ChemPubSoc Europe

DOI: 10.1002/chem.201602190

CHEMISTRY
A European Journal
Full Paper

Inhibitors

Mechanistic Insight into Heptosyltransferase Inhibition by using Kdo Multivalent Glycoclusters

Abdellatif Tikad,[a] Huixiao Fu,[a] Charlotte M. Sevrain,[a] Sophie Laurent,[b, c] Jean-François Nierengarten,[d] and Stéphane P. Vincent*[a]

Abstract: The synthesis of unprecedented multimeric Kdo glycoclusters based on fullerene and calix[4]arene central scaffolds is reported. The compounds were used to study the mechanism and scope of multivalent glycosyltransferase inhibition. Multimeric mannosides based on porphyrin and pillar[5]arenes were also generated in a controlled manner. Twelve glycoclusters and their monomeric ligands were thus assayed against heptosyltransferase WaaC, which is an important bacterial glycosyltransferase that is involved in lipopolysaccharide biosynthesis. It was first found that all the multimers interact solely with the *acceptor* binding site of the enzyme even when the multimeric ligands mimic the heptose *donor*. Second, the novel Kdo glycofullerenes displayed very potent inhibition ($K_i = 0.14$ μM for the best inhibitor); an inhibition level rarely observed with glycosyltransferases. Although the observed "multivalent effects" (i.e., the enhancement of affinity of a ligand when presented in a multimeric fashion) were in general modest, a dramatic effect of the central scaffold on the inhibition level was evidenced: the fullerene and the porphyrin scaffolds being by far superior to the calix- and pillar-arenes. We could also show, by dynamic light scattering analysis, that the best inhibitor had the propensity to form aggregates with the heptosyltransferase. This aggregative property may contribute to the global multivalent enzyme inhibition, but probably do not constitute the main origin of inhibition.

Introduction

Many important biological phenomena exploit an enhancement of binding affinities of a ligand for its receptor because of the presentation of the ligand in a multivalent fashion; that is, in a "clustered manner".[1] For instance, protein–carbohydrate interactions frequently mediate the first step of the infection process for many pathogens including viruses, fungi, bacteria, and bacterial toxins.[2] However, individual interactions between sugars and proteins are often very weak.[3] This poor binding affinity can be fully compensated for by multiple simultaneous interactions between carbohydrate ligands and their receptors.[2c,4] These affinity enhancements are usually coined "multivalent" or "cluster" effects.[5]

Carbohydrate processing enzymes being mostly monomeric, it was totally counter-intuitive to believe that these enzymes could be inhibited by multimeric species. Thus, the field of multivalent glycosidase inhibition has been overlooked for years.[6] However, multimeric iminosugars were recently assayed against a range of glycosidases and displayed significant multivalent effects against Jack Bean α-mannosidase and isomaltase.[7] Our laboratory described in 2012 the multivalent inhibition of a glycosyltransferase (GT), another major class of glycosyl processing enzymes.[8] Since then, multivalent inhibitors have been developed for other biologically relevant glycosidases such as β-glucocerebrosidase, trimming glucosidases and golgi α-mannosidase.[7d,9] Importantly, this unusual mode of inhibition is not restricted to glycosyl processing enzymes because early examples have proved that proteases[6b] and carbonic anhydrases (CAs),[10] including the tumor-associated CA IX,[11] are also prone to multivalent inhibition.

The biosynthesis of complex glycans involves the participation of a huge number of GTs, which catalyze the regio- and stereo-selective transfer of a saccharide from a donor to an acceptor. Many of these enzymes are involved in key biological processes such as cell adhesion and recognition, and signaling, and some of them represent attractive targets for the treatment of major pathologies such as cancer, inflammation, and infectious diseases.[12] Our initial investigation in this field was based on the inhibition of a therapeutically relevant[13] bacterial heptosyltransferase (WaaC) by glycofullerenes bearing 12

[a] Dr. A. Tikad, H. Fu, Dr. C. M. Sevrain, Prof. S. P. Vincent
University of Namur (UNamur), Département de Chimie
Laboratoire de Chimie Bio-Organique
rue de Bruxelles 61, 5000 Namur (Belgium)
E-mail: stephane.vincent@unamur.be

[b] Prof. S. Laurent
University of Mons (UMONS), Service de Chimie Générale
Organique et Biomédicale, Laboratoire de RMN et d'Imagerie Moléculaire
Avenue Maistriau 19, 7000 Mons (Blegium)

[c] Prof. S. Laurent
Center for Microscopy and Molecular Imaging (CMMI)
Avenue Adrienne Bolland 8, 6041 Gosselies (Belgium)

[d] Dr. J.-F. Nierengarten
Laboratoire de Chimie des Matériaux Moléculaires
Université de Strasbourg et CNRS (UMR 7509)
Ecole Européenne de Chimie, Polymères et Matériaux (ECPM) 25 rue Becquerel, 67087 Strasbourg Cedex 2 (France)

Supporting information and the ORCID number(s) for the author(s) of this article are available under http://dx.doi.org/10.1002/chem.201602190.

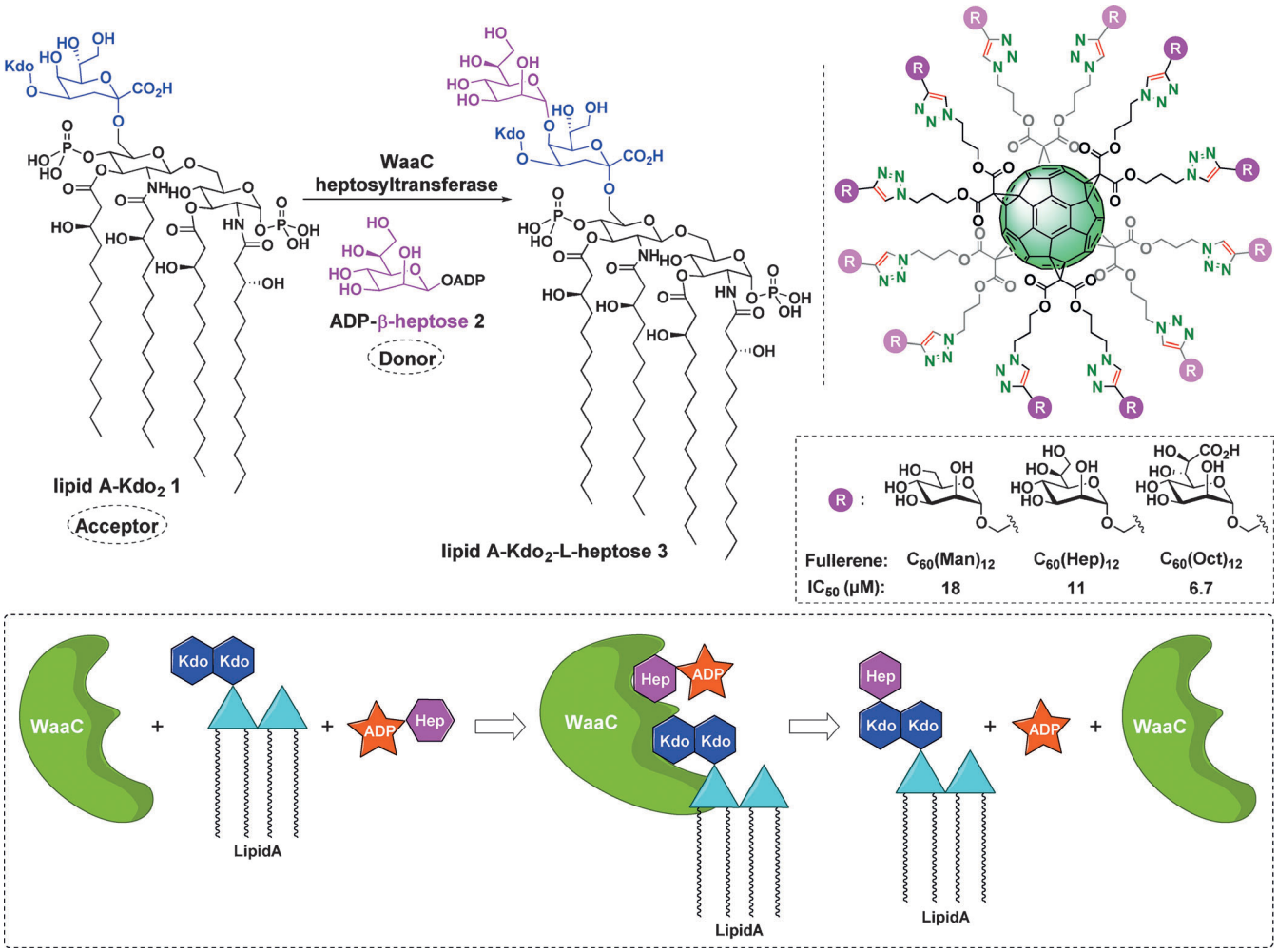


Figure 1. Heptosylation of Lipid A-Kdo2 catalyzed by heptosyltransferase WaaC. Man = mannose; Hep = heptose; Oct = octose.

monosaccharides such as $C_{60}(Hep)_{12}$ (Figure 1).[8] In this case, an inhibition enhancement of more than 45-fold was observed, compared with the monomeric α-heptoside (which corresponds to more than 3.7-fold affinity enhancement per sugar unit). WaaC is a monomeric enzyme that binds a glycolipid **1** (Lipid A-Kdo_2) as the acceptor substrate (Figure 1). Due to the lipidic nature of the substrate, the reaction takes place at a surface, more precisely at the cytoplasmic inner membrane. Therefore, clustering or local concentration effects might explain why multimeric structures such as glycofullerenes inhibit WaaC in a multivalent manner. Although the intimate mechanism by which WaaC is inhibited by these multimeric structures remains obscure, we are convinced that these results are significant because glycosyltransferases, in general, constitute a fantastic class of targets not only for drug development but also for deciphering their fundamental biochemical/biological functions. As recently reviewed, little is known on the possible mechanism(s) of multivalent enzyme inhibition.[6] As for lectins, the inhibition level of a multimer may be enhanced by quick rebinding processes, chelation and/or protein aggregation. Another interesting hypothesis is that multimeric species can bind several binding pockets of a single enzyme, simultaneously, or not.[7c] This assumption is particularly relevant for glycosyltransferases which, by essence, display two adjacent recognition sites: one for the so-called "acceptor", to which a sugar unit located in the "donor" binding site will be transferred. To shed light on the mechanism of multivalent enzyme inhibition, we decided (1) to identify first which binding pocket(s) is interacting with glycofullerenes; (2) to quantify the influence of the central scaffold on the affinity enhancement; (3) to determine whether glycoclusters inhibit WaaC by aggregating the protein. To answer these questions, we first planned to confirm that glycofullerenes $C_{60}(Man)_{12}$, $C_{60}(Hep)_{12}$, and $C_{60}(Oct)_{12}$, do bind in the WaaC *donor* pocket (Man = mannose; Hep = heptose; Oct = octose). Then, we designed a new series of glycofullerenes bearing multiple copies of the *acceptor* Kdo (see Scheme 2). This new series of glycoclusters was expected to allow us to compare the binding modes of acceptor or donor mimics and thus define the best strategy to inhibit a glycosyltransferase in a multivalent fashion. To probe the influence of the C_{60} central scaffold on the inhibition process, we also prepared a series of multimeric compounds with different core units, namely calix[4]arene, pillar[5]arene, and porphyrin (Scheme 3). These molecules were constructed to allow us to

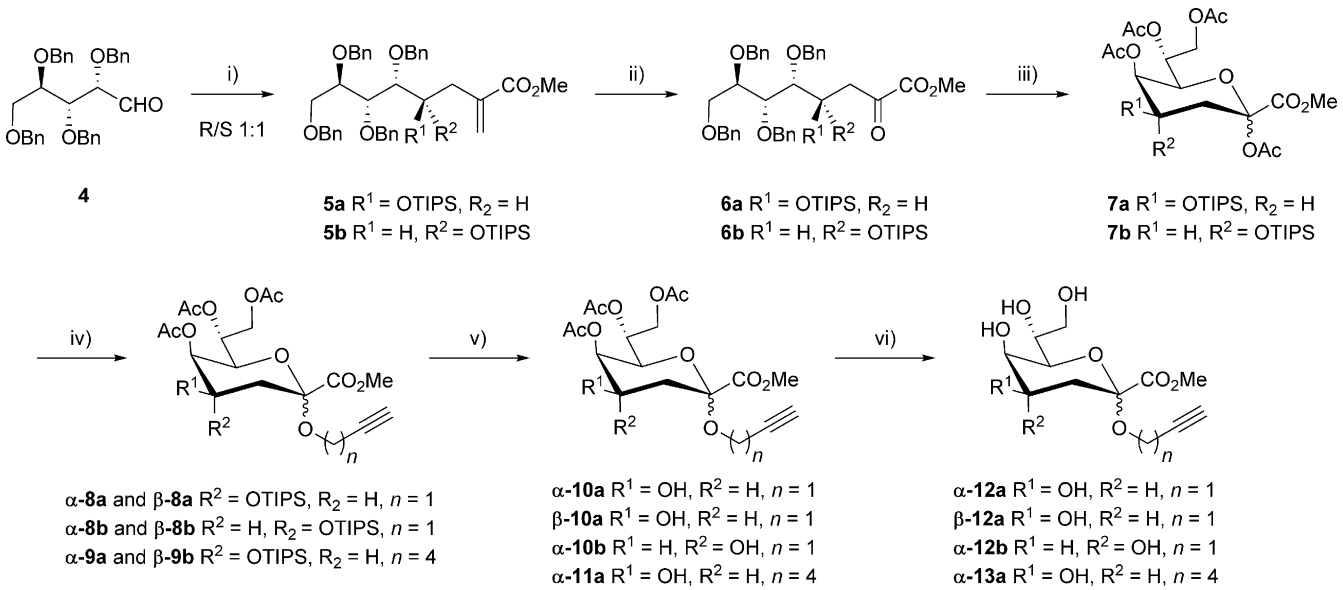
probe the effect of the sugar density/valency around the central scaffold on the binding affinity towards the targeted enzyme. In the next paragraphs, we will first describe the synthesis of the clickable Kdo derivatives, then their grafting onto a fullerene scaffold. Next, will be described the preparations of the novel multimers based on calix-, pillar-arenes and porphyrin scaffolds. The biochemical investigations on the binding modes and the mechanism of multivalent WaaC inhibition will be detailed afterwards.

Results and Discussion

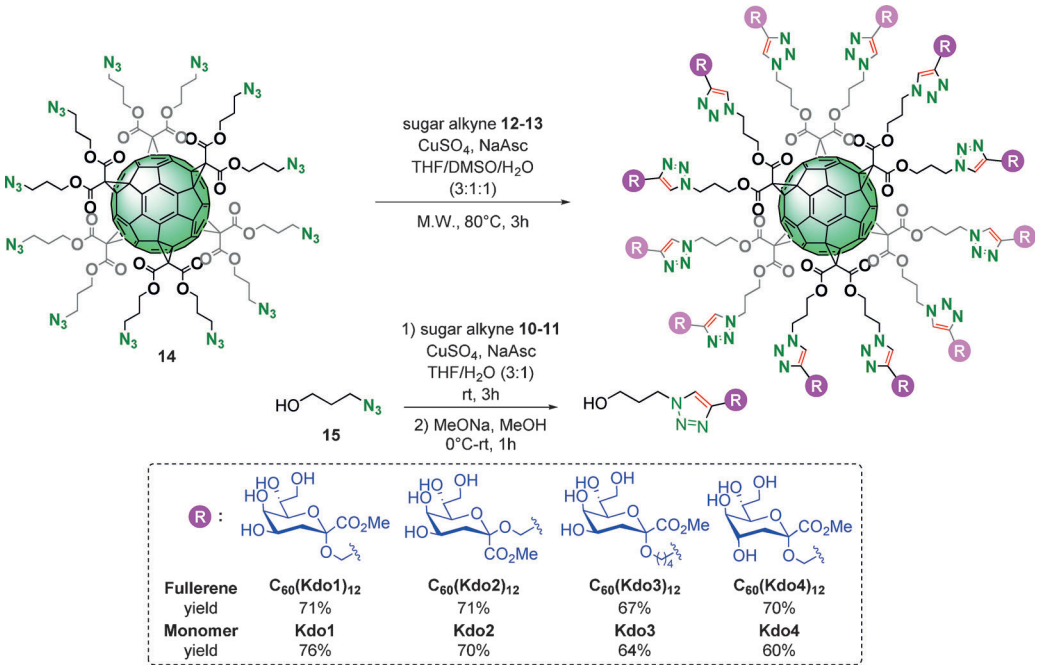
Synthesis of clickable Kdo derivatives

A family of unprecedented Kdo sugars bearing a terminal alkyne **α-12a**, **β-12a**, **α-12b**, and **α-13a** (Scheme 1) were thus designed to allow their grafting to azidofullerene **14** (Scheme 2). For the present project, we wished to exploit a synthetic pathway allowing the preparation of two distinct epimers at C-4 and the two anomers of Kdo to probe the selectivity of the binding process with the targeted glycosyltransferase. In the literature, two main synthetic strategies have been developed for the construction of Kdo octosides that are not commercially available: a (C_5+C_3) strategy from a pentose (D-arabinose) or a (C_6+C_2) strategy from D-mannose.[14] For this study, we followed a very appealing strategy initially developed by Schreiber and collaborators that involves the known acyclic perbenzylated D-arabinoside **4**,[15] and benzyl 2-(bromomethyl)acrylate (Scheme 1).[16]

The condensation was performed in the presence of chromium(II) chloride and gave the corresponding alcohols as an inseparable mixture of two diastereomers. The latter were then transformed into the corresponding triisopropylsilyl ethers to afford the pure methyl acrylates **5a** and **5b** in 82% global yield over two steps, as a near 1:1 ratio after purification by flash chromatography. Starting from compound **5a**, the first assay of ozonolysis in a mixture of solvents (CH_2Cl_2/MeOH, 5:1) at −78 °C, followed by reduction with Me_2S, afforded α-keto ester **6a** in only 30% yield. Fortunately, the use of CH_2Cl_2 as sole solvent allowed us to generate methyl pyruvate **6a** in 81% yield (Scheme 1). Deprotection of perbenzylated α-keto ester **6a** was accomplished by hydrogenolysis catalyzed by $Pd(OH)_2$/C in methanol, followed by peracetylation, to give peracetylated octoside **7a** in 93% yield over two steps. Compound **7a** was thus easily prepared on a multigram scale. Diastereoisomer **7b** was then prepared from **5b** by following the same synthetic route. The key step was the glycosylation of peracetylated Kdo **7a** and **7b** with propargyl alcohol and 5-hexyn-1-ol as acceptors. After optimization, we found that treatment of **7a** with propargyl alcohol in the presence of $BF_3{\cdot}OEt_2$ as a promoter in acetonitrile at 0 °C gave the desired alkynes **α,β-8a** along with a desilylated byproduct **α,β-10a** (Scheme 1). Whereas the separation of isomers **α-8a** and **β-8a** was easily achieved by silica gel chromatography, it was not the case for **α-10a** and **β-10a**. Hence, to obtain a larger amount of **α-8a** and **β-8a**, the mixture of **α,β-10a**, isolated from the glycosylation of **7a**, was immediately silylated under standard conditions. The crude material could then be purified



Scheme 1. Reagents and conditions: i) a) Methyl 2-(bromomethyl)acrylate, $CrCl_2$, THF, RT, 5 h; b) TIPSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C–RT, overnight, 82% (*R/S*, 1:1) [**5a** (35%), **5b** (31%) and (**5a**, **b**) (16%) as a mixture]; ii) a) O_3, CH_2Cl_2, −78 °C, 2 h; b) Me_2S, −78 °C to RT, 2 h, **6a** (from **5a**: 81%), **6b** (from **5b**: 87%); iii) a) $Pd(OH)_2$/C, H_2, 1 atm, MeOH, RT, overnight; b) Ac_2O, pyridine, 0 °C to RT, overnight, **7a** (from **6a**: 93%, α/β 7:3), **7b** (from **6b** 87%, α/β 7:3); iv) a) propargyl alcohol or 5-hexyn-1-ol, $BF_3{\cdot}OEt_2$, CH_3CN, MS (4 Å), 0 °C–RT, 1 h–3.5 h; b) TIPSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C–RT, 4 h–overnight, **α,β-8a** (from **7a**: 62%, α/β 6:4) over two steps [**α-8a** (37%), **β-8a** (25%)], **α,β-9a** (from **7a**: 56%, α/β 6:4) over two steps [**α-9a** (34%), **β-9a** (22%)], **α,β-8b** (from **7b**: 27%, α/β 6:4) over two steps [**α-8b** (16%), **β-8b** (11%)]; v) HF·py, THF, 0 °C-RT, 24 h, **α-10a** (from **α-8a**: 89%), **β-10a** (from **β-8a**: 85%), **α-11a** (from **α-9a**: 94%), **α-10b** (from **α-8b**: 71%); vi) MeONa, MeOH, 0 °C–rt, 30 min, **α-12a** (from **α-10a**: 98%), **β-12a** (from **β-10a**: 98%), **α-13a** (from **α-11a**: 96%), **α-12b** (from **α-10b**: 76%).



Scheme 2. Synthesis of the target glycofullerenes and their corresponding monomers by CuAAC.

by careful silica gel chromatography to provide **α-8a** and **β-8a** in better yield.

To study the effect of linker length on the inhibition of heptosyltransferase WaaC, 5-hexyn-1-ol was also used as an acceptor instead of propargyl alcohol to afford the desired separable compounds **α-9a** and **β-9a** in 56% global yield. The same sequence (glycosylation/silylation) starting from the epimeric Kdo **7b**, gave the two anomers **α-8b** and **β-8b** in a modest 27% yield, indicating a significant remote effect of the substituent at C-4 on the glycosylation process.

The first attempts at desilylation of **α-8a**, **β-8a**, **α-9a**, and **α-8b** using tetrabutylammonium fluoride (TBAF) were very disappointing because of concomitant acetate migrations. We eventually found that the final desilylations had to be carried out with HF·pyridine. Finally, deacetylation of **α-10a**, **β-10a**, **α-10b**, and **α-11a** was accomplished under Zemplén conditions to provide the expected products **α-12a**, **β-12a**, **α-12b**, and **α-13a** in good to excellent yields. The structures of all Kdo monomers were fully ascertained by NMR spectroscopy using ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY and ^{1}H-^{13}C (HMQC, HMBC) experiments. The anomeric configurations of **8a**, **8b**, and **9a** were unambiguously confirmed by NOE experiments that evidenced through-space correlations between H-1′, H-4, and H-6 for all α-configured glycosides.

Synthesis of multimeric Kdo glycofullerenes

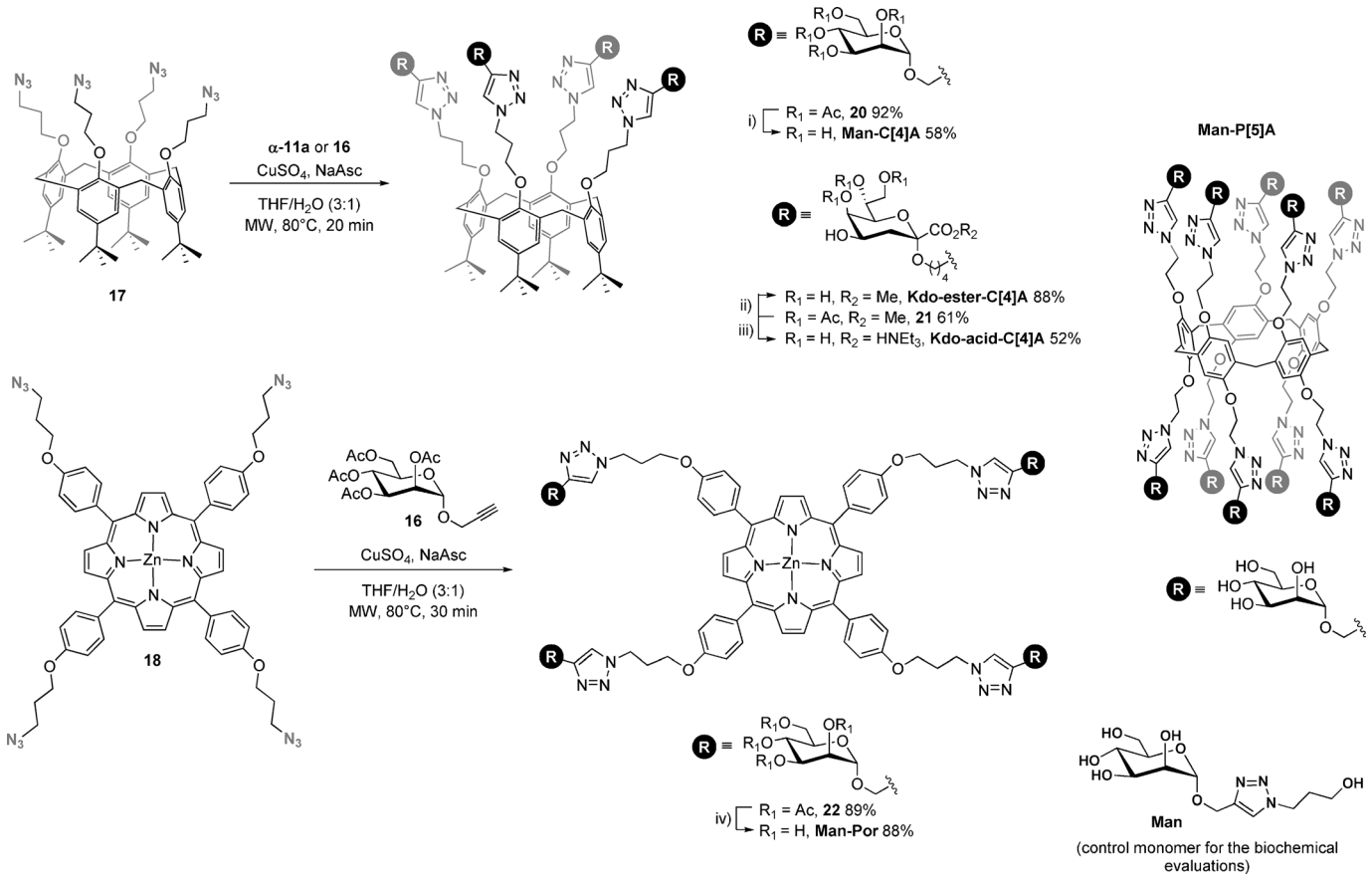
As previously reported for other glycofullerenes,[17] we took advantage of the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction for the grafting of twelve unprotected carbohydrates (**α-12a**, **β-12a**, **α-12b**, and **α-13a**) on the known fullerene core **14**[18] bearing twelve azides (Scheme 2). It was found that the use of an excess of terminal alkyne building block (13 equiv) with a catalytic amount of copper sulfate (1.2 equiv) and sodium L-ascorbate (2.4 equiv) in a ternary mixture of solvent (tetrahydrofuran (THF)/dimethyl sulfoxide (DMSO)/H_2O, 3:1:1) under microwave irradiation at 80 °C for 3 h, were the best conditions for complete conversion into the dodeca-Kdo fullerenes. The products were then precipitated with acetone and extensively centrifuged and washed with methanol and THF. The residual solid was then dissolved in water in the presence of a copper scavenger (QuadraSil™) to eliminate the residual traces of copper.

The scavenger was removed by simple filtration and the filtrate was further purified by size-exclusion chromatography (Sephadex™, G-25).[19]

All the final molecules displayed the characteristic ^{13}C NMR patterns for symmetrical fullerene hexa-adducts and were characterized by mass spectrometry. We could thus pursue our investigation by the synthesis of glycoclusters based on other central scaffolds than a fullerene.

Synthesis of the calix[4]arene, pillar[5]arene and porphyrin glycoclusters

We chose to synthesize novel glycoclusters based on a tetravalent calix[4]arene scaffold that would allow the multimers to be generated both as a Kdo methyl ester (KDO-ester-C[4]A, Scheme 3) and a free acid (KDO-acid-C[4]A) and directly compare them to the corresponding mannoside (Man-C[4]A) and the fullerene Kdo methyl esters $C_{60}(Kdo1–4)_{12}$. Multimeric mannosides were selected because we showed, in our preliminary study, that the dodecavalent mannoside $C_{60}(Man)_{12}$ (Figure 1) was already a potent WaaC inhibitor. Thus, we would be able to evaluate the effects of ligand distribution, valency and the ligand itself (Kdo versus D-mannose). Calix[4]arenes are cone-



Scheme 3. Synthesis of glycoclusters by CuAAC. Reagents and conditions: i) Amberlite resin OH^-, IRN78, MeOH/H_2O 3:1, RT, overnight; ii) a) MeONa, MeOH, RT; b) Dowex 50WX8-200 (H^+ form), MeOH/H_2O (1:1); iii) a) MeONa, MeOH, RT, 1 h; b) NaOH (1 M), THF/H_2O (1:1), RT, 1 h; c) Dowex 50WX8-200 (Et_3NH^+ form), MeOH/H_2O (1:1); iv) a) MeONa, THF/MeOH/H_2O (1:1:1), RT, 24 h; b) Dowex 50WX8-200 (H^+ form), THF/MeOH/H_2O (1:1:1).

shaped structures distributing four ligands. It is reasonable to assume that they may bind a protein very differently than the globular glycofullerenes. To push further this investigation, we also synthesized a planar tetravalent porphyrin (Man-Por) and a decavalent pillar[5]arene (Man-P[5]A) (Scheme 3). Indeed, the valency and the specific spatial orientations of the mannosides around the different selected scaffolds may impact the inhibition efficacy, as observed for other multivalent inhibitors.[6] Compared with calix[4]arenes, pillar[5]arenes distribute the ligands on both sides of their hydrophobic central core.[20] The synthesis of clickable Kdo derivatives being very demanding, we prepared the latter central scaffolds only in the mannose series.

Azidated calix[4]arene **17** and porphyrin **18** were obtained from the corresponding phenols and 3-azidopropyl tosylate in *N*,*N*-dimethylformamide (DMF), in 76 and 62% yields, respectively.[21] Pillar[5]arene bearing deprotected mannosides Man-P[5]A[20c] was prepared by following the procedure developed in our laboratory.[20a,b] The peracetylated propargyl mannoside **16** was synthesized by classical procedures.[22] Azidated scaffolds were coupled to the propargylated carbohydrates **α-11a** and **16** through CuAAC in the presence of copper sulfate and sodium L-ascorbate under microwave irradiation in THF/H_2O (3:1) at 80 °C. Peracetylated glycoclusters **20**–**22** were isolated in yields ranging from 61 to 92% depending on the difficulties of the purifications by silica gel chromatography. The deprotections of peracetylated scaffolds **21**–**22** were usually carried out under Zemplén conditions followed by sodium exchange with a Dowex® resin.

Calix[4]arene **20** was deprotected in one step by using a basic anion-exchange resin Amberlite™ IRN78 (hydroxide form) in (MeOH/H_2O, 1:1) at room temperature.

Biochemical investigation

Our first objective was to confirm that glycofullerenes $C_{60}(Man)_{12}$, $C_{60}(Hep)_{12}$, and $C_{60}(Oct)_{12}$ (represented in Figure 1), do bind the WaaC donor pocket because they mimic the heptose donor substrate. We thus began our study by measuring the inhibition constants K_i using Lineweaver–Burk plots. WaaC activity was monitored by a coupled enzyme assay involving pyruvate kinase (PK) and luciferase, as previously described.[13b] For each heptosylation reaction, the newly formed ADP is converted by PK into ATP, which is quantified by a luminescent luciferase-catalyzed reaction (see the Supporting Information).

The competition assays against ADP-heptose were performed at 1 μM of acceptor Re-LPS **3** ($K_m = 1$ μM)[13b] and ADP-heptose **2** at five different concentrations. The competition

assays against Re-LPS were performed at 1 μM of donor ADP-Heptose **2** ($K_m = 1$ μM)[13b] and Re-LPS **3**, also at five different concentrations. The initial velocities were determined from the time course of each reaction, thus yielding the K_i values (Table 1).

The Lineweaver–Burk plots (see Figure 2 and the Supporting Information) indicated the competitive/uncompetitive character of the inhibition process. As expected from our preliminary study, the K_i values measured for glycofullerenes $C_{60}(Man)_{12}$, $C_{60}(Hep)_{12}$, and $C_{60}(Oct)_{12}$, were found in the low micromolar range (Table 1).[8] However, to our great surprise, the inhibition modes of all these heptose-like multimers were clearly uncompetitive towards ADP-heptose although they were designed to mimic the *donor* substrate. Interestingly, they do bind the *acceptor* pocket in a competitive fashion, as clearly shown in Figure 2, despite the significant differences between the mannose-configured inhibitors and the Kdo acceptor structure. The characteristic plots of uncompetitive inhibition (plots B, D, F) indeed show that the glycofullerenes bind the enzyme already linked to the ADP-heptose donor substrate, whereas plots A, C, and E all show a competitive inhibition mode against the acceptor substrate. These unexpected results pushed us to investigate the mechanism of multivalent WaaC inhibition, using glycoclusters mimicking Kdo, with C_{60} and other multivalent scaffolds.

Table 1. K_i and inhibition modes of $C_{60}(Man)_{12}$, $C_{60}(Hep)_{12}$, and $C_{60}(Oct)_{12}$.

Entry	Inhibitor	K_i [μM]	Competitive inhibition against	
			ADP-heptose	LipidA-Kdo
1	$C_{60}(Man)_{12}$	52 ± 11	no	yes
2	$C_{60}(Hep)_{12}$	31 ± 3.7	no	yes
3	$C_{60}(Oct)_{12}$	8.6 ± 1.8	no	yes

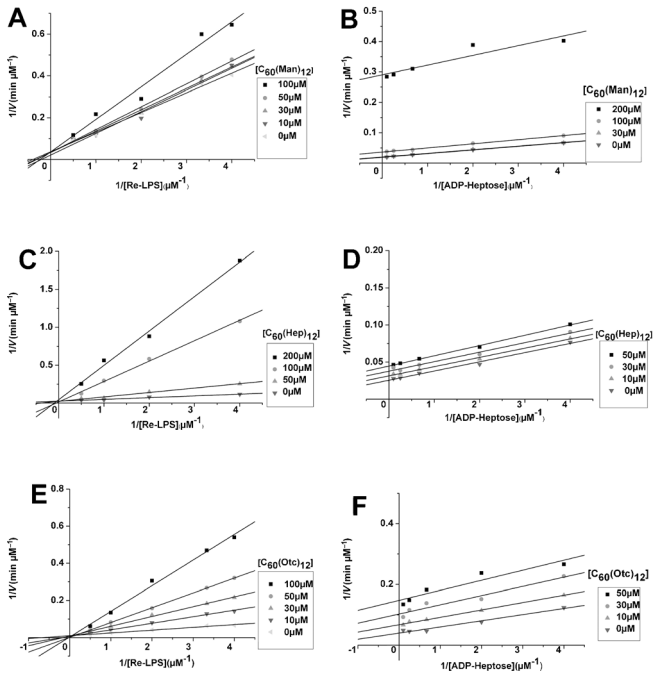


Figure 2. Competitive and uncompetitive inhibition of WaaC by $C_{60}(Man)_{12}$ (plots A, B), $C_{60}(Hep)_{12}$ (plots C, D), and $C_{60}(Oct)_{12}$ (plots E, F), with respect to Re-LPS (plots A, C, e) and ADP-heptose (plots B, D, F).

The relative affinities of the novel multimeric $C_{60}(Kdo1–4)_{12}$ for WaaC are reported in Table 2. For these experiments, ADP-heptose (1 μM) and Re-LPS (1 μM) were incubated at 25 °C for 30 min at concentrations close to their K_m. The reactions in the presence or absence of inhibitors were quenched thermally and the conversion into ADP was determined thanks to a readout mixture composed of PK, PEP (Phosphoenol pyruvate), luciferin, and luciferase (see the Supporting Information for experimental details). The results are gathered in Table 2.

Table 2. WaaC Inhibition data of the different glycoclusters and their corresponding monomers ([ADP-heptose] = 1 μM and [Re-LPS] = 1 μM).

Entry	Product	IC_{50} [μM]	Inhibition enhancement	Enhancement per sugar[a]
1	$C_{60}(Kdo1)_{12}$	0.34 ± 0.22	10.3	0.9
2	Kdo1	3.5 ± 1.3	–	–
3	$C_{60}(Kdo2)_{12}$	1.3 ± 0.33	12	1.0
4	Kdo2	15 ± 0.65	–	–
5	$C_{60}(Kdo3)_{12}$	0.31 ± 0.11	8.3	0.7
6	Kdo3	2.7 ± 1.1	–	–
7	$C_{60}(Kdo4)_{12}$	0.51 ± 0.1	28	2.3
8	Kod4	14 ± 1.8	–	–
9	Man-C[4]A	196 ± 14	2.1	0.5
10	Kdo-ester-C[4]A	140 ± 3.9	0.7	0.17
11	Kdo-acid-C[4]A	86 ± 10	–[b]	–[b]
12	Man-Por	2.0 ± 0.9	48	12
13	Man-P[5]A	34 ± 7.7	2.8	0.28
14	Man	94 ± 6.9	–	–

[a] Calculated on a per-sugar basis = IC_{50}monomer/(IC_{50}fullerene/valency). [b] Not determined.

Kdo fullerenes $C_{60}(Kdo1–4)_{12}$ displayed submicromolar inhibition levels against WaaC, which is an inhibition level rarely observed with glycosyltransferases.[12] As expected, compound $C_{60}(Kdo2)_{12}$, displaying the wrong β anomeric configuration, was a poorer ligand, with an IC_{50} value one order of magnitude lower when compared with the corresponding α epimer (Table 2, entries 1 and 3). The IC_{50} was also slightly increased for the Kdo epimer at C-4 $C_{60}(Kdo4)_{12}$ (entries 1 and 7) showing an influence of the C-4 absolute configuration on the binding process, especially for the monomers (entries 2 and 8). Similar IC_{50} values were obtained for multimers $C_{60}(Kdo1)_{12}$ and $C_{60}(Kdo3)_{12}$, demonstrating that the length of the spacer does not affect the affinity of the Kdo ligand for WaaC (entries 1 and 5). The IC_{50} values obtained with these four dodecavalent glycofullerenes correspond well with those measured for the corresponding monomers. Moreover, analysis of the enhancement of affinity per sugar units (based on valency corrected IC_{50} values) clearly shows that no or modest multivalent effect is

observed, except for glycofullerene $C_{60}(Kdo4)_{12}$, for which an enhancement factor of 2.3 was observed.

We then verified that the inhibitor $C_{60}(Kdo1)_{12}$ was indeed competitive against the acceptor Re-LPS: Lineweaver–Burk plots showed that it is effectively the case (see the Supporting Information). The K_i value for $C_{60}(Kdo1)_{12}$ was found to be 0.144 μM, showing that this novel glycofullerene has a significantly higher affinity for WaaC than the previously prepared glycoclusters $C_{60}(Man)_{12}$, $C_{60}(Hep)_{12}$, and $C_{60}(Oc)_{12}$ (Figure 1). Overall, these results showed that (i) potent inhibition levels could be obtained by using multimeric Kdo; (ii) the inhibition levels depend on the absolute configuration of both C-1 and C-4 of the Kdo ligand, especially for the monomers; (iii) the multimers are competitive inhibitors of the acceptor. Together, these results demonstrate that the new glycofullerenes tightly bind the Kdo acceptor pocket of the glycosyltransferase.

These results are consistent with the tridimensional structure of heptosyltransferase WaaC, which has been co-crystallized with ADP and a fluorinated analogue of ADP-heptose.[13b] The nucleotide-sugar binds the enzyme at the bottom of a deep cleft, which would be consistent with an ordered bi-bi mechanism in which ADP-heptose binds first, before the Kdo_2-lipid A molecule. Therefore, our results show that the synthetic glycofullerenes cannot easily reach and bind the deeply buried heptose pocket. They also evidence that multimeric glycosides can exhibit a high avidity towards the more accessible acceptor pocket. This enhanced avidity would also explain the somewhat lowered binding selectivity, possibly through the enhancement of nonspecific interactions (*manno*-configured glycoclusters bind the acceptor pocket).

We could now push further this investigation to determine whether this novel mode of GlycT inhibition is dependent on the central multimeric scaffold.

Influence of the central scaffold on the WaaC inhibition process

In general, the IC_{50} values of the glycoclusters synthesized in this study ranged from 0.3 to 140 μM and no or modest multivalent effects were observed for WaaC (Table 2). The enhancement is calculated on a "per-sugar" basis, taking into account both the IC_{50} values of the control monomers (entries 2, 4, 6, 8 for the Kdo derivatives and entry 14 for all mannosides) and the valency. The best enhancement was 12 for the tetravalent porphyrin Man-Por (entry 12). As expected, Kdo-acid-C[4]A showed a better inhibition than its ester congener Kdo-ester-C[4]A, showing that this interaction is important, although not essential in this particular case. Both Kdo-derived calix[4]arenes were also better inhibitors than their corresponding mannoside Man-C[4]A (entries 9–11), as expected for a binding process involving the glycosyltransferase acceptor pocket.

Inspection of the inhibition values showed that the tetravalent calix[4]arenes scaffold (Table 2, entries 9–11) gave much higher IC_{50} values ($>$ 86 μM) compared with the tetravalent porphyrin ManPor (2 μM; entry 12), which proved that two multimers of the same valency can display very different inhibition profiles. Interestingly, the three multimannosides of this series (the tetravalent Man-C[4]A and Man-Por, and the decavalent Man-P[5]A) gave very significantly different IC_{50} values, even when the inhibition values are valency-corrected. Globally, the inhibitory order of the different scaffolds was: porphyrin $\approx C_{60} >$ pillar[5]arene $>$ calix[4]arene.

Several mechanisms may explain how monomeric proteins, such as enzymes, are potently inhibited by multimeric ligands: ligand rebinding processes, chelating or aggregative effects are often invoked in the literature.[6] Some recent experimental data obtained on the multivalent inhibition of Jack Bean α-mannosidase suggested that multimeric inhibitors may operate by aggregating their target enzyme.[9d] For instance, Gouin and co-workers had shown that a porphyrin linked to four iminocyclitols was also a significantly better glycosidase inhibitor compared with other scaffolds presenting the same iminocyclitols.[9d] We noted that glycoporphyrin Man-Por displayed moderate solubilities in both water and DMSO, suggesting that the porphyrinic scaffold itself is prone to self-aggregation. This observation, along with the literature data, pushed us to investigate the size of the potential particles present in solution when the targeted enzyme and the glycoclusters are alone in solution and mixed together.

Probing an aggregative mechanism for WaaC inhibition

Dynamic light scattering experiments (DLS) were performed with representative monomeric and multimeric inhibitors of WaaC (Table 3). It is noteworthy that DLS values of Man-C[4]A, Kdo-acid-C[4]A, and Man-Por could not be measured because of their limited solubility under the assay conditions. DLS measurements of the multimers were performed in the absence (entries 2–5) and presence (entries 6–9) of enzyme and it was found that compounds $C_{60}(Man)_{12}$, $C_{60}(Oct)_{12}$, and Kdo-ester-C[4]A slightly aggregate WaaC, whereas strongest aggregation was observed with $C_{60}(Kdo3)_{12}$.

Table 3. Hydrodynamic diameters of WaaC with or without ligands.[a]

Entry	Molecule(s)	Diameter (nm)
1	WaaC	23 ± 3; 68 ± 12[b]
2	$C_{60}(Kdo3)_{12}$	4.8 ± 2
3	$C_{60}(Man)_{12}$	3.0 ± 2
4	$C_{60}(Oct)_{12}$	26.4 ± 7
5	Kdo-ester-C[4]A	14.1 ± 6
6	WaaC + $C_{60}(Kdo3)_{12}$	104 ± 47[b]; 750 ± 204
7	WaaC + $C_{60}(Man)_{12}$	35 ± 7; 138 ± 46[b]
8	WaaC + $C_{60}(Oct)_{12}$	34 ± 7; 140 ± 49[b]
9	WaaC + Kdo-ester-C[4]A	19 ± 3; 61 ± 27[b]; 213 ± 51

[a] Experimental conditions: The hydrodynamic diameter of protein/ligands complexes were determined in solution: Hepes (pH 7.5, 50 mM), KCl (50 mM), $MgCl_2$ (10 mM), Triton (0.1%), WaaC (2.5 μM), Inhibitor (250 μM), preincubation 30 min in buffer. [b] Highest distribution among the particle size detected.

This investigation showed first that the enzyme itself (Table 3, entry 1) and two of the multimers (entries 3 and 4) are self-aggregating. Thus, we first performed a kinetic study

that confirmed that the observed particle sizes were stable over time. Then, we analyzed the distribution of particle sizes when the multimers were mixed with WaaC.

The largest aggregates were observed at 104 and 750 nm after pre-incubation of $C_{60}(Kdo3)_{12}$ and WaaC for 30 min (Table 3, entry 6). These particles are significantly larger than the protein alone (entry 1), which unambiguously proves the ability of this glycofullerene to aggregate the enzyme. Given the self-aggregation propensity of the studied enzyme, it is difficult to precisely assess the exact number of protein molecules that could be aggregated by $C_{60}(Kdo3)_{12}$. Interestingly, this molecule is also one of the very best inhibitors of WaaC, showing a potential correlation between the inhibition power of this fullerene and its ability to form aggregates with its enzymatic target. These results show that aggregation can indeed contribute to multivalent WaaC inhibition, and confirmed the potency of some of the multimers to form cross-linked complexes with WaaC. However, these DLS experiments do not show that aggregation is the primary cause of inhibition and they do not exclude other inhibition mechanisms such as rapid rebinding of the ligands to the acceptor pockets or other chelation binding modes.

Very recently, Compain et al. described the synthesis of multivalent cyclopeptoid-iminosugars that displayed a high-level of multivalent inhibition effect against Jack Bean mannosidases.[23] Thanks to electron microscopy imaging, they could evidence the formation of a strong chelate complex in which two mannosidase molecules are cross-linked by one inhibitor. These results corroborate those obtained by Gouin et al. with the same mannosidase but also with a Golgi-localized α-mannosidase, and a homologue of human lysosomal α-mannosidases for which dynamic light scattering experiments and atomic force microscopy suggested a cross-linking mechanism.[9d] These results reveal that multivalent enzyme inhibition, when observed, really depend on the enzyme itself. Our group recently investigated the mechanism of multivalent glycosidase inhibition by using a collection of novel homo- and heterovalent glyco(mimetic)-fullerenes purposely conceived to probe the contribution of noncatalytic pockets in glysosidases to the multivalent inhibitory effect.[24] An original competitive glycosidase-lectin binding assay demonstrated that the multivalent derivatives and the substrate compete for low affinity non-glycone binding sites of the enzyme, leading to inhibition by a "recognition and blockage" mechanism. The latter study, which was performed on three structurally distinct glycosidases, showed that such an enhancement of nonspecific interactions depend on the accessibility and conformational flexibility of the catalytic pocket of the enzyme.

From the data detailed in the present study, with a glycosyltransferase, it is likely that the inhibition levels observed against WaaC with the inhibitors mimicking the donor substrate also result from the enhancement of nonspecific interactions.

Conclusion

We have reported the synthesis of unprecedented multimeric Kdo glycoclusters based on fullerene and calixarene central scaffolds to study the mechanism and the scope of multivalent glycosyltransferase inhibition. Multimeric mannosides based on porphyrin and pillar-arenes were also generated in a controlled manner. Twelve glycoclusters and their monomeric ligands were thus assayed against heptosyltransferase WaaC, which is an important bacterial glycosyltransferase involved in lipopolysaccharide (LPS) biosynthesis. Important mechanistic conclusions could be drawn from this study. First, the multimers interact solely with the acceptor binding site of the enzyme even if the multimeric ligand mimics the heptose donor. Second, the novel Kdo glycofullerenes displayed inhibition values in the nanomolar range, which is an inhibition level rarely observed with glycosyltransferases. Although the observed "multivalent effects" (i.e., the enhancement of the affinity of a ligand when presented in a multimeric fashion) were, in general, modest, a dramatic effect of the central scaffold on the inhibition level was evidenced: the fullerene and the porphyrin scaffolds being by far superior to the calix- and pillar-arene.

These results are consistent with the 3D structure of WaaC that show that the donor binding pocket is deeply buried and the acceptor pocket is much more accessible. The multivalent enzyme inhibition observed in this study would thus result from the enhancement of the affinity of ligands for the accessible acceptor binding pocket.

We could also show, by dynamic light scattering, that the best inhibitor had the propensity to form aggregates with the heptosyltransferase. This aggregative property may contribute to the global multivalent enzyme inhibition, without being the primary cause of inhibition.

Beyond the novel fundamental conclusions of this study, these results will contribute to the design of a new generation of inhibitors of glycosyltransferase, which is a central class of biocatalysts in glycosciences. Ours results could also be extrapolated to other kinds of enzymes possessing two or more binding or recognition pockets. In a more applied approach, multivalent enzyme inhibition may find therapeutic applications for instance when enzymes are exposed in a cell (or virus) outer membrane, as already demonstrated with cancer cells and HIV infection models.[6b]

Experimental Section

All experimental details (synthetic procedures, NMR spectra and biochemical data) are provided in the Supporting Information.

Acknowledgements

The authors thank the Fonds National de la Recherche Scientifique (FNRS, Mandat de Chargé de Recherche for A. T., PDR T.0170.13 for C.S.) and the China Scholarship Council (CSC, PhD grant NO.201308520012 to H.F.).

Keywords: fullerenes · glycoconjugates · inhibitors · receptors · transferases

[1] a) C. Fasting, C. A. Schalley, M. Weber, O. Seitz, S. Hecht, B. Koksch, J. Dernedde, C. Graf, E. W. Knapp, R. Haag, *Angew. Chem. Int. Ed.* **2012**, *51*, 10472 – 10498; *Angew. Chem.* **2012**, *124*, 10622 – 10650; b) E. Mahon, M. Barboiu, *Org. Biomol. Chem.* **2015**, *13*, 10590 – 10599.

[2] a) A. Bernardi, J. Jiménez-Barbero, A. Casnati, C. De Castro, T. Darbre, F. Fieschi, J. Finne, H. Funken, K.-E. Jaeger, M. Lahmann, T. K. Lindhorst, M. Marradi, P. Messner, A. Molinaro, P. Murphy, C. Nativi, S. Oscarson, S. Penadés, F. Peri, R. J. Pieters, O. Renaudet, J.-L. Reymond, B. Richichi, J. Rojo, F. Sansone, C. Schäffer, W. B. Turnbull, T. Velasco-Torrijos, S. Vidal, S. Vincent, T. Wennekes, H. Zuilhof, A. Imberty, *Chem. Soc. Rev.* **2013**, *42*, 4709 – 4727; b) A. Imberty, Y. M. Chabre, R. Roy, *Chem. Eur. J.* **2008**, *14*, 7490 – 7499; c) P. I. Kitov, J. M. Sadowska, G. Mulvey, G. D. Armstrong, H. Ling, N. S. Pannu, R. J. Read, D. R. Bundle, *Nature* **2000**, *403*, 669 – 672.

[3] a) Y. C. Lee, R. T. Lee, *Acc. Chem. Res.* **1995**, *28*, 321 – 326; b) S. Cecioni, A. Imberty, S. Vidal, *Chem. Rev.* **2015**, *115*, 525 – 561.

[4] R. J. Pieters, *Org. Biomol. Chem.* **2009**, *7*, 2013 – 2025.

[5] M. Lahmann, *Top Curr. Chem.* **2009**, *288*, 17 – 65.

[6] a) P. Compain, A. Bodlenner, *ChemBioChem* **2014**, *15*, 1239 – 1251; b) N. Kanfar, E. Bartolami, R. Zelli, A. Marra, J.-Y. Winum, S. Ulrich, P. Dumy, *Org. Biomol. Chem.* **2015**, *13*, 9894 – 9906; c) S. G. Gouin, *Chem. Eur. J.* **2014**, *20*, 11616 – 11628.

[7] a) J. Diot, M. I. Garcia-Moreno, S. G. Gouin, C. O. Mellet, K. Haupt, J. Kovensky, *Org. Biomol. Chem.* **2009**, *7*, 357 – 363; b) P. Compain, C. Decroocq, J. Iehl, M. Holler, D. Hazelard, T. M. Barragan, C. O. Mellet, J. F. Nierengarten, *Angew. Chem. Int. Ed.* **2010**, *49*, 5753 – 5756; *Angew. Chem.* **2010**, *122*, 5889 – 5892; c) R. Rísquez-Cuadro, J. M. Garcia Fernandez, J. F. Nierengarten, C. Ortiz Mellet, *Chem. Eur. J.* **2013**, *19*, 16791 – 16803; d) C. Decroocq, D. Rodriguez-Lucena, V. Russo, T. Mena Barragan, C. Ortiz Mellet, P. Compain, *Chem. Eur. J.* **2011**, *17*, 13825 – 13831.

[8] M. Durka, K. Buffet, J. Iehl, M. Holler, J.-F. Nierengarten, S. P. Vincent, *Chem. Eur. J.* **2012**, *18*, 641 – 651.

[9] a) P. Compain, C. Decroocq, A. Joosten, J. de Sousa, D. Rodriguez-Lucena, T. D. Butters, J. Bertrand, R. Clement, C. Boinot, F. Becq, C. Norez, *ChemBioChem* **2013**, *14*, 2050 – 2058; b) C. Decroocq, A. Joosten, R. Sergent, T. Mena Barragan, C. Ortiz Mellet, P. Compain, *ChemBioChem* **2013**, *14*, 2038 – 2049; c) A. Joosten, C. Decroocq, J. de Sousa, J. P. Schneider, E. Etame, A. Bodlenner, T. D. Butters, P. Compain, *ChemBioChem* **2014**, *15*, 309 – 319; d) Y. Brissonnet, C. Ortiz Mellet, S. Morandat, M. I. Garcia Moreno, D. Deniaud, S. E. Matthews, S. Vidal, S. Sestak, K. El Kirat, S. G. Gouin, *J. Am. Chem. Soc.* **2013**, *135*, 18427 – 18435.

[10] E. T. Mack, P. W. Snyder, R. Perez-Castillejos, B. Bilgicer, D. T. Moustakas, M. J. Butte, G. M. Whitesides, *J. Am. Chem. Soc.* **2012**, *134*, 333 – 345.

[11] a) M. Abellán-Flos, M. Tanç, C. T. Supuran, S. P. Vincent, *Org. Biomol. Chem.* **2015**, *13*, 7445 – 7451; b) N. Touisni, N. Kanfar, S. Ulrich, P. Dumy, C. T. Supuran, A. Mehdi, J.-Y. Winum, *Chem. Eur. J.* **2015**, *21*, 10306 – 10309; c) M. Stiti, A. Cecchi, M. Rami, M. Abdaoui, V. Barragan-Montero, A. Scozzafava, Y. Guari, J. Y. Winum, C. T. Supuran, *J. Am. Chem. Soc.* **2008**, *130*, 16130 – 16131.

[12] L. Tedaldi, G. K. Wagner, *Med. Chem. Commun.* **2014**, *5*, 1106 – 1125.

[13] a) H. Dohi, R. Périon, M. Durka, M. Bosco, Y. Roué, F. Moreau, S. Grizot, A. Ducruix, S. Escaich, S. P. Vincent, *Chem. Eur. J.* **2008**, *14*, 9530 – 9539; b) S. Grizot, M. Salem, V. Vongsouthi, L. Durand, F. Moreau, H. Dohi, S. Vincent, S. Escaich, A. Ducruix, *J. Mol. Biol.* **2006**, *363*, 383 – 394; c) M. Durka, M. Andaloussi, A. Tikad, R. Périon, M. Bosco, S. Floquet, E. Malacain, F. Moreau, M. Oxoby, V. Gerusz, S. P. Vincent, *Chem. Eur. J.* **2011**, *17*, 11305 – 11313.

[14] a) R. Winzar, J. Philips, M. J. Kiefel, *Synlett* **2010**, 583 – 586; b) J. Gao, R. Haerter, D. M. Gordon, G. M. Whitesides, *J. Org. Chem.* **1994**, *59*, 3714 – 3715.

[15] T. Storz, A. Vasella, *Helv. Chim. Acta* **1998**, *81*, 1896 – 1907.

[16] D. B. Smith, Z. Wang, S. L. Schreiber, *Tetrahedron* **1990**, *46*, 4793 – 4808.

[17] a) K. Buffet, E. Gillon, M. Holler, J.-F. Nierengarten, A. Imberty, S. P. Vincent, *Org. Biomol. Chem.* **2015**, *13*, 6482 – 6492; b) M. Sánchez-Navarro, A. Munoz, B. M. Illescas, J. Rojo, N. Martin, *Chem. Eur. J.* **2011**, *17*, 766 – 769; c) S. Cecioni, V. Oerthel, J. Iehl, M. Holler, D. Goyard, J. P. Praly, A. Imberty, J. F. Nierengarten, S. Vidal, *Chem. Eur. J.* **2011**, *17*, 3252 – 3261; d) A. Muñoz, D. Sigwalt, B. Illescas, J. Luczkowiak, L. Rodriguez, I. Nierengarten, M. Holler, J.-S. Remy, K. Buffet, S. Vincent, F. J. Rojo, R. Delgado, J.-F. Nierengarten, N. Martín, *Nat. Chem.* **2016**, *8*, 50 – 57.

[18] J.-F. Nierengarten, J. Iehl, V. Oerthel, M. Holler, B. M. Illescas, A. Munoz, N. Martin, J. Rojo, M. Sanchez-Navarro, S. Cecioni, S. Vidal, K. Buffet, M. Durka, S. P. Vincent, *Chem. Commun.* **2010**, *46*, 3860 – 3862.

[19] In our hands, all attempts to click a Kdo derivative bearing a free acid failed. The demethylation of the Kdo multimers also proved unsuccessful because of the presence of the malonyl esters in the functionalized fullerene structures. However, the effect of the presence of a carboxylic acid on the multimeric structures onto WaaC inhibition was addressed by using the following calixarenes derivatives (see the synthesis of calix-arene KDO-acid-C[4]A, and its biochemical evaluation).

[20] a) K. Buffet, I. Nierengarten, N. Galanos, E. Gillon, M. Holler, A. Imberty, S. E. Matthews, S. Vidal, S. P. Vincent, J.-F. Nierengarten, *Chem. Eur. J.* **2016**, *22*, 2955 – 2963; b) S. P. Vincent, K. Buffet, I. Nierengarten, A. Imberty, J.-F. Nierengarten, *Chem. Eur. J.* **2016**, *22*, 88 – 92; c) I. Nierengarten, K. Buffet, M. Holler, S. P. Vincent, J.-F. Nierengarten, *Tetrahedron Lett.* **2013**, *54*, 2398 – 2402.

[21] a) S. Smeets, H. Roex, W. Dehaen, *ARKIVOC (Gainesville, FL, U.S.)* **2003**, *4*, 83 – 92; b) J. K. Pak, M. Hesse, *J. Org. Chem.* **1998**, *63*, 8200 – 8204.

[22] M. Bergeron-Brlek, T. C. Shiao, M. C. Trono, R. Roy, *Carbohydr. Res.* **2011**, *346*, 1479 – 1489.

[23] M. L. Lepage, J. P. Schneider, A. Bodlenner, A. Meli, F. De Riccardis, M. Schmitt, C. I. Tarnus, N.-T. Nguyen-Huynh, Y.-N. Francois, E. Leize-Wagner, C. Birck, A. Cousido-Siah, A. Podjarny, I. Izzo, P. Compain, *Chem. Eur. J.* **2016**, *22*, 5151 – 5155.

[24] M. A. Flos, M. I. García Moreno, C. Ortiz Mellet, J. M. García Fernández, J.-F. Nierengarten, S. P. Vincent, *Chem. Eur. J.* **2016**, *22*, 11450 – 11460.

Received: May 9, 2016
Published online on ■■ ■■, 0000

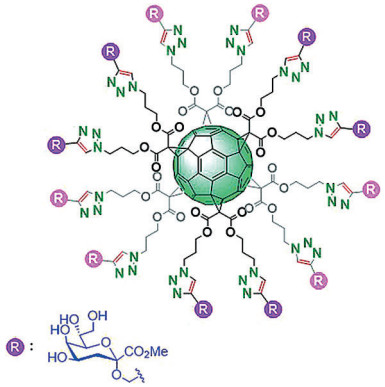
FULL PAPER

Inhibitors

*A. Tikad, H. Fu, C. M. Sevrain, S. Laurent, J.-F. Nierengarten, S. P. Vincent**

■■ – ■■

Mechanistic Insight into Heptosyltransferase Inhibition by using Kdo Multivalent Glycoclusters



Better together? The synthesis of unprecedented multimeric Kdo glycoclusters based on fullerene and calixarene central scaffolds is reported (see figure). The compounds were used to study the mechanism and the scope of multivalent heptosyltransferase inhibition, which is an important bacterial enzyme. The novel Kdo glycofullerenes displayed very potent inhibition ($K_i = 0.14$ μM), at a level that is rarely observed with glycosyltransferases.