took place with high efficiency to give the corresponding phosphonate **9** in excellent isolated yield (Table 2). Alternatively, reaction of **2** with **10** under the same conditions afforded expected phosphonate **11** in relatively low yield (45%). A glucosylmethyl phenyl ketone side product (ca. 10% yield) arising from reaction of **10** with the acyl radical PhC(O)$^{\cdot}$ derived from fragmentation of the photoinitiator DPAP also was noted. Reducing the amount of DPAP to 0.3 equiv. did not improve the yield of **11** although unreacted **10** could be recovered together with small amounts of the phenyl ketone byproduct.[24]

Table 2. Scope of the photoinduced (λ_{max} 365 nm) hydrophosphonylation[a] of enopyranoses with dimethyl *H*-phosphonate **2**.

Alkene	Product	Yield (%)[b]
 **1b**	 **3b**	91
 **1c**	 **3c**	90
 **1d**	 **3d**	94
 **6**	 **7**	92
 **8**	 **9**	88–98[c]
 **10**	 **11**	45
 **12**	 **13**	no reaction

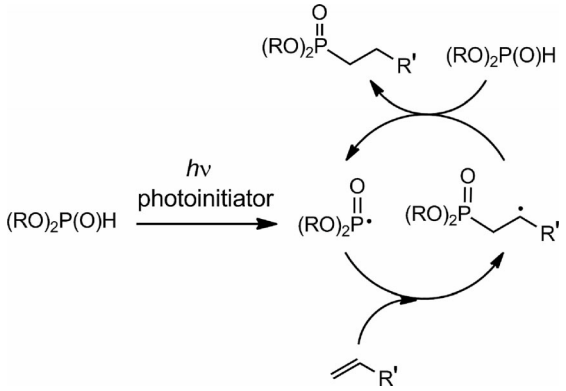
[a] General conditions: 100 equiv. of **2**, 0.5 equiv. of DPAP, 30 min, neat. [b] Isolated yield after column chromatography. [c] Yields obtained from two experiments under the same conditions.

A further extension of this hydrophosphonylation methodology to the 2-acetyl-D-glucal **12** featuring an endocyclic double bond failed since expected phosphonate **13** was not observed in the crude reaction mixture by TLC or MS analysis and the total amount of glucal **12** used in the reaction was recovered unaltered following chromatography of the reaction. This lack of reactivity contrasts the recently reported[25] hydrothiolation of **12** and the radical hydrophosphonylation of 3,4,6-tri-*O*-acetyl-D-glucal by diethyl *H*-phosphonate as described by Parsons and co-workers.[26] In light of these findings, some comments regarding the structural assignment of the prepared phosphonates are warranted. First, the terminal position of the phosphonyl group in the alkyl chain was assigned by the absence of a methyl group in all phosphonate derivatives described herein (on the basis of NMR data) as expected for an anti-Markovnikov hydrophosphonylation reaction of the C=C bond. Moreover, the configuration of the newly formed stereocenter of compounds **9** and **11** was established on the basis of the large coupling constants (ca. 10 Hz) between the H4-H5 and H1-H2 *trans*-diaxial protons, respectively, found in their NMR spectra.

Conclusions

Having considered the free-radical hydrophosphonylation of terminal alkenes by *H*-phosphonates under different conditions, it appears that the photoinduced process is quite promising as an efficient metal-free ligation tool. This C–P bond forming reaction, actually a phosphonylene coupling (PEC), owing to the mild and neutral conditions under which it occurs, enabled the introduction of the phosphonate group in sensitive biomolecules such as various alkenyl sugars. PEC appears to be endowed with several attributes that, if confirmed, may promote it to the rank of a new click process. In addition to the total atom economy, the reaction's 1,2-regioselectivity to give exclusively the anti-Markovnikov addition product is a powerful and enabling characteristic. Thus, a radical mechanism similar to that established for the photoinduced thiol-ene coupling (TEC)[7,8]

can be outlined (Scheme 3). In particular, PEC should involve light and photoinitiator-induced conversion of the *H*-phosphonate into a phosphonyl radical, a well-known reactive species in phosphorus chemistry,[11] which then adds to the terminal alkene to generate a carbon centered phosphoalkyl radical. This intermediate, by abstraction of a hydrogen radical from the unreacted *H*-phosphonate would lead to the final alkyl phosphonate and a new phosphonyl radical, thus propagating the radical chain. Based on this scheme it can be suggested that byproduct **4** is formed by addition of the phosphoalkyl radical to the starting alkene, a process that appears to be totally suppressed by the presence of a very large excess of *H*-phosphonate. Thus, efficient and side-product free phosphonylation by PEC of other important biomolecules such as modified peptides and proteins containing an olefinic amino acid tag,[27] can be safely foreseen.



Scheme 3. Proposed mechanism for the photoinduced hydrophosphonylation of alkenes.

Experimental Section

General Methods: Flash column chromatography was performed using silica gel 60 (40–63 mm). Optical rotations were measured at 20 ± 2 °C in the stated solvent. $[\alpha]_D$ values are given in deg mL g^{-1} dm^{-1}. ^{1}H NMR (300 and 400 MHz), ^{13}C NMR (75 and 100 MHz), and ^{31}P NMR (127 and 162 MHz) spectra were recorded in $CDCl_3$ solutions at room temperature unless otherwise specified. Peak assignments were aided by ^{1}H-^{1}H COSY and gradient-HMQC experiments. In the ^{1}H NMR spectra reported below, the *n* and *m* values quoted in geminal or vicinal proton–proton coupling constants $J_{n,m}$ refer to the number of the corresponding sugar protons. The hydrophosphonylation was carried out in a glass vial (diameter: 1 cm; wall thickness: 0.65 mm), closed with a natural rubber septum, located 2.5 cm away from the household UVA lamp apparatus equipped with four 15 W tubes (1.5 × 27 cm each). The commercially available dimethyl *H*-phosphonate **2** was distilled under vacuum and stored at –20 °C, the commercial photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DPAP) was used without further purification. Allyl *C*-galactopyranoside (**1a**),[28] *C*-mannopyranoside (**1b**),[28] *C*-glucopyranoside (**1c**),[28] and 2-acetamido-2-deoxy-*C*-glucopyranoside (**1d**),[29] galactose derived alkene **6**,[30] and the methylene *exo*-glycals **8**[31] and **10**[32] were prepared as previously reported.

Dimethyl [(2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl)propyl]-phosphonate (3a): A stirred solution of allyl *C*-galactoside **1a** (50 mg, 0.134 mmol) and DPAP (17 mg, 0.067 mmol) in dimethyl *H*-phosphonate **2** (1.2 mL, 13.4 mmol) was irradiated at r.t. for 30 min and then concentrated. The residue was eluted from a column of silica gel with 2:1 AcOEt/acetone (containing 0.5% of triethylamine) to give **3a** (59 mg, 91%) as a syrup. $[\alpha]_D$ = +59.8 (*c* = 1.0, $CHCl_3$). ^{1}H NMR (400 MHz): δ = 5.38 (dd, 1 H, $J_{3,4}$ = 3.4, $J_{4,5}$ = 2.1 Hz, H-4), 5.23 (dd, 1 H, $J_{1,2}$ = 5.1, $J_{2,3}$ = 9.5 Hz, H-2), 5.16 (dd, 1 H, H-3), 4.22 (dd, 1 H, $J_{5,6a}$ = 6.6, $J_{6a,6b}$ = 10.5 Hz, H-6a), 4.21–4.16 (m, 1 H, H-1), 4.05 (dd, 1 H, $J_{5,6b}$ = 5.0 Hz, H-6b), 4.02 (ddd, 1 H, H-5), 3.72 (d, 6 H, $J_{H,P}$ = 11.0 Hz, 2 OMe), 2.10, 2.05, 2.04, and 2.00 (4s, 12 H, 4 Ac), 1.82–1.70 and 1.65–1.47 (2m, 6 H, $CH_2CH_2CH_2$) ppm. ^{13}C NMR (100 MHz): δ = 170.5 (C), 170.0 (C), 169.8 (C), 169.7 (C), 71.5 (CH), 68.3 (CH), 68.2 (CH), 67.9 (CH), 67.5 (CH), 61.4 (CH_2), 52.24 and 52.17 (2d, CH_3, $J_{C,P}$ = 10.6 Hz, 2 OMe), 26.3 (d, CH_2, $J_{C,P}$ = 15.3 Hz), 24.1 (d, CH_2, $J_{C,P}$ = 140.7 Hz), 20.69 (CH_3), 20.63 (CH_3), 20.56 (CH_3), 20.54 (CH_3), 18.5 (d, CH_2, $J_{C,P}$ = 4.5 Hz) ppm. ^{31}P NMR (127 MHz): δ = 33.9 ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd. for $C_{19}H_{32}O_{12}P$ [M + H]$^+$ 483.1626, found 483.1614.

When the same reaction was carried out using 20 equiv. of dimethyl *H*-phosphonate **2**, sugar phosphonate **3a** was recovered in 46% yield. Also isolated was bis-*C*-glycoside **4** (25%) as an inseparable mixture of diastereomers. ^{1}H NMR (400 MHz) selected data: δ = 5.42–5.38 (m, 2 H, 2 H-4), 5.30–5.14 (m, 4 H, 2 H-2, 2 H-3), 3.78 and 3.74 (2d, 6 H, $J_{H,P}$ = 11.0 Hz, 2 OMe) ppm. ^{31}P NMR (162 MHz): δ = 33.9 ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd. for $C_{36}H_{56}O_{21}P$ [M + H]$^+$ 855.3052, found 855.3046.

Dimethyl [(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)propyl]phosphonate (3b): A solution of allyl *C*-mannoside **1b** (26 mg, 0.07 mmol) in **2** (670 μL, 7.00 mmol) was treated as described for the preparation of **3a** to give, after column chromatography on silica gel (2:1 AcOEt/acetone containing 0.5% of triethylamine), **3b** (30 mg, 90%) as syrup. $[\alpha]_D$ = +6.8 (*c* = 1.0, $CHCl_3$). ^{1}H NMR (400 MHz): δ = 5.21 (dd, 1 H, $J_{2,3}$ = 3.3, $J_{3,4}$ = 8.8 Hz, H-3), 5.16 (dd, 1 H, $J_{4,5}$ = 8.7 Hz, H-4), 5.11 (dd, 1 H, $J_{1,2}$ = 3.2 Hz, H-2), 4.34 (dd, 1 H, $J_{5,6a}$ = 6.5, $J_{6a,6b}$ = 12.0 Hz, H-6a), 4.08 (dd, 1 H, $J_{5,6b}$ = 4.0 Hz, H-6b), 3.93 (ddd, 1 H, *J* = 3.8, 10.6 Hz, H-1), 3.86 (ddd, 1 H, H-5), 3.72 (d, 6 H, $J_{H,P}$ = 11.0 Hz, 2 OMe), 2.11, 2.10, 2.04, and 2.00 (4s, 12 H, 4 Ac), 1.96–1.58 (m, 6 H, $CH_2CH_2CH_2$) ppm. ^{13}C NMR (100 MHz): δ = 170.7 (C), 170.2 (C), 169.9 (C), 169.6 (C), 74.0 (CH), 70.5 (2 CH), 68.8 (CH), 67.0 (CH), 62.3 (CH_2), 52.3 (d, $J_{C,P}$ = 9.1 Hz, CH_3, 2 OMe), 29.1 (d, $J_{C,P}$ = 15.2 Hz, CH_2), 24.0 (d, $J_{C,P}$ = 140.6 Hz, CH_2), 20.9 (CH_3), 20.71 (CH_3), 20.66 (2 CH_3), 18.6 (d, $J_{C,P}$ = 4.6 Hz, CH_2) ppm. ^{31}P NMR (127 MHz): δ = 33.7 ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd. for $C_{19}H_{32}O_{12}P$ [M + H]$^+$ 483.1626, found 483.1620.

Dimethyl [(2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl)propyl]phosphonate (3c): A solution of allyl *C*-glucoside **1c** (50 mg, 0.134 mmol) in **2** (1.2 mL, 13.4 mmol) was treated as described for the preparation of **3a** to give, after column chromatography on silica gel (2:1 AcOEt/acetone containing 0.5% of triethylamine), **3c** (58 mg, 90%) as white solid. $[\alpha]_D$ = +52.7 (*c* = 0.75, $CHCl_3$). ^{1}H NMR (400 MHz): δ = 5.29 (dd, 1 H, $J_{2,3}$ = 9.5, $J_{3,4}$ = 8.7 Hz, H-3), 5.05 (dd, 1 H, $J_{1,2}$ = 5.8 Hz, H-2), 4.96 (dd, 1 H, $J_{4,5}$ = 9.6 Hz, H-4), 4.23 (dd, 1 H, $J_{5,6a}$ = 5.7, $J_{6a,6b}$ = 12.1 Hz, H-6a), 4.16 (ddd, 1 H, H-1), 4.08 (dd, 1 H, $J_{5,6b}$ = 2.6 Hz, H-6b), 3.82 (ddd, 1 H, H-5), 3.72 (d, 6 H, $J_{H,P}$ = 11.0 Hz, 2 OMe), 2.10, 2.05, 2.01 and 2.00 (4s, 12 H, 4 Ac), 1.90–1.55 (m, 6 H, $CH_2CH_2CH_2$) ppm. ^{13}C NMR (75 MHz): δ = 170.9 (C), 170.3 (C), 169.9 (C), 169.8 (C), 72.3 (CH), 70.5 (2 CH), 69.0 (2 CH), 62.5 (CH_2), 52.5 (d, $J_{C,P}$ = 6.1 Hz, CH_3, 2 OMe), 26.1 (d, $J_{C,P}$ = 15.7 Hz, CH_2), 24.3 (d, $J_{C,P}$ = 140.7 Hz, CH_2), 20.9 (4 CH_3), 18.5 (d, $J_{C,P}$ = 4.9 Hz, CH_2) ppm. ^{31}P NMR

(127 MHz): δ = 33.8 ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd. for $C_{19}H_{32}O_{12}P$ [M + H]$^+$ 483.1626, found 483.1626.

Dimethyl [(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)propyl]phosphonate (3d): A solution of allyl *C*-glycoside **1d** (37 mg, 0.10 mmol) in **2** (0.9 mL, 10.0 mmol) was treated as described for the preparation of **3a** to give, after chromatography on silica gel (1:1 AcOEt/acetone), **3d** (45 mg, 94%) as a syrup. $[\alpha]_D$ = +29.3 (*c* = 0.8, $CHCl_3$). ^{1}H NMR (400 MHz): δ = 6.08 (bd, 1 H, *J* = 8.6 Hz, NH), 5.03 (dd, 1 H, $J_{2,3}$ = 8.5, $J_{3,4}$ = 7.5 Hz, H-3), 4.93 (dd, 1 H, $J_{4,5}$ = 7.0 Hz, H-4), 4.31 (dd, 1 H, $J_{5,6a}$ = 6.5, $J_{6a,6b}$ = 12.0 Hz, H-6a), 4.25 (ddd, 1 H, $J_{1,2}$ = 4.7 Hz, H-2), 4.13–4.08 (m, 1 H, H-1), 4.10 (dd, 1 H, $J_{5,6b}$ = 3.5 Hz, H-6b), 3.84 (ddd, 1 H, H-5), 3.72 (d, 6 H, $J_{H,P}$ = 11.0 Hz, 2 OMe), 2.09, 2.07, 2.05, and 1.96 (4s, 12 H, 4 Ac) ppm. ^{13}C NMR (100 MHz): δ = 171.0 (C), 170.7 (C), 169.9 (C), 169.1 (C), 71.3 (CH), 70.3 (CH), 70.1 (CH), 68.8 (CH), 61.7 (CH_2), 52.4 (br. s, 2 OCH_3), 50.7 (CH), 27.3 (d, $J_{C,P}$ = 13.7 Hz, CH_2), 24.0 (d, $J_{C,P}$ = 139.9 Hz, CH_2), 23.1 (CH_3), 20.8 (CH_3), 20.72 (CH_3), 20.67 (CH_3), 18.5 (d, $J_{C,P}$ = 5.3 Hz, CH_2) ppm. ^{31}P NMR (127 MHz): δ = 33.9 ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd. for $C_{19}H_{33}NO_{11}P$ [M + H]$^+$ 482.1786, found 482.1778.

[(α-D-Galactopyranosyl)propyl]phosphonic Acid (5a): To a stirred, warmed (40 °C) solution of **3a** (23 mg, 0.048 mmol) and NaI (21 mg, 0.143 mmol) in CH_3CN (75 μL) was added Me_3SiCl (20 μL, 0.143 mmol). The mixture was stirred at 40 °C for 1 h (a precipitate formed) and at r.t. for 2 h, then filtered, the solid was washed with $CHCl_3$ and the solution was concentrated. A solution of the residue in 5:1 H_2O/AcOEt (3 mL) was stirred at r.t. for 2 h then concentrated. A solution of the crude phosphonic acid in a 0.2 M solution of MeONa in MeOH (2 mL, prepared from Na and MeOH immediately before the use) was kept at r.t. for 2 h, then neutralized with Amberlite IR-120 resin resin (H^+ form, activated and washed with H_2O and MeOH immediately before the use), and filtered through a sintered glass filter. The resin was washed with MeOH, and the yellow solution was concentrated. The residue was eluted from a column of Sephadex LH-20 (2 × 25 cm) with MeOH to give **5a** (13.5 mg, 99%) as an amorphous solid. $[\alpha]_D$ = +43.4 (*c* = 0.9, H_2O). ^{1}H NMR (D_2O, 400 MHz): δ = 3.88 (ddd, 1 H, $J_{1,2}$ = 6.0, $J_{1,CH2}$ = 3.3, 11.0 Hz, H-1), 3.80 (dd, 1 H, $J_{2,3}$ = 9.8 Hz, H-2), 3.77 (dd, 1 H, $J_{3,4}$ = 3.5, $J_{4,5}$ = 0.8 Hz, H-4), 3.61 (dd, 1 H, H-3), 3.60–3.50 (m, 3 H, H-5, 2 H-6), 1.71–1.35 (m, 6 H, $CH_2CH_2CH_2$) ppm. ^{13}C NMR (CD_3OD, 100 MHz): δ = 75.6 (CH), 73.9 (CH), 71.9 (CH), 70.2 (2 CH), 62.3 (CH_2), 27.8 (d, $J_{C,P}$ = 134.6 Hz, CH_2), 26.8 (d, $J_{C,P}$ = 16.0 Hz, CH_2), 20.5 (br. s, CH_2) ppm. ^{31}P NMR (D_2O, 127 MHz): δ = 30.5 ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd. for $C_9H_{20}O_8P$ [M + H]$^+$ 287.0896, found 287.0900.

6,7-Dideoxy-1,2:3,4-di-*O*-isopropylidene-7-dimethoxyphosphanyl-α-D-*galacto*-heptopyranose (7): A solution of **6** (37 mg, 0.144 mmol) in **2** (1.3 mL, 14.4 mmol) was treated as described for the preparation of **3a** to give, after column chromatography on silica gel (15:1 AcOEt/acetone containing 0.5% of triethylamine), **7** (49 mg, 92%) as a white solid. $[\alpha]_D$ = –45.8 (*c* = 0.6, $CHCl_3$). ^{1}H NMR (400 MHz): δ = 5.50 (d, 1 H, $J_{1,2}$ = 5.1 Hz, H-1), 4.57 (dd, 1 H, $J_{2,3}$ = 2.4, $J_{3,4}$ = 8.0 Hz, H-3), 4.28 (dd, 1 H, H-2), 4.11 (dd, 1 H, $J_{4,5}$ = 1.8 Hz, H-4), 3.75–3.70 (m, 1 H, H-5), 3.72 (d, 6 H, $J_{H,P}$ = 10.8 Hz, 2 OMe), 2.08–1.66 (m, 4 H, 2 H-6, 2 H-7), 1.50, 1.43, 1.32, 1.31 (4 s, 12 H, 4 Me) ppm. ^{13}C NMR (100 MHz): δ = 109.2 (C), 108.4 (C), 96.4 (C-1), 72.5 (C-4), 70.9 (C-3), 70.5 (C-2), 67.4 (d, $J_{C,P}$ = 16.0 Hz, CH, C-5), 52.3 (br. s, 2 OCH_3), 25.9 (2 CH_3), 24.9 (CH_3), 24.3 (CH_3), 23.3 (br. s, C-6), 20.9 (d, $J_{C,P}$ = 141.0 Hz, CH_2, C-7) ppm. ^{31}P NMR (162 MHz): δ = 34.7 ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd. for $C_{15}H_{28}O_8P$ [M + H]$^+$ 367.1516, found 367.1514.

Methyl 2,3,4-Tri-*O*-acetyl-6-deoxy-6-dimethoxyphosphanyl-α-D-glucopyranoside (9): A solution of **8** (57 mg, 0.188 mmol) in **2** (1.7 mL, 18.9 mmol) was treated as described for the preparation of **3a**. A solution of the crude product in pyridine (2 mL) and Ac_2O (2 mL) was kept at r.t. for 3 h and then concentrated to give, after column chromatography on silica gel (2:1 AcOEt/acetone containing 0.5% of triethylamine), **9** (76 mg, 98%) as a white solid. $[\alpha]_D$ = +92.7 (*c* = 0.8, $CHCl_3$). ^{1}H NMR (400 MHz): δ = 5.43 (dd, 1 H, $J_{2,3}$ = 10.4, $J_{3,4}$ = 9.1 Hz, H-3), 4.91 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.83 (dd, 1 H, H-2), 4.82 (dd, 1 H, $J_{4,5}$ = 10.3 Hz, H-4), 4.18 (ddd, 1 H, $J_{5,6a}$ = 5.5, $J_{5,6b}$ = 8.0 Hz, H-5), 3.74 and 3.71 (2d, 6 H, $J_{H,P}$ = 11.0 Hz, 2 POMe), 3.49 (s, 3 H, OMe), 2.06, 2.03, and 1.98 (3s, 9 H, 3 Ac), 2.01–1.95 (m, 2 H, 2 H-6) ppm. ^{13}C NMR (100 MHz): δ = 170.2 (C), 169.9 (2 C), 96.6 (C-1), 72.8 (d, $J_{C,P}$ = 16.8 Hz, CH, C-4), 70.9 (C-2), 69.8 (C-3), 64.3 (d, $J_{C,P}$ = 6.1 Hz, CH, C-5), 55.7 (OMe), 52.7 (d, $J_{C,P}$ = 5.4 Hz, CH_3, POMe), 52.0 (d, $J_{C,P}$ = 6.1 Hz, CH_3, POMe), 27.6 (d, $J_{C,P}$ = 143.8 Hz, CH_2, C-6), 20.71 (CH_3), 20.68 (CH_3), 20.64 (CH_3) ppm. ^{31}P NMR (162 MHz): δ = 29.8 ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd. for $C_{15}H_{26}O_{11}P$ [M + H]$^+$ 413.1207, found 413.1135.

Dimethyl [(2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl)methyl]phosphonate (11): A solution of **10** (50 mg, 0.145 mmol) in **2** (1.3 mL, 14.5 mmol) was treated as described for the preparation of **3a**. A solution of the crude product in pyridine (2 mL) and Ac_2O (2 mL) was kept at r.t. for 1 h and then concentrated to give, after column chromatography on silica gel (AcOEt then 1:1 AcOEt/acetone containing 0.5% of triethylamine), first 4,5,6,8-tetra-*O*-acetyl-3,7-anhydro-2-deoxy-1-*C*-phenyl-*aldehydo*-D-*glycero*-D-*gulo*-octose contaminated by uncharacterized byproducts (20 mg). The pure compound (6.5 mg, 10%) was obtained by column chromatography on silica gel (2:1 cyclohexane-AcOEt). $[\alpha]_D$ = –12.3 (*c* = 0.1, $CHCl_3$). ^{1}H NMR (400 MHz): δ = 7.87–7.85 (m, 2 H, *o*-Ar), 7.54–7.48(m, 1 H, *p*-Ar), 7.43–7.37 (m, 2 H, *m*-Ar), 5.18 (dd, 1 H, $J_{4,5}$ = 9.3, $J_{5,6}$ = 9.4 Hz, H-5), 5.02 (dd, 1 H, $J_{6,7}$ = 10.2 Hz, H-6), 4.96 (dd, 1 H, $J_{3,4}$ = 10.0 Hz, H-4), 4.18 (dd, 1 H, $J_{7,8a}$ = 4.8, $J_{8a,8b}$ = 12.3 Hz, H-8a), 4.16 (ddd, 1 H, $J_{2a,3}$ = 8.3, $J_{2b,3}$ = 3.2 Hz, H-3), 3.93 (dd, 1 H, $J_{7,8b}$ = 2.3 Hz, H-8b), 3.67 (ddd, 1 H, H-7), 3.28 (dd, 1 H, $J_{2a,2b}$ = 16.9 Hz, H-2a), 2.87 (dd, 1 H, H-2b), 1.96, 1.94, 1.93, and 1.91 (4s, 12 H, 4 Ac) ppm. ^{13}C NMR (100 MHz): δ = 196.1 (C), 170.7 (C), 170.3 (C), 170.0 (C), 169.6 (C), 136.2 (C), 133.5 (CH), 128.7 (CH), 128.3 (CH), 75.8 (CH), 74.2 (CH), 74.0 (CH), 71.8 (CH), 68.5 (CH), 62.0 (CH_2), 40.6 (CH_2), 20.7 (4 CH_3) ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd. for $C_{22}H_{27}O_{10}$ [M + H]$^+$ 451.1604, found 451.1618.

Eluted second was **11** (29 mg, 45%) as a white solid. $[\alpha]_D$ = +5.0 (*c* = 0.6, $CHCl_3$). ^{1}H NMR (400 MHz): δ = 5.10 (dd, 1 H, $J_{2,3}$ = 8.9, $J_{3,4}$ = 9.8 Hz, H-3), 4.99 (dd, 1 H, $J_{4,5}$ = 9.6 Hz, H-4), 4.82 (dd, 1 H, $J_{1,2}$ = 10.2 Hz, H-2), 4.14 (dd, 1 H, $J_{5,6a}$ = 5.0, $J_{6a,6b}$ = 12.5 Hz, H-6a), 4.07 (dd, 1 H, $J_{5,6b}$ = 2.3 Hz, H-6b), 3.85–3.76 (m, 1 H, H-1), 3.68 and 3.65 (2d, 6 H, $J_{H,P}$ = 11.0 Hz, 2 OMe), 3.66 (ddd, 1 H, H-5), 2.02–1.91 (m, 2 H, CH_2P), 2.01, 1.98, 1.96, and 1.93 (4s, 12 H, 4 Ac) ppm. ^{13}C NMR (100 MHz): δ = 170.6 (C), 170.2 (C), 169.8 (C), 169.5 (C), 75.9 (CH), 73.9 (d, $J_{C,P}$ = 3.3 Hz, CH), 73.2 (d, $J_{C,P}$ = 5.5 Hz, CH), 72.1 (d, $J_{C,P}$ = 15.7 Hz, CH), 68.3 (CH), 62.1 (CH_2), 52.9 (d, $J_{C,P}$ = 6.2 Hz, CH_3, OMe), 52.1 (d, $J_{C,P}$ = 6.6 Hz, CH_3, OMe), 28.0 (d, $J_{C,P}$ = 144.2 Hz, CH_2), 20.73 (CH_3), 20.71 (CH_3), 20.6 (2 CH_3) ppm. ^{31}P NMR (162 MHz): δ = 29.6 ppm. HRMS (ESI/Q-TOF) *m*/*z* calcd. for $C_{17}H_{28}O_{12}P$ [M + H]$^+$ 455.1318, found 455.1337.

Supporting Information (see footnote on the first page of this article): Copies of the ^{1}H NMR and ^{13}C NMR spectra for compounds **3a**–**3d**, **5a**, **7**, **9**, and **11**.

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PAPER

Glycoside and peptide clustering around the octasilsesquioxane scaffold *via* photoinduced free-radical thiol–ene coupling. The observation of a striking glycoside cluster effect†

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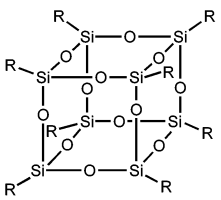
Two series of multivalent octasilsesquioxane glyco- and peptido-conjugates were synthesized using the photoinduced free-radical thiol–ene coupling (TEC). The first series was obtained by coupling *C*-glycosylpropyl thiols and cysteine containing peptides with the known octavinyl octasilsesquioxane while the second series was obtained by reacting glycosyl thiols with a new octasilsesquioxane derivative displaying eight PEGylated chains functionalized with terminal allyl groups. The evaluation of the binding properties of mannoside and glucoside clusters toward Concanavalin A by Enzyme-Linked Lectin Assay (ELLA) revealed a modest glycoside cluster effect. On the other hand, the PEGylated POSS-based glycocluster featuring eight *N*-acetyl-glucosamine residues showed high affinity toward Wheat Germ Agglutinin to give a measured IC_{50} at 3 nM. The calculated relative potency per number of sugar unit (rp/*n*) was superior to a value of 10^6, thus revealing the occurrence of a striking glycoside cluster effect.

Introduction

The cube-octameric silsesquioxanes (COSS, $R_8Si_8O_{12}$), most often referred to as polyhedral oligomeric silsesquioxanes (POSS),[1] the molecular equivalents to the cubic symmetric platonic polyhedron, are receiving considerable attention because of their rigid globular architecture displaying a precise clustering of eight ligand molecules in space. Thus, POSS can serve as nanobuilding blocks for constructing functional materials,[2] as supports for organometallic catalysts,[3] and as biocompatible drug carriers.[4] POSS-derived materials exhibited no significant cell toxicity demonstrating their potential as biomaterials.[5] Starting materials for the construction of complex POSS derivatives are compounds **1a–d** (Fig. 1) bearing reactive functional groups at the periphery such as amino, azido, vinyl and chloro. These compounds are commercially available or can be prepared from inexpensive organosilicon precursors.[1]



1a R = $(CH_2)_3NH_2$
1b R = $(CH_2)_3N_3$
1c R = $CH{=}CH_2$
1d R = $(CH_2)_3Cl$

Fig. 1 Functionalized POSS derivatives **1a–d**.

Thus, in the late 1900s Feher *et al.* reported the synthesis of peptidyl and glycosyl POSS by standard amide coupling of octa (aminopropyl) POSS **1a** with *N*-protected peptides and sugar lactones, respectively.[6] It now appears that this pioneering approach was plagued by two main drawbacks, one being the scarce availability of octaamine **1a** (35% from aminopropyl silane), the other being the low yields of amide coupling (20–60%). The need for efficient approaches to POSS leading to a complete and uniform conjugation at each apex to avoid the troublesome separation of partially functionalized derivatives and/or reaction intermediates quite recently led two independent research groups, one headed by Fessner[7] and the other by Chiara,[8] to use the most popular click reaction, *i.e.* the Cu-catalyzed azide-alkyne cycloaddition (CuAAC),[9] for the synthesis of triazole-linked POSS glycoconjugates. Both research groups employed the octaazide silsesquioxane **1b** as a scaffold. Unfortunately, the

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†Electronic supplementary information (ESI) available: Syntheses of sugar thiols **3a** and **3b**. ^1H and ^{13}C NMR spectra of all new compounds, ^{29}Si NMR spectrum of **4b**, stacked ^1H NMR spectra of **1c** and **4a**, **4b**, **8** and **9**, stacked ^1H NMR spectra of **12** and **13a**, **13b**, **13c**, **14** and **15**. See DOI: 10.1039/c2ob07078b

preparation of this densely nitrogenated compound presented some hazards due to the formation of azidomethane as a by-product. Moreover, while the potency and synthetic utility of CuAAC is undeniable, there is a diffuse concern about the use of this ligation tool in bioorganic synthesis due to the toxic copper catalyst as potential contaminant of the reaction product. This drawback has been recently reported in dendrimer formation[10] so that the strained-promoted azide-alkyne cycloaddition (SPAAC) approach[11] had to be employed. Fortunately enough, the click chemistry space is unlimited,[12] so that many other metal-free ligation reactions are available for the solution of specific problems.[13] One of these reactions is the century old free-radical hydrothiolation of terminal alkenes,[14] referred to as thiol–ene coupling (TEC), that is emerging as a valuable click process[15] in bioorganic[16] and polymer/dendrimer chemistry[17] as well as biomaterial synthesis.[18] Quite remarkably TEC can be initiated by using a simple initiator such as 2,2-dimethoxy-2-phenylacetophenone (DPAP) and irradiation at wavelength close to visible light, *e.g.* λ_{max} 365 nm, the latter being a condition that excludes any photodamage of biomolecules such as carbohydrates and proteins. The main features of TEC that support its click status are high efficiency, total atom economy, orthogonality to a broad range of reagents, and compatibility with water and oxygen. Notably, when an excess of thiol with respect to alkene is used, the only side product is the readily removable disulfide which in turn can be reduced back to thiol by using, for instance, dithiothreitol (DTT).[19] The only study on the use of TEC for the synthesis of POSS glycoconjugates was reported in 2004 by Lee and co-workers[20] *via* photoinduced reaction of *N*-mannosyl and *N*-lactosyl γ-thiobutyramides with octavinyl POSS **1c**. While the preparation of this manuscript was in progress, a paper has appeared describing the introduction of glucose residues on a heptavinyl POSS-polylactide conjugate (VPOSS-PLLA) *via* thiol–ene coupling.[21] Thus, we would like to report here validation/extension of TEC-based approach toward peptide and glycoside cube-shaped clusters using the commercially available **1c** and a new octaene reagent derived from it as POSS starting materials. The evaluation of the inhibition properties of selected glycoclusters thus prepared toward lectins will be also reported for the first time. This study follows our recent work on the use of TEC as a ligation tool for glycoclustering on the rigidified platform of calix[4]arene.[16c]

Results and discussion

We first set out to study the photoinduced coupling of **1c** with the simple sugar thiol 1-thio-β-D-glucopyranose[22] **2a** (Fig. 2) under previously established standard conditions for multiple TEC on calix[4]arene scaffold,[16c] *i.e.* irradiation for 1 h at λ_{max} 365 nm in the presence of DPAP as the initiator (entry 1, Table 1). The reaction was conducted at room temperature in a glass vial and no care was taken to exclude air and moisture. Despite the use of excess of **2a** from 1.5 to 4 equiv./ene of **1c**, a partial hydrothiolation of the latter was observed as evidenced by the presence of residual alkene proton signals in the 5–6 ppm region of the NMR spectrum (CD_3OD) of the crude reaction mixture.

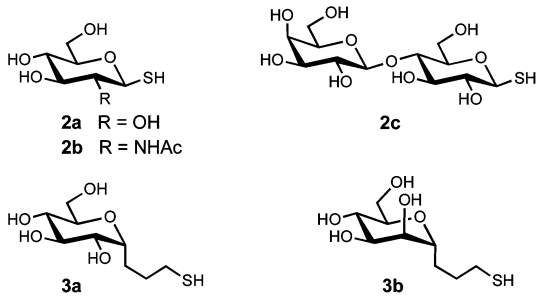


Fig. 2 Sugar thiols employed for the hydrothiolation of POSS.

Table 1 Hydrothiolation of POSS **1c** at λ_{max} 365 nm in the presence of DPAP (0.1 equiv./thiol)

Entry	Thiol	Thiol equiv./ene	Solvent	Time	Product	Yield (%)
1	**2a**	1.5–4	DMF	1 h	—	—
2	**3a**	2	DMF–THF	1 h	**4a**	94
3	**3b**	2	DMF–THF	1 h	**4b**	93
4	**5**	1.5	DMF	45 min	**8**	84
5	**6**	1.5	DMF	45 min	**9**	75
6	**7**	3	DMF–H_2O	2 h	—	—

We felt that the steric congestion around the octasilsesquioxane scaffold produced by the sequential attachment of thioglycoside fragments was responsible for these findings. Therefore, we set out to circumvent this limitation by introducing suitable tethers holding the alkenyl groups of the scaffold or the sulfhydryl group of the carbohydrate. At first we decided to test the latter possibility. To this end we prepared the *C*-glucosylpropyl thiol **3a** (Fig. 2) by thiol–ene coupling of known[23] allyl *C*-glucopyranoside with thioacetic acid and transesterification (MeONa–MeOH) of the resulting thioacetate (see ESI, Fig. S1†). Quite rewardingly the photoinduced hydrothiolation of **1c** by **3a** in the presence of DPAP was complete after 1 h as evidenced by the total disappearance of alkene proton signals in the NMR spectrum of the crude reaction mixture (Fig. 3). This indicated that all vinyl groups of the octasilsesquioxane **1c** had been saturated through eight concomitant TEC reactions.

Chromatography over Sephadex LH-20 allowed the isolation of the POSS-based octavalent glycocluster **4a** (Fig. 4) in excellent yield (entry 2, Table 1). No side reactions were observed as most of the excess of thiol was recovered unaltered while the only side product was the corresponding disulfide. A complete hydrothiolation of **1c** was also carried out using the *C*-mannosyl thiol **3b** (prepared from the known allyl *C*-mannopyranoside,[23] see ESI, Fig. S1†) to give the corresponding POSS-based glycocluster **4b** in an almost identical yield of **4a** (entry 3, Table 1). Evidence for the conservation of the structural integrity of the POSS cage in **4b** upon irradiation at λ_{max} 365 nm was unambiguously provided by ^{29}Si NMR spectroscopy showing a sharp peak at −66.2 ppm.

While a recent paper by Kolmar and co-workers reported on the preparation of POSS-peptide conjugates *via* CuAAC using

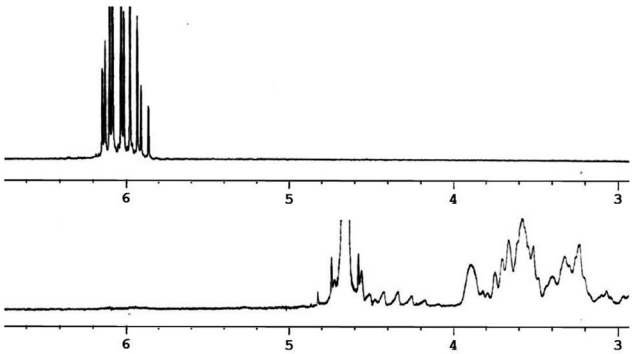


Fig. 3 ^{1}H NMR spectra of octavinyl POSS **1c** (300 MHz, $CDCl_3$) (top) and the crude reaction mixture of the coupling of **1c** with **3a** (300 MHz, D_2O) (bottom).

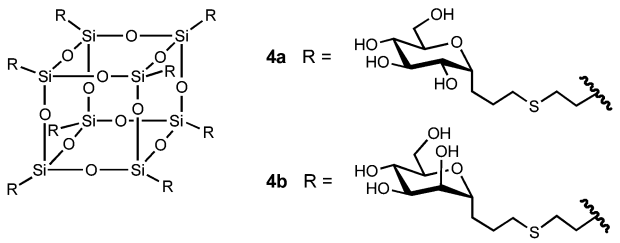


Fig. 4 Glycoconjugates prepared from octavinyl POSS **1c**.

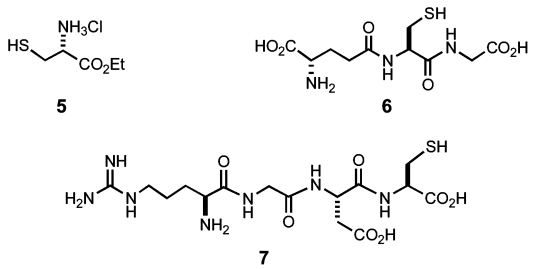


Fig. 5 Cysteine derivative and cysteine containing peptides used for the hydrothiolation of POSS.

octaazide silsesquioxane **1b** as the reagent,[24] we decided to develop a complementary metal-free approach *via* TEC using octavinyl POSS **1c**. As we intended to use cysteine-containing peptides as thiol partners, we first explored the feasibility of the photoinduced coupling of **1c** with cysteine. Specifically, we used the commercially available cysteine hydrochloride ethyl ester **5** (Fig. 5) because this compound was fairly soluble in DMF, a solvent also capable of dissolving **1c** and the photoinitiator DPAP.

Thus, the photoinduced coupling between **1c** and excess of **5** (1.5 equiv./ene of **1c**) in the presence of DPAP was successfully carried out to give the POSS-cysteine conjugate **8** (Fig. 6) in high isolated yield (entry 4, Table 1). Then, the coupling of **1c** with the natural tripeptide glutathione Glu-Cys-Gly (GSH) **6** (Fig. 5) was performed as well and also in this case complete hydrothiolation of POSS substrate was observed by ^{1}H-NMR analysis to give the POSS-GSH conjugate **9** (Fig. 6) in 75% isolated yield (entry 5, Table 1). The attempt to conjugate **1c** with a larger peptide, namely the tetrapeptide Arg-Gly-Asp-Cys

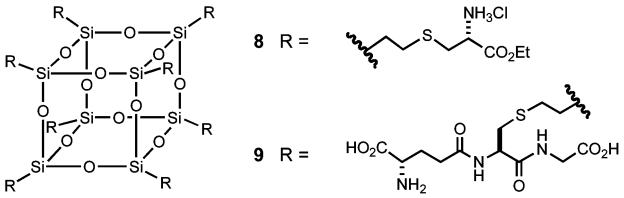
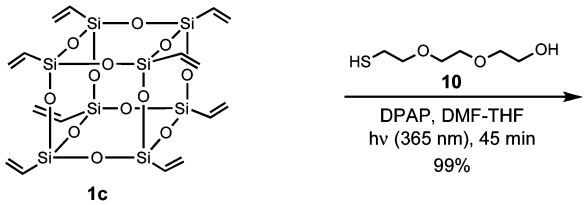
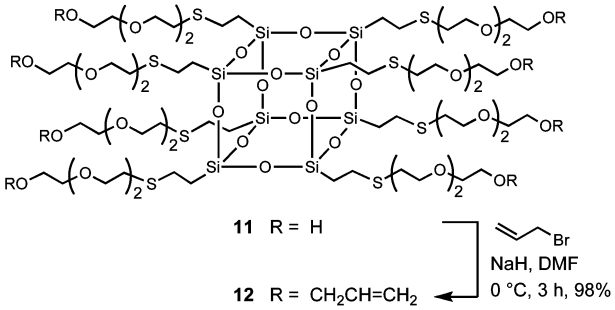


Fig. 6 POSS-peptide conjugates prepared from **1c**.





Scheme 1 Synthesis of PEGylated octaallyl POSS **12**.

(RGDC) **7** (Fig. 5) gave less satisfactory results. Although a considerable excess of **7** was employed (3 equiv./ene of **1c**), only partial hydrothiolation of **1c** was achieved as revealed by the presence of unreacted vinyl groups by NMR analysis of the crude reaction mixture (entry 6, Table 1). Therefore no efforts were made to optimize this reaction. On the other hand, the ^{1}H and ^{13}C NMR spectra of all glyco- and peptido-conjugates reported above showed the absence of olefinic signals while there was some line broadening of the other signals, very likely due to various conformations of the ligands. Moreover, MS analysis of products **4a**, **4b**, **8**, and **9** confirmed their structure.

In a second instance we set out to circumvent the incomplete conjugation due to steric hindrance by using an octaene POSS derivative in which alkene groups were attached to the scaffold through a spacer. To this end we decided to use a PEGylated tether because this hydrophilic chain is known to improve water solubility and biocompatibility. The PEG fragment was introduced by photoinduced coupling of **1c** with the known[25] thiol **10** bearing a PEG chain with a terminal hydroxyl group, to give the octahydroxy functionalized POSS **11** (Scheme 1). This in turn was treated with allyl bromide and NaH to afford the target PEG-linked octaene silsesquioxane **12** in almost quantitative yield. Notably the ^{1}H NMR spectrum of this new POSS-based reagent revealed a single set of olefinic protons in accordance with the T_8 symmetry of the system. We considered this observation as an additional evidence of the conservation of the structural integrity of the POSS cage under the conditions of photoinduced TEC.

Next, the photoinduced coupling of **12** with glycosyl thiols, *i.e.* sugars bearing the sulfhydryl group directly linked to the anomeric carbon, was explored. Thus, it was quite rewarding to find that the irradiation (λ_{max} 365 nm) of a mixture constituted of **12**, 1-thio-β-D-glucopyranose **2a** (Fig. 2) and DPAP in an aqueous solvent (MeOH–DMF–H_2O) induced the complete consumption of **12** as shown by ^{1}H NMR analysis of the crude mixture. Column chromatography of the latter allowed the isolation of pure POSS-based glycoconjugate **13a** (Fig. 7) in very good yield (entry 1, Table 2). Effective conjugation was achieved also from the reaction of **12** with the 2-acetamido-2-deoxy-1-thio-β-D-glucopyranose **2b** and the sterically more demanding disaccharide 1-thio-β-D-lactopyranose[22] **2c** (Fig. 2). In both cases the reaction afforded the corresponding glycoconjugate, being product **13b** and **13c** (Fig. 7) isolated in very good and fair yield, respectively (entries 2 and 3, Table 2). In a second instance, the photoinduced reactions of **12** with the tripeptide glutathione **6** and tetrapeptide RGDC **7** (Fig. 5) were carried out under the above conditions. These reactions did not present any problems apart the need of using the hydrochloride of **6** to achieve complete solubility of reagents and product in the selected solvent (MeOH). In both cases the silsesquioxane **12** was completely hydrothiolated after 1 h irradiation as shown by NMR analysis of the reaction mixtures. Suitable work-up and chromatography over Sephadex LH-20 afforded the corresponding peptidyl conjugates **14** and **15** (Fig. 7) in very good yields (entries 4 and 5, Table 2). Also the thioconjugates derived from **12**, *i.e.* **13a–c**, **14**, and **15**, were characterized by NMR as well as mass spectrometry. Only product **13b** failed to give a satisfactory MALDI-TOF MS spectrum (the experimental mass differed by 1.7 Da from the calculated value) but this was characterized by consistent elemental analysis of its hydrated form.

In order to ascertain whether the prepared POSS-based glycoclusters exhibited to some extent a glycoside cluster effect[26] in lectin recognition, the binding properties of some of them were studied with two lectins, one from *Canavalia ensiformis* (Concanavalin A, ConA), which is specific for the α-D-mannopyranosides and, to a lesser extent, the α-D-glucopyranosides, the other from *Triticum vulgaris* (wheat germ agglutinin, WGA), which is specific for *N*-acetyl-D-glucosamine (D-GlcNAc). First, the ability of glucosylated and mannosylated glycoclusters **4a** and **4b** to inhibit the binding of horseradish peroxidase-labelled ConA (ConA-HRP) to an α-D-mannose-polyacrylamide

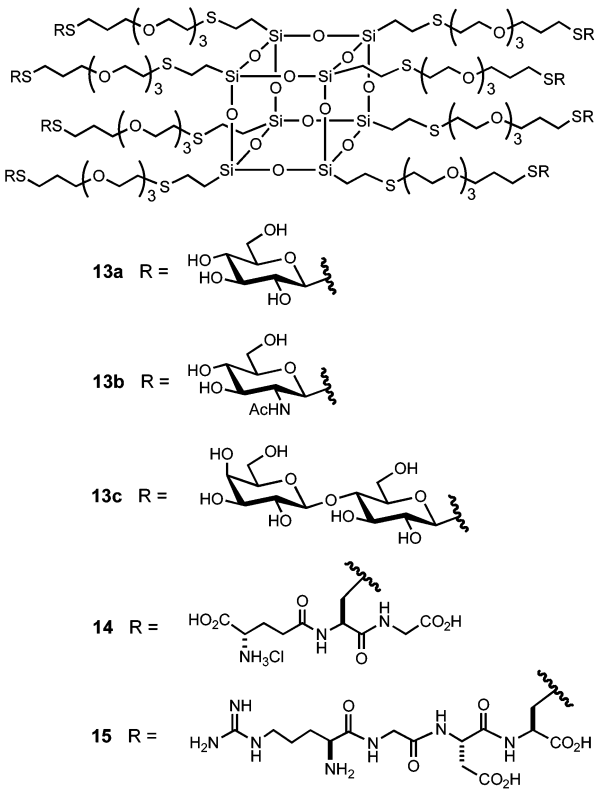


Fig. 7 Glyco- and peptido-conjugates prepared from PEGylated POSS **12**.

Table 2 Hydrothiolation of POSS **12** at λ_{max} 365 nm in the presence of DPAP (0.1 equiv./thiol)

Entry	Thiol	Thiol equiv./ene	Solvent	Time (h)	Product	Yield (%)
1	**2a**	3	MeOH–DMF–H_2O	1	**13a**	79
2	**2b**	3	DMF–H_2O	1	**13b**	82
3	**2c**	3	DMF–H_2O	1.5	**13c**	50
4	**6·HCl**	3	MeOH	1	**14**	78
5	**7**	3	MeOH	1	**15**	61

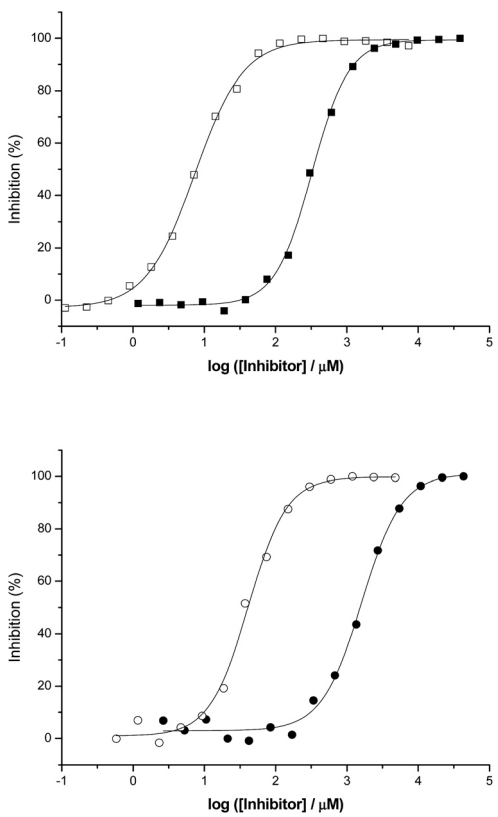


Fig. 8 Inhibition curves of methyl α-D-mannopyranoside (■) and mannosylated glycocluster **4b** (□) (top) or methyl α-D-glucopyranoside (●) and glucosylated glycocluster **4a** (○) (bottom).

Table 3 ELLA data for the inhibition of the binding of ConA-HRP to α-D-Man-PAA with glucosylated (**4a**) or mannosylated (**4b**) glycoclusters[a]

Entry	Product	n^b	IC_{50} (μM)	rp[c]	rp/n^d
1	Me α-D-Glc	1	1422 ± 129	1	1
2	**4a**	8	40.4 ± 0.7	35.2	4.4
3	Me α-D-Man	1	328 ± 27	1	1
4	**4b**	8	6.8 ± 0.9	48.2	6

[a] Each experiment was carried out in triplicate. [b] Number of sugar units in the molecule. [c] Relative potency = IC_{50}(monosaccharide)/IC_{50}(glycocluster). [d] Relative potency/number of sugar units.

Table 4 ELLA data for the inhibition of the binding of WGA-HRP to D-GlcNAc-PAA with PEGylated POSS-based glycoclusters **13a** and **13b**[a]

Entry	Product	n^b	IC_{50} (μM)	rp[c]	rp/n^d
1	D-GlcNAc	1	28 000 ± 2500	1	1
2	**13b**	8	0.003 ± 0.0006	9.3×10^6	10^6
3	**13a**	8	No inhibition[e]	—	—

[a] Each experiment was carried out in triplicate. [b] Number of sugar units in the molecule. [c] Relative potency = IC_{50}(monosaccharide)/IC_{50}(glycocluster). [d] Relative potency/number of sugar units. [e] No inhibition detected at 100 μM.

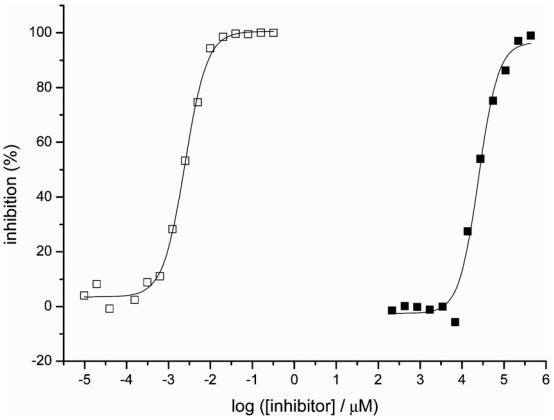


Fig. 9 Inhibition curves for the binding of WGA-HRP to D-GlcNAc-PAA by GlcNAc (■) and glycocluster **13b** (□).

conjugate (α-D-Man-PAA) was measured by an Enzyme-Linked Lectin Assay (ELLA) following a previously reported procedure[27] (Fig. 8). Methyl α-D-mannopyranoside (Me α-D-Man) and methyl α-D-glucopyranoside (Me α-D-Glc) were used as monovalent references.

As indicated in Table 3, both compounds showed modest inhibitory properties with IC_{50} values of 40 and 7 μM for **4a** and **4b**, respectively, which correspond to a relative potency (rp) of 35 (**4a**) and 48 (**4b**) in reference to the corresponding monosaccharide. When reported to the number of sugar unit (rp/*n*), the inhibition enhancement was 4.4 (**4a**) and 6-fold (**4b**) higher, indicating a weak glycoside cluster effect. It is likely that the rather short spacers between the sugars and the platform in glycoclusters **4a** and **4b** did not allow a multivalent interaction with Concanavalin A, which displays four binding sites located far away from each other (*ca.* 65 Å). These findings are in good agreement with the moderate binding affinity to ConA that is usually shown by low molecular weight glycoclusters.[28] It has to be noted, however, that this is not a general result, as in some cases higher affinity was observed.[29]

A similar assay was performed with WGA and PEGylated POSS-based GlcNAc cluster **13b** as the inhibitor while 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and glucosylated glycocluster **13a** were used as the monovalent reference and the negative control, respectively (Fig. 9).

GlcNAc cluster **13b** showed a strong inhibition effect (IC_{50} 3 nM) whereas no inhibition was observed with the glucosylated derivative **13a** at a concentration 100 μM, thus precluding unspecific binding between WGA and the silsesquioxane core (Table 4). In contrast to the results obtained from the assays with ConA, the IC_{50} found for **13b** corresponds to an extremely high relative potency when compared to the monosaccharidic GlcNAc (rp = 9.3×10^6, rp/*n* = 10^6). These unprecedented values for the inhibition of WGA by a synthetic glycocluster clearly indicated a strong multivalent effect, very likely due to a chelate binding mode.[30] Indeed WGA is a dimeric lectin containing a total of eight binding sites separated by approximately 14 Å.[31] These structural features appear fully compatible with the tridimensional orientation and the length of the spacers linking the GlcNAc moieties to the silsesquioxane platform in the glycocluster **13b**. Therefore, the multiple and simultaneous interactions of the sugar ligands with the WGA binding sites take place efficiently.

It is worth noting that ELLA experiments measure the ability of a ligand to inhibit the binding of a lectin to an immobilized glycopolymer. Therefore, the IC_{50} value is only indicative of the binding potency of the ligand to the lectin in reference to the immobilized compound. In order to fully assess the binding properties of **13b** toward WGA lectin, other assays, *e.g.* by Isothermal Titration Calorimetry (ITC) or Surface Plasmon Resonance (SPR), should be performed.

Conclusions

In conclusion, the above results demonstrate the versatility and fidelity of the free-radical thiol–ene coupling (TEC) as a tool for the introduction of sugars and peptide residues into octasilsesquioxane scaffolds to give bioorganic–inorganic hybrid materials. As exhaustive hydrothiolation of the eight vinyl groups of the octasilsesquioxanes employed did occur in all cases examined, the modest yields of some isolated products can be ascribed to the difficulty in their purification. Hence, the efficiency of TEC as a metal-free click process that can be initiated by visible light appears to be confirmed. Moreover, TEC proved to be also a useful methodology for the high yield preparation of a new functionalized octasilsesquioxane, *i.e.* the PEG-linked octaene silsesquioxane **12**. The use of this compound appears to overcome the problem of incomplete silsesquioxane conjugation due to steric hindrance. The striking glycoside cluster effect registered in inhibition experiments of a specific lectin by a glycocluster derived from **12** is notable. This particular issue needs further studies for establishing the key

structural factors of the glycocluster responsible for such effect. These studies are under way in our laboratories.

Experimental

General experimental section

Flash column chromatography was performed on silica gel 60 (40–63 mm). Optical rotations were measured at 20 ± 2 °C in the stated solvent; $[\alpha]_D$ values are given in deg mL g^{-1} dm^{-1}. ^1H NMR (300 and 400 MHz), ^{13}C NMR spectra (75 and 100 MHz), and ^{29}Si NMR (79.5 MHz) were recorded from D_2O solutions at room temperature unless otherwise specified. Peak assignments were aided by ^1H-^1H COSY and gradient-HMQC experiments. In the ^1H NMR spectra reported below, the *n* and *m* values quoted in geminal or vicinal proton–proton coupling constants $J_{n,m}$ refer to the number of the corresponding sugar protons.

The commercially available octavinyl POSS **1c**, photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DPAP), cysteine hydrochloride **5**, and glutathione **6** were used without further purification. The tetrapeptide Arg-Gly-Asp-Cys (RGDC, **7**) was supplied by GL Biochem Ltd (Shangai, China). Horseradish peroxidase-labelled Concanavalin A (ConA-HRP) and *Triticum vulgaris* lectin (wheat germ agglutinin) (WGA-HRP), Bovine Serum Albumin (BSA), and SIGMA*FAST* *O*-phenylenediamine dihydrochloride (OPD) were purchased from Sigma-Aldrich. The α-D-mannose-polyacrylamide (α-D-Man-PAA) and 2-acetamido-2-deoxy-D-glucose-polyacrylamide (D-GlcNAc-PAA) were obtained from Lectinity Holding, Inc., Moscow.

The thiol–ene coupling was carried out in a glass vial (diameter: 1 cm; wall thickness: 0.65 mm), sealed with a natural rubber septum, located 2.5 cm away from the household UVA lamp apparatus equipped with four 15 W tubes (1.5 × 27 cm each).

High resolution MS analysis

For accurate mass measurements the compounds were analyzed in positive ion mode by electrospray hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF) fitted with a Z-spray electrospray ion source. The capillary source voltage and the cone voltage were set at 3500 V and 35 V, respectively; the source temperature was kept at 80 °C; nitrogen was used as a drying gas at a flow rate of *ca.* 50 L h^{-1}. The time-of-flight analyzer was externally calibrated with NaI from *m*/*z* 300 to 2000 to yield an accuracy near to 5 ppm. When necessary an internal lock mass was used to further increase the mass accuracy. Accurate mass data were collected by directly infusing samples (1.5 pmol μL^{-1} in 1 : 1 CH_3CN–H_2O) into the system at a flow rate of 5 μL min^{-1}. The acquisition and data processing were performed with the MassLynx 4.1 software. Compounds **4a**, **9**, **11**, **12**, **13a–c**, **14**, and **15** were analyzed by MALDI TOF mass spectrometry using a pulsed nitrogen laser (λ = 337 nm) and α-cyano-4-hydroxycinnamic acid or sinapinic acid as the matrix. The instrument was operated in positive ion reflectron mode with the source voltage set to 12 kV. The pulse voltage was optimized at 1999 V, and the detector and reflectron voltages were set to 5200 and 2350 V, respectively. Measurements were performed in the mass range *m*/*z* 800–5000 with a suppression mass gate set to *m*/*z* 500 to prevent detector saturation from matrix cluster peaks and an extraction delay of 600 ns. The instrument was externally calibrated using a polyethylene glycol mix as standard. A mass accuracy near to the nominal (50 ppm) was achieved for each standard.

Glycoconjugate 4a. A solution of octavinyl POSS **1c** (10 mg, 15.8 μmol), thiol **3a** (60 mg, 0.25 mmol), and DPAP (6.5 mg, 25.3 μmol) in DMF (300 μL) and THF (100 μL) was irradiated at r.t. for 1 h under magnetic stirring and then concentrated. The residue was eluted from a column of Sephadex LH-20 with 3 : 1 MeOH–H_2O to give **4a** (38 mg, 94%) as a syrup; $[\alpha]_D$ = +54.4 (*c* 1.5, H_2O). ^1H NMR (300 MHz): δ 3.96–3.83 (m, 8H), 3.80–3.43 (m, 32H), 3.42–3.20 (m, 16H), 2.70–2.45 (m, 32H), 1.85–1.45 (m, 32H), 1.10–0.90 (m, 16H). ^{13}C NMR (75 MHz): δ 75.9 (CH), 73.8 (CH), 72.8 (CH), 71.6 (CH), 70.4 (CH), 61.4 (CH_2), 31.5 (CH_2), 25.6 (CH_2), 25.5 (CH_2), 23.5 (CH_2), 14.0 (CH_2). MALDI-TOF MS: *m*/*z* calcd for $C_{88}H_{168}NaO_{52}S_8Si_8$ $(M + Na)^+$ 2562.47, found 2562.47.

Glycoconjugate 4b. The octavinyl POSS **1c** (10 mg, 15.8 μmol) was treated with thiol **3b** (60 mg, 0.25 mmol) as described for the preparation of **4a** to give **4b** (37.5 mg, 93%) as a syrup; $[\alpha]_D$ = +16.4 (*c* 1.6, H_2O). ^1H NMR (300 MHz): δ 3.90–3.51 (m, 48H), 3.45–3.33 (m, 8H), 2.73–2.50 (m, 32H), 1.96–1.41 (m, 32H), 1.15–0.95 (m, 16H). ^{13}C NMR (75 MHz): δ 78.1 (CH), 73.7 (CH), 71.9 (CH), 71.2 (CH), 67.2 (CH), 61.4 (CH_2), 31.4 (CH_2), 27.1 (CH_2), 25.7 (CH_2), 25.6 (CH_2), 12.8 (CH_2). ^{29}Si NMR (79.5 MHz): δ −66.2. HRMS (ESI/Q-TOF): *m*/*z* calcd for $(C_{88}H_{170}O_{52}S_8Si_8)/2$ $(M + 2H)^{2+}$ 1269.3289, found 1269.3259.

POSS-cysteine conjugate 8. A solution of **1c** (10 mg, 15.8 μmol), cysteine hydrochloride **5** (35 mg, 0.19 mmol), and DPAP (5 mg, 19.0 μmol) in DMF (1.6 mL) was irradiated at r.t. for 45 min under magnetic stirring and then concentrated. The residue was eluted from a column of Sephadex LH-20 with 1 : 1 MeOH–H_2O to give **8** (28 mg, 84%) as a syrup; $[\alpha]_D$ = +6.7 (*c* 0.8, MeOH). ^1H NMR (300 MHz): δ 4.25 (dd, 8H, *J* = 5.0, 5.5 Hz, 8 CHN), 4.22 (q, 16H, *J* = 7.2 Hz, 8 CH_2CH_3), 3.12 (dd, 8H, *J* = 5.5, 15.0 Hz, 8 H of CH_2S), 3.04 (dd, 8H, *J* = 5.0, 15.0 Hz, 8 H of CH_2S), 2.59 (t, 16H, *J* = 8.0 Hz, 8 CH_2S), 1.22 (t, 24H, *J* = 7.2 Hz, 8 CH_2CH_3), 1.05 (dd, 8H, *J* = 8.0, 15.0 Hz, 8 H of CH_2Si), 0.96 (dd, 8H, *J* = 8.0, 15.0 Hz, 8 H of CH_2Si). ^{13}C NMR (75 MHz): δ 169.2 (C), 63.6 (CH_2), 52.3 (CH), 31.3 (CH_2), 25.9 (CH_2), 13.4 (CH_3), 11.7 (CH_2). HRMS (ESI/Q-TOF): *m*/*z* calcd for $(C_{56}H_{114}N_8O_{28}S_8Si_8)/2$ $(M + 2H)^{2+}$ 913.1831, found 913.1842.

POSS-glutathione conjugate 9. The octavinyl POSS **1c** (10 mg, 15.8 μmol) was treated with glutathione **6** (58 mg, 0.19 mmol) as described for the preparation of **8** to give, after column chromatography on Sephadex LH-20 (2 : 1 H_2O–MeOH), **9** (36.5 mg, 75%) as a syrup; $[\alpha]_D$ = −17.9 (*c* 0.8, H_2O). ^1H NMR (300 MHz): δ 4.44 (bt, 8H, *J* = 5.8 Hz), 3.84 (bs, 16H), 3.70 (t, 8H, *J* = 6.2 Hz), 2.98–2.86 (m, 8H), 2.84–2.71 (m, 8H), 2.62–2.53 (m, 16H), 2.45–2.36 (m, 16H), 2.08–1.98 (m, 16H), 1.04–0.91 (m, 16H). ^{13}C NMR (100 MHz): δ 174.5 (C), 173.6 (C), 172.5 (C), 53.7 (CH), 53.0 (CH), 41.7

(CH_2), 32.7 (CH_2), 31.2 (CH_2), 26.0 (CH_2), 12.0 (CH_2). MALDI-TOF MS: *m/z* calcd for $C_{96}H_{161}N_{24}O_{60}S_8Si_8$ $(M + H)^+$ 3092.67, found 3092.66.

PEGylated POSS 11. A solution of **1c** (80 mg, 126.4 μmol), 2-[2-(2-hydroxyethoxy)ethoxy]-1-ethanethiol (**10**, 336 mg, 2.02 mmol), and DPAP (16 mg, 63.2 μmol) in DMF (1.4 mL) and THF (0.7 mL) was irradiated at r.t. for 45 min under magnetic stirring and then concentrated. The residue was eluted from a column of Sephadex LH-20 with 1 : 1 MeOH–H_2O to give **11** (245 mg, 99%) as a syrup. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.76 (t, 16H, J = 4.7 Hz, 8 CH_2O), 3.72–3.65 (m, 48H, 24 CH_2O), 3.62 (t, 16H, J = 4.3 Hz, 8 CH_2O), 2.77 (t, 16H, J = 6.9 Hz, 8 CH_2S), 2.71–2.64 (m, 16H, 8 CH_2S), 2.60 (bs, 8H, 8 OH), 1.09–1.02 (m, 16H, 8 CH_2Si). ^{13}C NMR (75 MHz, $CDCl_3$): δ 72.5 (CH_2), 70.6 (CH_2), 70.3 (CH_2), 61.6 (CH_2), 31.1 (CH_2), 26.4 (CH_2), 13.0 (CH_2). MALDI-TOF MS: *m/z* calcd for $C_{64}H_{136}NaO_{36}S_8Si_8$ $(M + Na)^+$ 1985.96, found 1985.95.

PEGylated octaallyl POSS 12. NaH (16 mg, 0.40 mmol, of a 60% dispersion in oil) and then allyl bromide (35 μL, 0.40 mmol) were added to a stirred, cooled (0 °C) solution of **11** (49 mg, 25.0 μmol) in anhydrous DMF (2 mL). The mixture was stirred at 0 °C for 3 h, then diluted with 1 M phosphate buffer at pH 7 (0.5 mL), warmed to r.t., diluted with H_2O (15 mL), and extracted with AcOEt (3 × 30 mL). The combined organic phases were dried (Na_2SO_4) and concentrated. The residue was eluted from a column of Sephadex LH-20 with MeOH to give **12** (56 mg, 98%) as a syrup. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.94 (ddt, 8H, J = 5.6, 10.7, 16.5 Hz, 8 C*H*═CH_2), 5.30 (bd, 8H, J = 16.5 Hz, CH═C*H*$_2$), 5.21 (bd, 8H, J = 10.7 Hz, CH═C*H*$_2$), 4.05 (d, 16H, J = 5.6 Hz, 4 C*H*$_2$–CH═), 3.78–3.60 (m, 80H, 40 CH_2O), 2.82–2.62 (m, 32H, 16 CH_2S), 1.14–0.98 (m, 16H, 8 CH_2Si). ^{13}C NMR (75 MHz, $CDCl_3$): δ 134.7 (CH), 117.1 (CH_2), 72.2 (CH_2), 70.6 (CH_2), 70.3 (CH_2), 69.4 (CH_2), 31.3 (CH_2), 26.7 (CH_2), 14.1 (CH_2). MALDI-TOF MS: *m/z* calcd for $C_{88}H_{168}NaO_{36}S_8Si_8$ $(M + Na)^+$ 2303.71, found 2303.71.

Glycoconjugate 13a. A solution of **12** (14 mg, 6.1 μmol), glucosyl thiol **2a** (29 mg, 147.3 μmol), and DPAP (3.8 mg, 14.8 μmol) in 4 : 2 : 1 MeOH–DMF–H_2O (1.5 mL) was irradiated at r.t. for 1 h under magnetic stirring and then concentrated. The residue was eluted from a column of Sephadex LH-20 with 1 : 1 MeOH–H_2O to give **13a** (18.5 mg, 79%) as a syrup; $[\alpha]_D$ = −45.5 (*c* 0.7, H_2O). ^{1}H NMR (300 MHz): δ 4.42 (d, 8H, $J_{1,2}$ = 9.8 Hz, 8 H-1), 3.78 (bd, 8H, $J_{6a,6b}$ = 12.5 Hz, 8 H-6a), 3.72–3.50 (m, 104H, 48 CH_2O, 8 H-6b), 3.42–3.26 (m, 24H), 3.20 (t, 8H, J = 8.8 Hz), 2.80–2.58 (m, 48H, 24 CH_2S), 1.90–1.77 (m, 16H, 8 $OCH_2CH_2CH_2S$), 1.15–0.93 (m, 16H, 8 CH_2Si). ^{13}C NMR (75 MHz): δ 89.8 (CH), 80.7 (CH), 77.4 (CH), 72.8 (CH_2), 71.6 (CH), 70.1 (CH_2), 70.0 (CH_2), 69.6 (CH), 61.2 (CH_2), 31.4 (CH_2), 29.8 (CH_2), 26.4 (CH_2), 14.2 (CH_2). MALDI-TOF MS: *m/z* calcd for $C_{136}H_{264}CaO_{76}S_{16}Si_8$ $(M + Ca)^+$ 3893.36, found 3893.33.

Glycoconjugate 13b. A solution of **12** (14 mg, 6.1 μmol), thiol **2b** (35 mg, 147.3 μmol), and DPAP (3.8 mg, 14.8 μmol) in DMF (200 μL) and H_2O (50 μL) was irradiated at r.t. for 1 h under magnetic stirring and then concentrated. The residue was eluted from a column of Sephadex LH-20 with 1 : 1 MeOH–H_2O to give **13b** (21 mg, 82%) as a syrup; $[\alpha]_D$ = −12.5 (*c* 1.0, H_2O). ^{1}H NMR (300 MHz): δ 4.47 (d, 8H, $J_{1,2}$ = 10.5 Hz, 8 H-1), 3.76 (bd, 8H, $J_{6a,6b}$ = 12.3 Hz, 8 H-6a), 3.65–3.34 (m, 120H), 3.33–3.29 (m, 16H), 2.78–2.52 (m, 48H, 24 CH_2S), 1.90 (s, 24H, 8 Ac), 1.83–1.68 (m, 16H, 8 $OCH_2CH_2CH_2S$), 1.10–0.86 (m, 16H, 8 CH_2Si). ^{13}C NMR (75 MHz): δ 174.1 (C), 84.5 (CH), 80.1 (CH), 75.4 (CH), 70.0 (CH_2), 69.7 (CH_2), 69.6 (CH_2), 61.1 (CH_2), 55.0 (CH), 30.9 (CH_2), 29.3 (CH_2), 29.1 (CH_2), 27.1 (CH_2), 26.4 (CH_2), 22.5 (CH_3), 14.2 (CH_2). MALDI-TOF MS: *m/z* calcd for $C_{152}H_{288}N_8NaO_{76}S_{16}Si_8$ $(M + Na)^+$ 4204.70, found 4203.00. Anal. Calcd for $C_{152}H_{288}N_8O_{76}S_{16}Si_8{\cdot}8H_2O$: C, 42.20; H, 7.08; N, 2.59; S, 11.86. Found: C, 42.08; H, 6.88; N, 2.38; S, 11.42.

Glycoconjugate 13c. A solution of **12** (14 mg, 6.1 μmol), lactosyl thiol **2c** (53 mg, 147.3 μmol), and DPAP (3.8 mg, 14.8 μmol) in DMF (200 μL) and H_2O (50 μL) was irradiated at r.t. for 1.5 h under magnetic stirring and then concentrated. The residue was eluted from a column of Sephadex LH-20 with 3 : 1 H_2O–MeOH to give **13c** (16 mg, 50%) as a syrup; $[\alpha]_D$ = +6.0 (*c* 0.4, DMSO). ^{1}H NMR (300 MHz): δ 4.43 (bd, 8H, $J_{1,2}$ = 9.8 Hz, 8 H-1), 4.34 (d, 8H, $J_{1',2'}$ = 7.8 Hz, 8 H-1′), 3.83–3.79 (m, 16H), 3.72–3.40 (m, 168H), 3.26 (t, 8H, J = 8.8 Hz), 2.76–2.52 (m, 48H, 24 CH_2S), 1.88–1.76 (m, 16H, 8 $OCH_2CH_2CH_2S$), 1.10–0.91 (m, 16H, 8 CH_2Si). ^{13}C NMR (75 MHz): δ 103.4 (CH), 85.7 (CH), 79.1 (CH), 78.8 (CH), 76.3 (CH), 75.8 (CH), 75.6 (CH), 73.0 (CH), 72.5 (CH), 72.1 (CH), 71.4 (CH), 70.2 (CH_2), 69.8 (CH_2), 69.5 (CH), 69.0 (CH), 68.7 (CH), 61.5 (CH_2), 60.8 (CH_2), 38.2 (CH_2), 29.8 (CH_2), 27.1 (CH_2), 25.0 (CH_2), 22.7 (CH_2). MALDI-TOF MS: *m/z* calcd for $C_{184}H_{344}O_{116}S_{16}Si_8$ $(M)^+$ 5150.35, found 5150.53.

POSS-glutathione conjugate 14. A solution of **12** (10 mg, 4.4 μmol), glutathione chloridrate **6·HCl** (36 mg, 105.2 μmol, prepared by freeze-drying a solution of **6** in aqueous HCl), and DPAP (2.7 mg, 10.5 μmol) in MeOH (600 μL) was irradiated at r.t. for 1 h under magnetic stirring and then concentrated. The residue was eluted from a column of Sephadex LH-20 with 3 : 1 MeOH–H_2O to give **14** (17.2 mg, 78%) as a syrup; $[\alpha]_D$ = −13.9 (*c* 0.9, H_2O). ^{1}H NMR (300 MHz): δ 4.42–4.36 (m, 8H), 3.78 (bs, 16H), 3.64–3.40 (m, 104H), 2.94–2.83 (m, 16H), 2.76–2.53 (m, 32H), 2.50 (bt, 16H, J = 7.0 Hz), 2.42–2.32 (m, 16H), 2.04–1.95 (m, 16H), 1.76–1.65 (m, 16H), 1.04–0.89 (m, 16H). ^{13}C NMR (75 MHz): δ 175.1 (C), 174.1 (C), 172.8 (C), 172.1 (C), 72.3 (CH_2), 70.1 (CH_2), 69.8 (CH_2), 63.9 (CH_2), 60.9 (CH_2), 54.4 (CH), 53.6 (CH), 53.2 (CH), 42.5 (CH_2), 41.6 (CH_2), 33.3 (CH_2), 31.8 (CH_2), 31.0 (CH), 29.1 (CH_2), 28.7 (CH_2), 26.6 (CH_2), 14.1 (CH_2). MALDI-TOF MS: *m/z* calcd for $C_{184}H_{344}O_{116}S_{16}Si_8$: *m/z* calcd for $C_{168}H_{305}N_{24}O_{84}S_{16}Si_8$ $(M + H)^+$ 4738.40, found 4738.72.

POSS-RGDC conjugate 15. A solution of **12** (5 mg, 2.2 μmol), tetrapeptide RGDC **7** (23.5 mg, 52.6 μmol), and DPAP (1.3 mg, 5.3 μmol) in MeOH (300 μL) was irradiated at r.t. for 1 h under magnetic stirring and then concentrated. The residue was eluted from a column of Sephadex LH-20 with MeOH to give **15** (7.9 mg, 61%) as a syrup; $[\alpha]_D$ = −9.9 (*c* 0.3, H_2O). ^{1}H NMR (300 MHz) selected data: δ 3.66–3.44 (m, 96H, 48 CH_2O), 3.14–3.07 (m, 16H), 1.88–1.78 (m, 16H), 1.76–1.68

(m, 16H), 1.62–1.50 (m, 16H), 1.07–0.90 (m, 16H, 8 CH_2Si). ^{13}C NMR (75 MHz) selected data: δ 172.3 (C), 170.6 (C), 170.1 (C), 69.7 (CH_2), 69.5 (CH_2), 42.5 (CH_2), 40.4 (CH_2), 33.7 (CH_2), 28.3 (CH_2), 28.0 (CH_2), 23.5 (CH_2). MALDI-TOF MS: m/z calcd for $(C_{208}H_{384}Na_2O_{92}S_{16}Si_8)/2$ $(M + 2Na)^{2+}$ 2962.65, found 2961.94.

Enzyme-linked lectin assay (ELLA). 96-well microtiter Nunc-Immuno plates (Maxi-Sorp) were coated with α-D-Man-PAA or D-GlcNAc-PAA [100 µL per well, diluted from a stock solution of 5 µg mL^{-1} in 0.01 M phosphate-buffered saline (PBS) pH 7.4 (containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+} for ConA assay)] for 1 h at 37 °C. The wells were then washed with T-PBS (3 × 100 µL per well, PBS containing 0.05% (v/v) Tween 20). The washing procedure was repeated after each incubation. The wells were then blocked with BSA in PBS (3% w/v, 100 µL per well) at 37 °C for 1 h. After washing, the wells were filled with 100 µL of serial dilutions of ConA-HRP or WGA-HRP (100 µL, from 10^{-1} to 10^{-7} mg mL^{-1} in PBS (pH 7.4) or PBS containing 0.1 mM Ca^{2+}, 0.1 mM Mn^{2+} (for ConA) and BSA (0.3% w/v)) and were incubated at 37 °C for 1 h. The plates were washed with T-PBS (3 × 100 µL per well), then the colour was developed using OPD (100 µL per well, 0.4 mg mL^{-1} in 0.05 M phosphate-citrate buffer) and urea hydrogen peroxide (0.4 mg mL^{-1}). The reaction was stopped after 10 min by adding H_2SO_4 (30% v/v, 50 µL per well) and the absorbance was measured at 490 nm. The concentration of ConA-HRP or WGA-HRP that gives absorbance between 0.8 and 1 was used for inhibition experiments.

Inhibition experiments. The microtiter plates were coated with α-D-Man-PAA or D-GlcNAc-PAA as described previously. Serial two-fold dilutions of each inhibitor was incubated 1 h at 37 °C in PBS on Nunclon (Delta) microtiter plates (60 µL per well) in the presence of ConA-HRP or WGA-HRP (60 µL) at the desired concentration. The above solutions (100 µL) were then transferred to the coated microtiter plates which were incubated for 1 h at 37 °C. After incubation, the plates were washed with T-PBS and the colour was revealed described above. The percentage of inhibition was plotted against the logarithm of the concentration of the sugar derivatives. The sigmoidal curves were fitted and the concentration at 50% inhibition of binding of the ConA-HRP to α-D-Man-PAA or WGA-HRP to D-GlcNAc-PAA coated plates were determined (IC_{50}). The percentages of inhibition were calculated as given in the equation below, where A = absorbance.

$$\% \text{ inhibition} = (A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})})/A_{(\text{no inhibitor})} \times 100$$

The IC_{50} values were obtained from several independently performed tests in the range of ±17%. Nevertheless, the relative inhibition values calculated from independent series of data were highly reproducible.

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Thiyl Glycosylation of Propargylated Octasilsesquioxane: Synthesis and Lectin-Binding Properties of Densely Glycosylated Clusters on a Cubic Platform

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A new polyhedral oligomeric silsesquioxane (POSS) derivative with a periphery of eight PEGylated chains functionalized with terminal propargyl groups was synthesized starting from commercially available octavinyl-POSS. The photoinduced free-radical coupling of this octapropargyl POSS derivative with various sugar thiols enabled the preparation of globular hexadecavalent glycoclusters. Thus, it appears that according to the alkyne hydrothiolation mechanism, two thiyl radicals were added across each triple bond of the POSS scaffold side-chains. The affinities of some of the densely glycosylated clusters towards certain lectins were measured by the Enzyme-Linked Lectin Assay (ELLA). The binding selectivity of Concanavalin A between the hexadecavalent mannosylated and glucosylated clusters was much higher than the selectivity observed for the corresponding octavalent glycoclusters (ref.[4]). Moreover, the affinity of the *N*-acetylglucosamine-based cluster towards wheat germ agglutinin (WGA) revealed a remarkable glycoside cluster effect with up to a 9.0×10^5-fold increase in binding compared to monovalent GlcNAc. As a multivalent effect of the same order of magnitude was reported for an octavalent GlcNAc cluster towards the same lectin (ref.[4]), it is concluded that increasing the number of sugar units around the cubic platform does not lead systematically to an enhancement of binding affinity.

Introduction

The polyhedral oligomeric silsesquioxane (POSS), actually a rigid silica cube, is mostly known as a nanoscale building block for constructing functional materials.[1] The clustering of biomolecules onto POSS as a chemically and thermally stable platform is also an interesting topic which, however, has scarcely been investigated to date. Nevertheless, quite recently, POSS glycoconjugates (*glyco*-POSS) have received significant attention due to their rigid globular architecture, in which a precise clustering of eight sugar molecules in space is displayed. Thus, these hybrid inorganic–organic multivalent systems are emerging as interesting substrates for exploratory biological studies, such as, for example, studies of carbohydrate–lectin interactions. Synthetic routes to *glyco*-POSS were reported in 2010 by Fessner[2] and Chiara[3] and their co-workers, using the click copper-catalysed cycloaddition of an octaazide silsesquioxane with alkynyl glycosides. A more recent contribution towards this goal was provided by our own group using another click reaction, i.e., the photoinduced free radical coupling of octaene silsesquioxanes (POSS-ene) with sugar thiols to give *S*-linked octavalent POSS-based glycoclusters[4] (Figure 1).

With these glycoclusters, the binding of *glyco*-POSS to specific lectins was determined for the first time. Notably, in one case, an IC_{50} value of 3 nM (glycocluster concentration) was observed. Thus, the calculated relative potency per number of sugar units (*rp*/*n*) was of the order of 10^6, which reveals a striking glycoside cluster effect.[5] We were stimulated by this exciting result to synthesize further POSS-based glycoclusters and to evaluate their lectin-binding properties. In this paper, we will describe the synthesis of more densely glycosylated POSS derivatives, all featuring 16 sugar fragments (Figure 1), by the addition of sugar thiols to a new POSS-based reagent bearing alkynylated side-arms.[6] The reaction that we planned to use for the ligation was the classical free-radical double hydrothiolation of terminal alkynes, a process that is generally referred to as

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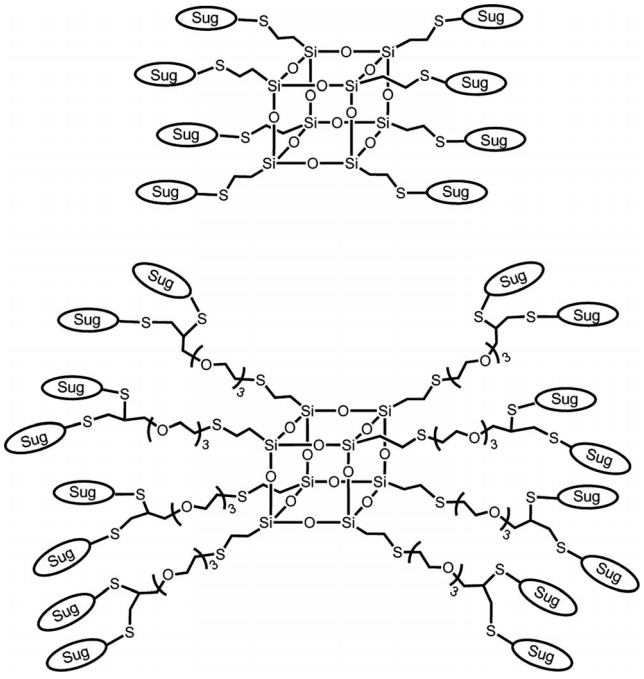
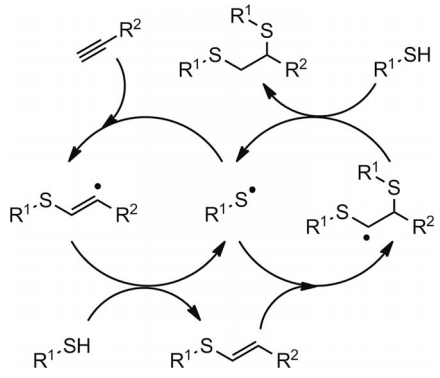


Figure 1. Schematic representation of *glyco*-POSS prepared (top, ref.[4]) and targeted in the present work (bottom).

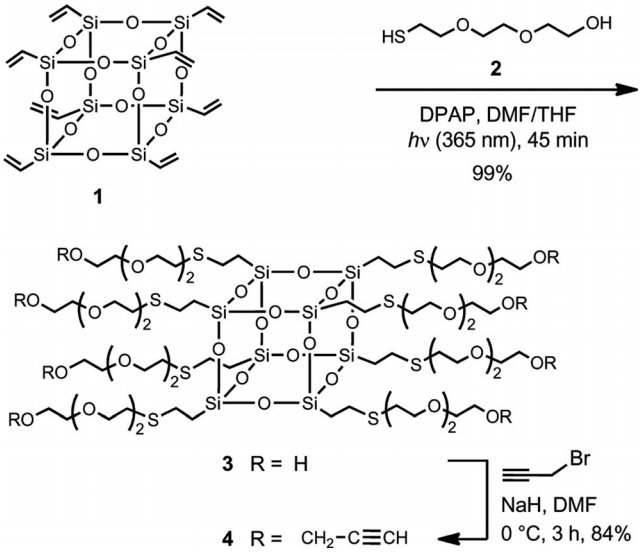
thiol–yne coupling (TYC). It has been well established that TYC can be initiated photochemically, and that it then proceeds by a free-radical chain mechanism that involves a vinyl thioether as a key intermediate[7] (Scheme 1). Notably, TYC leads to the formation of bis-addition products, i.e. dithioethers, by exclusive 1,2-addition of two thiyl radicals across the alkyne triple bond. This reaction, which is a sister reaction to the more commonly used free-radical hydrothiolation of terminal alkenes (thiol–ene coupling, TEC),[8] is emerging as a new metal-free click process, due to its high efficiency, regioselectivity, atom economy, and orthogonality to a great variety of other reactive groups.[9] While the use of TYC has been well documented in polymer and materials synthesis,[7,10] recent reports have described interesting extensions to bioorganic chemistry.[11] A wide range of applications of TYC in this field can be predicted, owing to the numerous alkynyl tagged biomolecules that have been prepared for use in Cu-catalysed alkyne–azide cycloadditions (CuAAC).[12]



Scheme 1. Mechanism of free-radical thiol–yne coupling.

Results and Discussion

We approached the preparation of octapropargyl silsesquioxane derivative **4** (POSS-yne) by following the synthetic scheme that was recently established for the synthesis of the analogous octaallyl derivative (POSS-ene).[4] Accordingly, also POSS-yne **4** was designed to contain PEGylated spacers, as these would avoid steric congestion around the scaffold and induce water solubility and biocompatibility. Briefly, commercially available octavinyl-POSS (**1**) was transformed as described[4] into octahydroxy functionalized POSS **3** in an almost quantitative yield by TEC with known PEGylated thiol **2**[13] (Scheme 2). Upon treatment of polyalcohol **3** with propargyl bromide and NaH in DMF as a solvent, it gave the target POSS-yne (i.e., **4**) in 84% yield. The ^{1}H NMR spectrum of this new POSS-based reagent showed a single set of signals at ca. 4.2 ppm (the methylene protons of the propargyl group), consistent with the T_8 symmetry of the system.

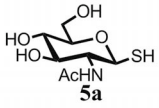
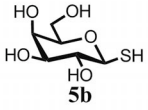
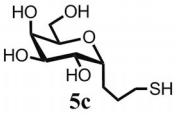
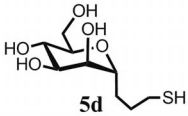
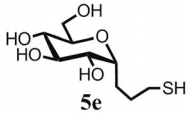


Scheme 2. Synthesis of octapropargyl POSS derivative **4**.

With substantial amounts of **4** in hand, we set out to investigate its photoinduced reactions with sugar thiols. Conditions similar to those used in previous TEC and TYC reactions reported by our group were adopted, i.e., irradiation with a household UVA lamp apparatus (λ_{max} = 365 nm) in the presence of the photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DPAP). The experiments were carried out in glass vials at room temperature, and no care was taken to exclude air and moisture. In the event, irradiation of a stirred solution of POSS-yne **4**, an excess of 2-acetamido-2-deoxy-1-thio-β-D-glucopyranose[14] **5a**, and DPAP in DMF (Table 1, Figure 2) gave within 1 h a reaction mixture that, when analysed by ^{1}H NMR spectroscopy,

showed no signals in the regions corresponding to the resonances of propargyl and alkenyl protons (4.2 and 5.5–6.5 ppm, respectively).[15]

Table 1. Photoinduced hydrothiolation[a] of POSS-yne **4**[b] by thiols **5a**–**e**.

Thiol	Thiol equiv./yne	DPAP[c] equiv./yne	Solvent	Product[d] (yield %)[e]
 **5a**	4	0.3	DMF	**6a** (60)
 **5b**	8	0.3	MeOH-DMF (1:1)	**6b** (50)
 **5c**	4	0.3	MeOH-DMF (1:1)	**6c** (70)
 **5d**	4	0.3	MeOH-DMF (1:1)	**6d** (82)
 **5e**	4	0.3	MeOH-DMF (1:1)	**6e** (80)

[a] Conditions: irradiation at λ_{max} = 365 nm for 1 h. [b] Scale: 14–21 mg (6–9 μmol). [c] 2,2-Dimethoxy-2-phenylacetophenone. [d] See structure in Figure 2. [e] Isolated yields after chromatography.

This indicated an essentially complete conversion of all of the propargyl groups of **4**, and the absence of any residual vinyl thioether intermediate. Chromatography of the crude reaction mixture gave the target bis adduct *glyco*-POSS (i.e., **6a**; 60% yield) featuring a hexadecavalent glycocluster installed onto the PEGylated silsesquioxane scaffold by thiother linkages. ^{1}H and ^{13}C NMR spectroscopic data were consistent with the assigned structure. In particular, retention of the anomeric configuration was established by the presence of doublets ($J_{1,2}$ = 10.6 Hz) at ca. 4.50 ppm in the ^{1}H NMR spectrum of **6a**. No reactions other than the double addition of thiol **5a** to the propargyl groups of **4** were observed, and most of the excess of **5a** was recovered either unaltered or in the form of the corresponding disulfide.

Encouraged by these results, we decided to investigate the substrate scope of sugar thiols. Hence, we carried out the photoinduced coupling of **4** with galactosyl thiol **5b**.[14] This reaction also gave the corresponding hexadecavalent glycocluster (i.e., **6b**) in good isolated yield (Table 1). In this case, however, a large excess of **5b** (8 equiv. per alkyne group) was used in order to complete the double hydrothiolation of all eight of the propargyl groups of **4**. A reaction in which 4 equiv. of **5b** per alkyne group of **4** was used did not reach completion, as shown by ^{1}H NMR spectroscopy. This decrease in reactivity may be ascribed to shielding of

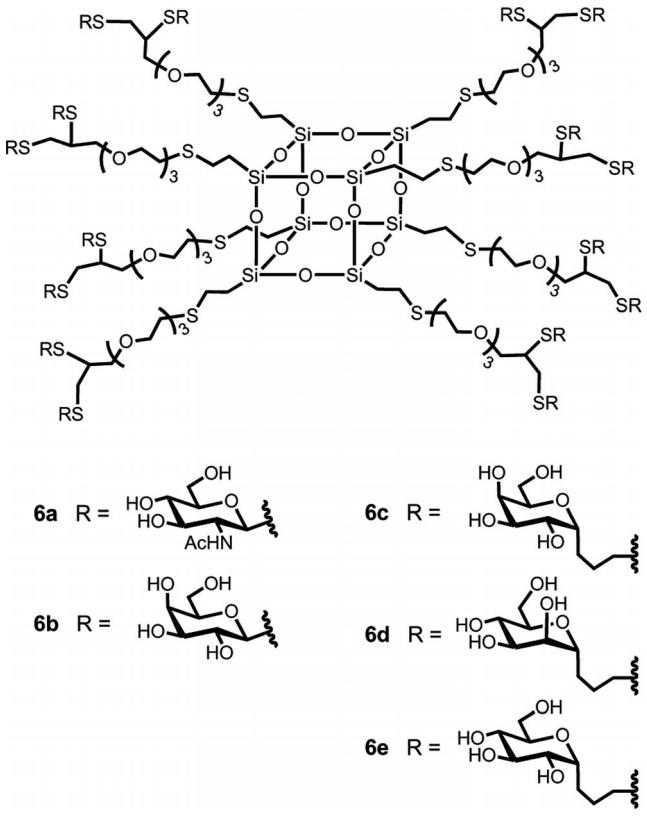


Figure 2. POSS-*glyco* **6a**–**e** prepared from PEGylated POSS-yne **4** and sugar thiols **5a**–**e**.

the free sulfhydryl group of **5b** by the axial hydroxy group at C-4 of the sugar moiety. Therefore, we decided to use a less encumbered thiol in which the sulfhydryl group was separated from the sugar moiety by an alkyl spacer. The photoinduced reaction of thiopropyl *C*-galactoside **5c** with **4** was essentially complete using the usual excess of thiol (4 equiv. per alkynyl group), and the target bis adduct (i.e., **6c**) was formed in good yield (Table 1). Successful results were also obtained by coupling **4** with thiopropyl *C*-mannoside **5d**[4] and thiopropyl *C*-glucoside **5e**[4] to give the corresponding POSS-based glycoclusters (i.e., **6d** and **6e**) in high yields (Table 1).

The binding properties of the hexadecavalent glycoclusters **6a**, **6d**, and **6e** towards certain lectins were determined with an Enzyme-Linked Lectin Assay (ELLA) to assess whether the higher number of sugar moieties would improve the affinity found for the octavalent POSS-based glycoclusters reported in our previous paper.[4] However, given the recent finding by Sundberg, Chiara, and co-workers[16] on the disassembling of triazole-linked glycoconjugates with a POSS core in aqueous media, we wondered whether this would also occur for our compounds under the ELLA test conditions. To investigate this, a solution of **6d** in deuterated 0.01 M phosphate-buffered saline (PBS) at pH 7.4 was heated at 37 °C for 16 h and monitored at various intervals by ^{1}H NMR spectroscopy. To our delight, the NMR spectra remained almost unchanged (see Figure S2), thus showing the stability of the POSS-based glycoclusters under

the conditions of the ELLA tests (pH 7.4, 37 °C, 2 h). Mannosylated and glucosylated clusters **6d** and **6e** were submitted to the ELLA experiments using Concanavalin A (ConA), a lectin from *Canavalia ensiformis*, which is specific for α-D-mannopyranosides and α-D-glucopyranosides. Compound **6d** showed good inhibition of the binding of horseradish-peroxidase-labelled ConA (ConA-HRP) to an α-D-mannose-polyacrylamide conjugate (α-D-Man-PAA) with an IC_{50} of 0.179 μM, which corresponds to a relative potency (*rp*) of 2564 with respect to methyl α-D-mannopyranoside, which was used as a monovalent reference (Table 2, Figure S1). This *rp* value was significantly higher (53 times) than that found for the corresponding octavalent glycocluster[4] (*rp* = 48.2). When reported relative to the number of sugar units (*rp*/*n* = 160), the inhibition enhancement was 27-fold, indicating a moderate glycoside cluster effect (Table 2). However, the experimental data cannot establish whether this effect was mainly due to the higher sugar valency (i.e., 16 vs. 8), or to the presence of a PEGylated linker between the mannose moieties and the platform. Moreover, it has to be mentioned that only a few studies have reported higher binding enhancements for ConA.[17–19]

Table 2. ELLA data for the inhibition of the binding of the lectin ConA-HRP to α-D-Man-PAA by glycoclusters **6d** and **6e**, and the inhibition of the binding of the lectin WGA-HRP to D-GlcNAc-PAA by glycocluster **6a**.[a]

	n[b]	IC_{50} [μM]	*rp*[c]	*rp*/*n*[d]
Me α-D-Man	1	459 ± 26	1	1
6d	16	0.179 ± 0.011	2564	160
Me α-D-Glc	1	2108 ± 75	1	1
6e	16	4.4 ± 0.6	479	30
D-GlcNAc	1	28800 ± 2200	1	1
6a	16	0.002 ± 5^{-5}	14.4×10^6	9.0×10^5

[a] Each experiment was performed in triplicate. [b] Number of sugar units. [c] *rp*: relative potency = IC_{50}(monosaccharide)/IC_{50}(glycocluster). [d] *rp*/*n*: relative potency/number of sugar units.

Similarly, hexadecavalent glucosylated cluster **6e** showed stronger inhibition (IC_{50} = 4.4 μM; *rp* = 479) than its octavalent counterpart[4] (IC_{50} = 40.4 μM; *rp* = 35.2), although the enhancement of inhibition (13-fold) was lower than that observed in the D-mannose series (Table 2, Figure S1). It is worth noting that the selectivity of ConA between hexadecavalent clusters **6d** and **6e** [IC_{50}(**6e**)/IC_{50}(**6d**) = 24.6] was much higher than the selectivity observed for the corresponding octavalent glycoclusters (IC_{50} ratio = 5.9). This finding suggests that the spatial orientation of sugars is more favourable in **6d**.

In a previous paper,[4] we reported the unprecedented inhibition of wheat germ agglutinin (WGA), a lectin from *Triticum vulgaris* that is specific for *N*-acetyl-D-glucosamine (GlcNAc), by a POSS-based octavalent GlcNAc cluster (IC_{50} = 3 nM; $rp = 9.3 \times 10^6$; $rp/n = 10^6$). Hexadecavalent *N*-acetyl-D-glucosamine cluster **6a** also showed remarkably strong inhibition properties towards WGA when submitted to similar ELLA experiments (IC_{50} = 2 nM), although the *rp* (14.4×10^6) and *rp*/*n* (9.0×10^5) values were only slightly higher or even lower, respectively, than those found for the corresponding octavalent cluster (Table 2). This observation indicated that increasing the number of sugar units around the cubic platform does not lead systematically to an affinity enhancement, in particular when the affinity of the multivalent ligand is in the nanomolar range.[18,20,21]

Conclusions

We have succeeded in preparing five densely glycosylated silsesquioxanes by TYC, all featuring 16 carbohydrate fragments attached to the POSS scaffold by thioether linkages. The pure compounds were isolated in very good yields, although some material was lost upon chromatography on Sephadex LH-20. The compounds are *S*- and *C*-glycosides, and are therefore expected to be endowed with enhanced chemical stability and enzymatic resistance. Finally, it must also be pointed out that all of the isolated products were mixtures of diastereoisomers, very likely in 1:1 ratios, due to the lack of stereoselectivity of the thiyl radical addition to the vinyl thioether intermediate. No attempts were made to separate individual stereoisomers, as this matter was beyond the scope of the present work. Therefore, it cannot ruled out that separation or stereoselective synthesis of pure stereoisomers could allow the identification of even stronger and more selective lectin ligands.

Experimental Section

General Methods: Flash column chromatography was performed on silica gel 60 (40–63 mm). ^1H (300 and 400 MHz) and ^{13}C (75 and 100 MHz) NMR spectra were recorded from D_2O solutions at room temperature unless otherwise specified. Peak assignments were aided by ^1H–^1H COSY and gradient-HMQC experiments. In the ^1H NMR spectra reported below, the *n* and *m* values quoted in geminal or vicinal proton–proton coupling constants $J_{n,m}$ refer to the numbers of the corresponding sugar protons.

Commercially available octavinyl-POSS (**1**) and photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DPAP) were used without further purification. Horseradish-peroxidase-labelled Concanavalin A (ConA-HRP) and *Triticum vulgaris* lectin (wheat germ agglutinin; WGA-HRP), Bovine Serum Albumin (BSA), and SIGMA*FAST* *O*-phenylenediamine dihydrochloride (OPD) were purchased from Sigma–Aldrich. The α-D-mannose-polyacrylamide (α-D-Man-PAA) and 2-acetamido-2-deoxy-D-glucose-polyacrylamide (D-GlcNAc-PAA) were obtained from Lectinity Holding, Inc., Moscow.

The thiol–yne coupling was carried out in a glass vial (diameter: 1 cm; wall thickness: 0.65 mm), sealed with a natural rubber septum, located 2.5 cm away from a household UVA lamp apparatus equipped with four 15 W tubes (1.5 × 27 cm each).

Octapropargyl-Substituted Silsesquioxane (4): NaH (64 mg, 1.60 mmol, of a 60% dispersion in oil) and then freshly distilled propargyl bromide (120 μL, 1.60 mmol) were added to a stirred, cooled (0 °C) solution of **3** (98 mg, 50.0 μmol) in anhydrous DMF (4 mL). The mixture was stirred at 0 °C for 3 h, then diluted with phosphate buffer at pH 7 (1 M; 2 mL), warmed to r.t., diluted with H_2O (30 mL), and extracted with EtOAc (3 × 50 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated. The residue was eluted from a column of Sephadex LH-20 with MeOH to give **4** (95 mg, 84%) as a syrup. ^1H NMR (400 MHz, $CDCl_3$): δ

= 4.20 (s, 16 H, 8 CH_2C≡CH), 3.76–3.57 (m, 80 H, 40 CH_2O), 2.78–2.56 (m, 32 H, 16 CH_2S), 2.45 (br. s, 8 H, 8 C≡CH) 1.12–0.94 (m, 16 H, 8 CH_2Si) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 79.7 (CH), 74.7 (CH), 70.5 (CH_2), 70.3 (CH_2), 69.1 (CH_2), 58.4 (CH_2), 31.3 (CH_2), 26.7 (CH_2), 14.2 (CH_2) ppm. MS (MALDI-TOF): calcd. for $C_{88}H_{152}NaO_{36}S_8Si_8$ $[M + Na]^+$ 2287.59; found 2287.60.

4,8-Anhydro-1,2,3-trideoxy-1-thio-D-*glycero*-L-*gluco*-nonitol (5c): A solution of allyl 2,3,4,6-tetra-*O*-acetyl-*C*-α-D-galactopyranoside (186 mg, 0.50 mmol), thioacetic acid (53 μL, 0.75 mmol), and DPAP (19 mg, 75 μmol) in MeOH (0.5 mL) was irradiated at r.t. for 1 h with magnetic stirring, and then the mixture was concentrated. The residue was eluted from a column of silica gel with 2:1 cyclohexane/EtOAc to give 1,5,6,7,9-penta-*O*-acetyl-4,8-anhydro-1,2,3-trideoxy-1-thio-D-*glycero*-L-*gluco*-nonitol (190 mg, 85%) as a syrup. $[\alpha]_D$ = +76.6 (c = 0.8, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$): δ = 5.40 (dd, $J_{6,7}$ = 3.0, $J_{7,8}$ = 2.0 Hz, 1 H, 7-H), 5.24 (dd, $J_{4,5}$ = 5.0, $J_{5,6}$ = 9.8 Hz, 1 H, 5-H), 5.17 (dd, 1 H, 6-H), 4.24–4.15 and 4.12–3.98 (2 m, 4 H, 4-H, 8-H, 2 9-H), 2.98–2.82 (m, 2 H, 2 1-H), 2.34 (s, 3 H, SAc), 2.12, 2.08, 2.05, and 2.02 (4 s, 12 H, 4 Ac), 1.82–1.44 (m, 4 H, 2 2-H, 3-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 195.5 (C), 170.6 (C), 170.1 (C), 170.0 (C), 169.8 (C), 71.6 (CH), 68.3 (CH), 68.1 (CH), 67.9 (CH), 67.6 (CH), 61.5 (CH_2), 30.6 (CH_3), 28.6 (CH_2), 25.5 (CH_2), 24.7 (CH_2), 20.8 (CH_3), 20.7 (CH_3) ppm. HRMS (ESI/Q-TOF): calcd. for $C_{19}H_{28}NaO_{10}S$ $[M + Na]^+$ 471.1301; found 471.1315.

NaOMe (0.2 M solution in MeOH, prepared from Na and MeOH immediately before use; 1 mL) was added to a solution of the thioacetate (161 mg, 0.36 mmol) in MeOH (3 mL). The solution was kept at r.t. for 2 h under a nitrogen atmosphere, then it was neutralized with Amberlite IR-120 resin (H^+ form, activated and washed with H_2O and MeOH immediately before use), and filtered through a sinteredglass filter. The resin was washed with MeOH, and the solution was concentrated. The residue was eluted from a C-18 silica gel cartridge with 1:1 H_2O/MeOH to give **5c** (60 mg, 70%) as a syrup. $[\alpha]_D$ = +91.0 (c = 1.0, H_2O). ^1H NMR (300 MHz): δ = 3.94–3.27 and 3.66–3.52 (2 m, 7 H, 5-H, 6-H, 7-H, 8-H, 2 9-H), 2.52–2.38 (m, 2 H, 2 1-H), 1.76–1.43 (m, 4 H, 2 2-H, 2 3-H) ppm. ^{13}C NMR (75 MHz): δ = 75.4 (CH), 71.8 (CH), 69.9 (CH), 69.3 (CH), 68.5 (CH), 61.3 (CH_2), 37.9 (CH_2), 24.7 (CH_2), 22.4 (CH_2) ppm. HRMS (ESI/Q-TOF): calcd. for $C_9H_{18}NaO_5S$ $[M + Na]^+$ 261.0773; found 261.0780.

Glycoconjugate 6a: A solution of **4** (15 mg, 6.6 μmol), thiol **5a** (50 mg, 211.7 μmol), and DPAP (4.1 mg, 15.9 μmol) in DMF (300 μL) was irradiated at r.t. for 1 h with magnetic stirring, and then the mixture was concentrated. The residue was eluted from a column of Sephadex LH-20 with 1:1 MeOH/H_2O to give **6a** (24 mg, 60%) as a syrup. ^1H NMR (400 MHz) selected data: δ = 4.57 and 4.51 (2 d, $J_{1,2}$ = 10.6 Hz, 16 H, 16 1-H), 3.79 (br. d, $J_{6a,6b}$ = 12.0 Hz, 16 H, 16 6a-H), 3.02–2.56 (m, 64 H, 8 CHS, 24 CH_2S), 1.93 (s, 48 H, 16 Ac), 1.05–0.90 (m, 16 H, 8 CH_2Si) ppm. ^{13}C NMR (100 MHz): δ = 174.0 (C), 85.5 (CH), 83.8 (CH), 79.9 (CH), 75.0 (CH), 69.7 (CH_2), 60.9 (CH_2), 54.7 (CH), 30.6 (CH_2), 22.2 (CH_3), 14.2 (CH_2) ppm. $C_{216}H_{392}N_{16}O_{116}S_{24}Si_8{\cdot}8H_2O$ (6063.64): calcd. C 41.79, H 6.62, N 3.61, S 12.40; found C 41.52, H 6.50, N 3.67, S 12.77.

Glycoconjugate 6b: A solution of **4** (14 mg, 6.2 μmol), galactosyl thiol **5b** (77 mg, 395.2 μmol), and DPAP (3.8 mg, 14.8 μmol) in 1:1 MeOH-DMF (300 μL) was irradiated at r.t. for 1 h with magnetic stirring, and then the mixture was concentrated. The residue was eluted from a column of Sephadex LH-20 with 1:1 MeOH/H_2O to give **6b** (16.7 mg, 50%) as a syrup. ^1H NMR (400 MHz) selected data: δ = 4.45, 4.44, 4.38, and 4.34 (4 d, $J_{1,2}$ = 9.5 Hz, 16 H, 16 1-H), 3.04–2.84 and 2.68–2.50 (2m, 64 H, 8 CHS, 24 CH_2S), 1.02–0.85 (m, 16 H, 8 CH_2Si) ppm. ^{13}C NMR (100 MHz) selected data: δ = 87.1 (CH), 85.8 (CH), 85.2 (CH), 78.9 (CH), 73.8 (CH), 71.7 (CH_2), 69.8 (CH_2), 69.6 (CH_2), 68.7 (CH), 61.0 (CH_2) ppm. $C_{184}H_{344}O_{116}S_{24}Si_8{\cdot}8H_2O$ (5406.80): calcd. C 39.81, H 6.54, S 13.86; found C 39.46, H 6.24, S 13.33.

Glycoconjugate 6c: A solution of thiol **5c** (71 mg, 296.4 μmol) and DPAP (5.7 mg, 22.2 μmol) in MeOH (200 μL) was added to a solution of **4** (21 mg, 9.3 μmol) in DMF (200 μL). The solution was irradiated at r.t. for 1 h with magnetic stirring, and then it was concentrated. The residue was eluted from a column of Sephadex LH-20 with 1:1 MeOH/H_2O to give **6c** (39.5 mg, 70%) as a syrup. ^1H NMR (400 MHz) selected data: δ = 2.80–2.48 (m, 88 H, 8 CHS, 40 CH_2S), 1.70–1.45 (m, 64 H, 16 CH_2CH_2), 1.02–0.82 (m, 16 H, 8 CH_2Si) ppm. ^{13}C NMR (100 MHz) selected data: δ = 75.0 (CH), 71.5 (CH), 69.8 (CH_2), 68.9 (CH), 68.2 (CH), 61.0 (CH_2), 30.6 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.3 (CH_2) ppm. $C_{232}H_{440}O_{116}S_{24}Si_8{\cdot}8H_2O$ (6080.09): calcd. C 44.77, H 7.38, S 12.36; found C 44.70, H 6.81, S 12.42.

Glycoconjugate 6d: Octapropargyl POSS **4** (21 mg, 9.3 μmol) was treated with thiol **5d** (71 mg, 296.4 mmol) as described for the preparation of **6c** to give, after chromatography on Sephadex LH-20 (1:1 MeOH/H_2O), **6d** (46 mg, 82%) as a syrup. ^1H NMR (400 MHz) selected data: δ = 3.00–2.51 (m, 88 H, 8 CHS, 40 CH_2S), 1.84–1.43 (m, 64 H, 16 CH_2CH_2), 1.07–0.91 (m, 16 H, 8 CH_2Si) ppm. ^{13}C NMR (75 MHz): δ = 78.1 (CH), 73.8 (CH), 72.6 (CH_2), 71.7 (CH), 71.1 (CH), 70.2 (CH_2), 67.4 (CH), 61.4 (CH_2), 45.1 (CH), 34.3 (CH_2), 32.2 (CH_2), 30.6 (CH_2), 26.9 (CH_2), 25.9 (CH_2), 22.8 (CH_2), 14.2 (CH_2) ppm. $C_{232}H_{440}O_{116}S_{24}Si_8{\cdot}8H_2O$ (6080.09): calcd. C 44.77, H 7.38, S 12.36; found C 45.11, H 7.41, S 12.77.

Glycoconjugate 6e: Octapropargyl POSS **4** (21 mg, 9.3 μmol) was treated with thiol **5e** (71 mg, 296.4 mmol) as described for the preparation of **6c** to give, after chromatography on Sephadex LH-20 (1:1 MeOH/H_2O), **6e** (45 mg, 80%) as a syrup. ^1H NMR (400 MHz) selected data: δ = 3.04–2.46 (m, 88 H, 8 CHS, 40 CH_2S), 1.77–1.40 (m, 64 H, 16 CH_2CH_2), 1.10–0.89 (m, 16 H, 8 CH_2Si) ppm. ^{13}C NMR (75 MHz): δ = 75.7 (CH), 73.6 (CH), 72.6 (CH), 71.4 (CH), 70.4 (CH), 70.2 (CH_2), 61.3 (CH_2), 45.1 (CH), 34.3 (CH_2), 32.3 (CH_2), 30.7 (CH_2), 26.3 (CH_2), 25.6 (CH_2), 23.2 (CH_2), 14.1 (CH_2) ppm. $C_{232}H_{440}O_{116}S_{24}Si_8{\cdot}8H_2O$ (6080.09): calcd. C 44.77, H 7.38, S 12.36; found C 44.56, H 7.39, S 12.62.

Enzyme-Linked Lectin Assay (ELLA): Microtitre Nunc-Immuno plates (96-well; Maxi-Sorp) were coated with α-D-Man-PAA or D-GlcNAc-PAA [100 μL per well, diluted from a stock solution of 5 μg mL^{-1} in 0.01 M phosphate-buffered saline (PBS) at pH 7.4 (containing 0.1 mM Ca^{2+} and 0.1 mM Mn^{2+} for the ConA assay)] for 1 h at 37 °C. The wells were then washed with T-PBS [3 × 100 μL per well, PBS containing 0.05% (v/v) Tween 20]. The washing procedure was repeated after each incubation. The wells were then blocked with BSA in PBS (3% w/v, 100 μL per well) at 37 °C for 1 h. After washing, the wells were filled with 100 μL of serial dilutions of ConA-HRP or WGA-HRP [100 μL, from 10^{-1} to 10^{-7} mg mL^{-1} in PBS (pH 7.4) or PBS containing 0.1 mM Ca^{2+}, 0.1 mM Mn^{2+} (for ConA) and BSA (0.3% w/v)] and were incubated at 37 °C for 1 h. The plates were washed with T-PBS (3 × 100 μL per well), then the colour was developed using OPD (100 μL per well, 0.4 mg mL^{-1} in 0.05 M phosphate-citrate buffer) and urea hydrogen peroxide (0.4 mg mL^{-1}). The reaction was stopped after 10 min by adding H_2SO_4 (30% v/v, 50 μL per well), and the absorbance was measured at 490 nm. The concentration of ConA-

HRP or WGA-HRP that gave an absorbance of between 0.8 and 1 was used for inhibition experiments.

Inhibition Experiments: The microtitre plates were coated with α-D-Man-PAA or D-GlcNAc-PAA as described previously. Serial two-fold dilutions of each inhibitor were incubated for 1 h at 37 °C in PBS on Nunclon (Delta) microtitre plates (60 μL per well) in the presence of ConA-HRP or WGA-HRP (60 μL) at the desired concentration. The above solutions (100 μL) were then transferred to the coated microtitre plates, which were then incubated for 1 h at 37 °C. After incubation, the plates were washed with T-PBS, and the colour was revealed as described above. The percentage of inhibition was plotted against the logarithm of the concentration of the sugar derivatives. The sigmoidal curves were fitted, and the concentration at 50% inhibition of binding of the ConA-HRP to α-D-Man-PAA or WGA-HRP to D-GlcNAc-PAA coated plates were determined (IC_{50}). The percentages of inhibition were calculated as given in the equation below, where A = absorbance.

$$\%\text{inhibition} = (A_{(\text{no inhibitor})} - A_{(\text{with inhibitor})})/A_{(\text{no inhibitor})} \times 100$$

Supporting Information (see footnote on the first page of this article): ^{1}H and ^{13}C NMR spectra of new compounds.

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COMMUNICATION

Multi-molecule reaction of serum albumin can occur through thiol-yne† coupling‡

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The free-radical hydrothiolation of alkynes (thiol-yne† coupling, TYC) unites two thiol fragments across the carbon-carbon triple bond to give a dithioether derivative with exclusive 1,2-addition; this reaction can be used for modification of peptides and proteins allowing glycoconjugation and fluorescent labeling. These results have implications not only as a flexible strategy for attaching two modifications at a single site in proteins but also for unanticipated side-reactions of reagents (such as cycloalkynes) used in other protein coupling reactions.

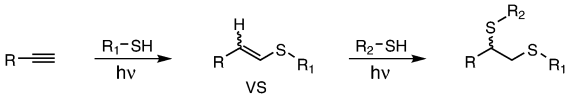
Protein modification and strategies for achieving such modifications with precision have seen tremendous development in the last decade.[1–4] Several reactions have been developed as part of strategies for allowing positional and molecular control and have even been developed to allow not only complex protein multisite protein alteration[5] but even cell surface[6] and *in vivo* conjugations.[7]

Part of the utility of such methods is in the study of natural protein alterations such as post-translational modification, a process that occurs after protein biosynthesis and folding and that incorporates a wide range of chemical moieties including phosphate, sugars, lipids, alkyl and acyl groups.[1,8] Glycosylation is by far the most common and complex of these modifications and it is known to affect both protein structure and function.[9] This is manifested in a variety of biological recognition events such as cell-cell communication, cell growth and differentiation, as well as viral infection. The microheterogeneity of native glycoproteins due to the presence of various glycoforms complicates their characterization and functional determination.

Methods allowing access to either labelled proteins and proteins that contain such post-translational modifications therefore remain in high demand.[10] Among the various chemical and enzymatic glycoprotein synthetic approaches,[11] those entailing the introduction of a functional tag into a protein by site-directed mutagenesis and then treatment with a suitably functionalized glycosyl reagent appears to be quite attractive.[12] Examples include the synthesis of disulfide-linked glycoproteins from proteins containing a cysteine residue as a thiol tag.[13] Desulfurization of these readily available disulfides can also afford thioether-linked glycoproteins.[14] While other examples of this "tag-and-modify" approach have been duly reviewed,[15] one of our groups reported also the free-radical coupling of ene-tagged proteins with glycosyl thiols to give *S*-linked protein glycoconjugates.[16] In this context, another of our groups reported a complementary approach in which *unmodified* native protein bovine serum albumin (BSA) displaying a single cysteine residue was coupled with allyl α-D-*C*-galactoside *via* a photoinduced thiol-ene free-radical reaction.[17] These and other important examples[18,19] have highlighted the selectivity and reactivity of the thiyl radical in protein modification approaches. In some examples of the thiol-ene reaction, however, multiple modifications have been observed that have been attributed to photocleavage of the disulfide bridge of cystine; these suggest that multi-site-selective protein modification can be induced in this way.

The thiol-yne† coupling (TYC), *i.e.* the free-radical addition of two thiol residues to a terminal alkyne,[20] has not yet been explored as a possible tool in protein modification. We have demonstrated[21] for small molecules in organic solvent that the photoinduced hydrothiolation of the triple bond can be carried out by the sequential addition of two different thiols. Thus, under suitable reaction conditions the vinyl sulfide (VS) intermediate formed by addition of a first thiol can be trapped by a second and different thiol *via* a thiol-ene†[22] type coupling process (Scheme 1).

To demonstrate the viability of this approach to more complex and biologically relevant molecules we first examined



Scheme 1

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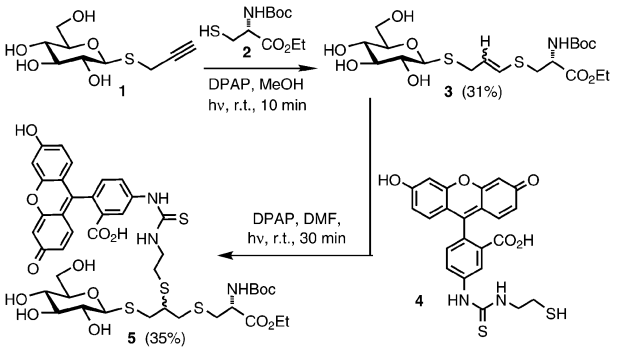
† The naming of reaction-type chosen in this paper is dictated by overall stoichiometry as opposed to being indicative of mechanism. Although, we suggest that this reaction is likely to proceed *via* a thiyl radical intermediate and might therefore be more appropriately described as a 'thiyl-yne', this is yet to be determined. The use of the term 'thiol-yne' is also inkeeping with more prevalent current usage of related reactions.

‡ Electronic supplementary information (ESI) available: Syntheses and characterization data of all new compounds. See DOI: 10.1039/c1cc14402b

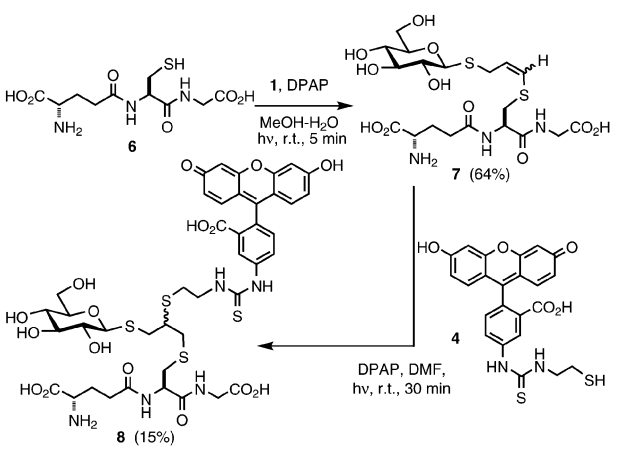


Scheme 2

a model system that used a representative L-cysteine derivative (**2**) and carbohydrate **1** (Scheme 2). Conditions were sought that would allow one equivalent only of cysteine derivative **2** to add to the alkynyl sugar **1**. Thus, coupling of **2** with a significant excess of **1** (4 equiv) was carried out at room temperature in a protic solvent (MeOH) by irradiation with a UV-lamp (λ_{max} 365 nm) using 2,2-dimethoxy-2-phenylacetophenone (DPAP, 10%) as a radical initiator. The reaction was carried out in a glass vial and no care was taken to exclude either air or moisture. After 10 min irradiation, the NMR spectrum of the reaction mixture revealed the presence of olefinic proton signals in the 5–6.5 ppm region. The excess of sugar alkyne **1** was recovered almost quantitatively (3 equiv.) by chromatography with pure vinyl sulfide **3** being isolated in fair yield (31%) as a 1 : 1 mixture of *E* and *Z* isomers. This key initial experiment confirmed the feasibility of step one of our intended two-step process.

Next, a solution of this intermediate **3**, fluorescein thiol **4** (4 equiv.) and DPAP in DMF was irradiated at λ_{max} 365 nm for 30 min. The ^{1}H NMR spectrum of the reaction mixture showed the complete conversion of alkene (as judged by ^{1}H NMR of **3**); chromatography over Sephadex LH-20 allowed the isolation of conjugated cysteine derivative **5**.

Excited by these results with a model amino acid, the same strategy was next explored in tripeptide glutathione GSH **6** (Scheme 3). Again photoinduced reaction of **1** with **6** under the same conditions afforded an alkene intermediate **7** (64% isolated yield) which was successfully reacted with fluorescein thiol **4** to give a corresponding doubly-conjugated product **8** as

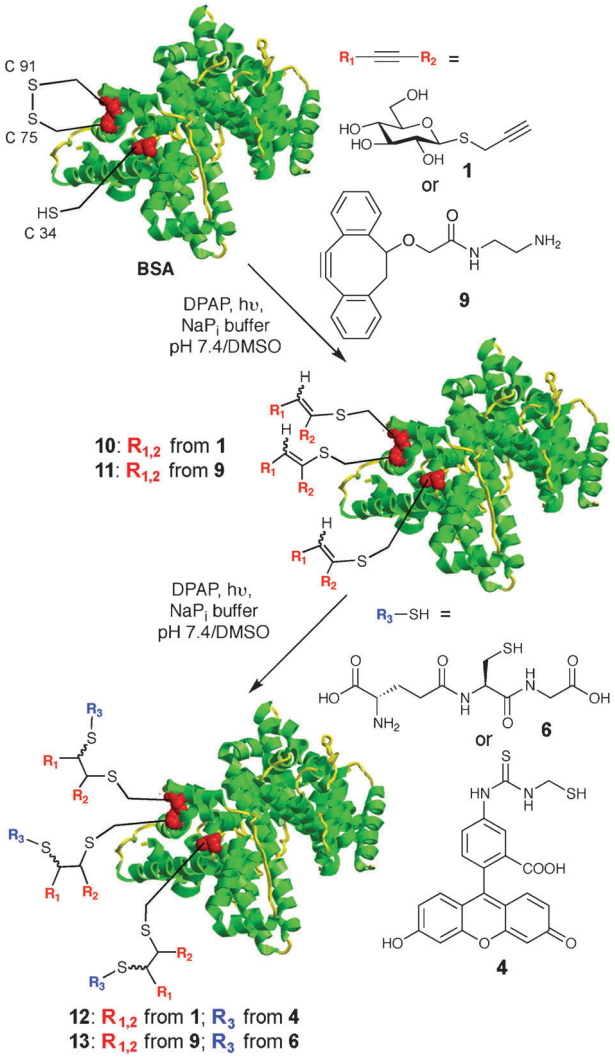


Scheme 3

a mixture of diastereomers with good conversion (>95% by NMR); isolated yield was more modest (15%). In all of the above cases, conversions were higher than isolated yields due to difficulties in purification associated with the amphiphilicity of these compounds. These key proof-of-principle experiments illustrated that a strategy could be developed for the dual modification at the same site of representative cysteinyl derivatives with both a biologically-relevant biomolecule (carbohydrate) and a label that is contingent on this first modification (here fluorescein).

Guided by the above preliminary experiments on small molecules, the same dual glycoconjugation and fluorolabeling of a representative thiol-containing protein, bovine serum albumin (BSA), was examined. Experimentation established an optimized procedure: a mixture of BSA, excess alkynyl sugar **1** (33 equiv) and photoinitiator DPAP (3 equiv) were dissolved in DMSO/phosphate buffer at pH 7.4 (5% v/v) and irradiated at λ_{max} 365 nm for 5 min. Again the experiment was conducted at room temperature without any caution to exclude air. The crude reaction mixture was purified by size-exclusion centrifugation to remove small molecule reagent sugar alkyne **1** and then the resulting solution containing the protein intermediate **10** was mixed with phosphate buffer (pH = 7.4) and an excess of fluorescein thiol **4** (160 equiv.) and DPAP (16 equiv.) dissolved in DMSO. The resulting solution was again irradiated at λ_{max} 365 nm for 10 min. MS (MALDI-TOF) analysis of the resulting synthetic conjugate **12** (found 68546 Da; calculated 68565 Da Fig. S3 ESI) indicated overall incorporation of three molecules of **1** and three molecules of **4** consistent with sequential dual modification at three cysteinyl sites (Scheme 4): the free cysteine at 34 and two thiyls 75 and 91 created by the photoinduced opening of the corresponding 75–91 cystine, as observed previously.[17] Consistent with these observations the fluorescence spectrum (Fig. S1 ESI) confirmed the incorporation of fluorescein residues. It should be noted that if disulfides provide structural integrity critical to function then this type of cystine cleavage and modification may therefore clearly prove detrimental to protein activity.

These key experiments revealed that a representative mammalian serum albumin (here BSA) reacts by undergoing representative dual modifications (*e.g.*, glycosylation and fluorolabeling) at up to three different positions *via* a TYC-based strategy. Not only does this valuably allow the dual conjugation of proteins at single sites in proteins containing cysteine residues it highlighted the possibility that other reagents that contain alkynes may react analogously. Foremost amongst these are the cycloalkynes used in so-called 'Cu-free CLICK' reactions that take place between azides and strained cycloalkynes to yield triazole diastereomeric products.[23–26] To test this possibility cycloalkyne **9** (commercially available as a Click-iT® reagent from Invitrogen), which is the core structure of several so-called DIBO-alkynes that have been used in strain-promoted reactions with azides, was reacted (Scheme 4) with BSA. The resulting intermediate **11** (found 67451 Da; calculated 67425 Da) was reacted with glutathione **6** as a representative thiol that can be present in significant levels *in vivo*. Ready conversion to the corresponding dually-modified conjugate **13** was observed (found 68359 Da; calculated 68346 Da). Furthermore, when cycloalkyne **9** was reacted alone in the



Scheme 4

absence of glutathione **6**, light and initiator the formation of a conjugate **14** (see ESI‡) that is the product of the addition of a single copy of **9** was observed (found 66 786 Da; calculated 66 783 Da). Tryptic digest followed by MS/MS analysis of the resultant peptides suggested reaction at the free cysteine that is present in serum albumin at position 34 (see ESI).

Although conditions for *in vitro* experiments can never adequately reproduce those *in vivo* our results confirm that alternative reactive pathways exist for such strained alkyne reagents. Indeed, our results are consistent with the 'dark' reactions of simple aliphatic thiols with cyclooctyne[27] and Bertozzi *et al.* have recently noted[25] that such alkynes show unusual high affinity for murine serum albumin, possibly consistent with the formation of a covalent linkage that is not due to reaction with an azide. Taken together with our results we suggest that thiols in such albumins may act as potential unwanted reaction partners during such experiments in the manner we disclose here. It should be noted that other reactions that involve the use of excessive double-bond containing reagents (such as so-called photoclick variants[28]) may also suffer from similar limitations. Further utility of our dual site conjugation methods using the TYC are under exploration.

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