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Probing multivalency for the inhibition of an enzyme: glycogen phosphorylase as a case study†

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Glycogen phosphorylase is involved in the hepatic glucose production and appears an emerging biological target for the treatment of type 2 diabetes. Two distinct trivalent inhibitors of GP were synthesized either through Cu(I)-assisted 1,3-dipolar cycloaddition or through formation of a tris-oxadiazole derivative. A biological study of the inhibiting properties of these trivalent inhibitors of GP have shown that the valency of the molecules influences slightly the inhibition of the enzyme whereas the presence of a spacer arm between the core and the pharmacophore moieties does not. The possible modes of binding of these multivalent inhibitors to the enzyme are discussed.

Introduction

Glycogen phosphorylase and diabetes

Diabetus mellitus affects about 3% of the world population and up to 6% for the adult population of developed countries. Diabetes, one of the major causes of death worldwide, is characterized by elevated glycaemia causing heart and kidney failures as well as visual impairment problems.[1] Type 2 diabetes is non-insulino-dependent and arises from insulin signalling inefficiency resulting in insufficient or even late insulin secretion. A series of biological targets have been identified for anti-diabetic therapy[2] such as peroxisome proliferator-activated receptors α/γ (PPARs α/γ),[3] glucagon-like peptide-1 (GLP-1),[4] dipeptidyl peptidase IV (DPP-IV)[5] or protein tyrosine phosphatase 1B (PTP 1B).[6] Glycogen phosphorylase (GP) has recently appeared as an enzyme of interest for the treatment of type 2 diabetes.[7] This enzyme catalyses glycogen depolymerisation to release glucose-1-phosphate according to the schematic equation:

$$(\text{Glucose})_n \rightarrow (\text{Glucose})_{n-1} + \text{Glucose-1-phosphate}$$

The inhibition of GP is expected to slow down glycogenolysis and to lower the production of glucose from the liver therefore allowing for a better control over hyperglycaemia. GP has been extensively studied and crystallographic data analyses are displaying a large number of sites for the inhibition of this enzyme.[8] A series of GP inhibitors have been described previously with various heterocyclic structures[9] but our work[10] is focusing on carbohydrate-based inhibitors of GP[7b,c,f] which are capable of binding selectively at the catalytic site of GP. These glycomimetic approaches are based on structural modifications at the molecular level for improving the binding to the enzyme and therefore affording valuable GP inhibitors.

Multivalency and inhibition of enzymes

Another approach for the inhibition of GP could take advantage of multivalency. This strategy may provide additional opportunities in the field of drug discovery for the design of potent enzyme inhibitors particularly by reaching higher affinities and probably better selectivities. A few examples of multivalent inhibition of an enzyme are reported in the literature where multimeric species of a specific drug are capable of improving the inhibition in comparison to the monomeric molecule.[11] The binding of one ligand subunit from the multivalent molecule to the enzyme generates an increase in local concentration of ligands, thus creating an apparent cooperativity causing an enhancement in inhibition. Dimeric inhibitors of influenza virus neuraminidase have been developed by MacDonald *et al.*[12] and displayed up to a 100-fold increase in inhibition along with improved pharmacokinetic properties. In an additional study of the same group,[13] a set of trimeric and tetrameric inhibitors displayed improved antiviral activities and long-lasting protective activities against influenza virus. More recently, the group of J. Gervay-Hague has described the synthesis of a series of trivalent zanamivir derivatives *via* click chemistry although no biological activity has been reported yet.[14] Inhibition of acetylcholinesterase by dimeric molecules resulted in up to 3000-fold increases in potency and selectivity compared to the monomeric inhibitor.[15] Glycosidases inhibition[16] with tethered dimeric azasugars was investigated and the molecules displayed interesting inhibitions of these enzymes but more

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importantly a selectivity for two enzymes out of the seven tested.[16a] However, tetravalent 1-azafagomine inhibitors displayed no improved inhibition compared to the monovalent structure but rather a strong decrease in activity.[16b] Finally, bivalent inhibitors of tetrameric β-tryptase constructed on a cyclodextrin scaffold have shown increased inhibitions for this enzyme.[17]

Inspired by the above mentioned results for the multivalent neuraminidase inhibition and by the fact that a multimeric enzyme offers additional possibilities for improved inhibition through multivalency, we have designed synthetic routes to trivalent carbohydrate-based GP inhibitors. A single case of a dimeric inhibitor of GP has been reported with a bis(5-chloroindole-2-carboxamide) derivative inhibiting human liver GP*a* (HLGP*a*) with an IC_{50} value of 6 nM compared to 12.5 μM for the parent monovalent inhibitor (2000-fold increase).[18] This result highlights a productive and cooperative binding of both ends of the bivalent inhibitor to two binding sites. The co-crystallization of HLGP*a* with the divalent inhibitor demonstrated that a single molecule of inhibitor was capable of interacting with each monomeric unit of GP by linking the two binding sites across the interface of GP homodimeric structure. This result encouraged us to further investigate this approach for the design of multivalent inhibitors of GP.

A closer look at the modes of binding of multivalent inhibitors to an enzyme reveals several possibilities as depicted in Fig. 1. A dimeric enzyme such as GP can interact with two monomeric molecules of inhibitor in a 1:1 complex (2:2 at the molecular level) providing a reference IC_{50mono} value for monovalent inhibitors. When considering the same inhibitor repeated three times on a molecular scaffold, four main possible cases can then be envisaged. The inhibition of a trivalent inhibitor must be divided by three in order to consider the contribution of each residue in its comparison to a monovalent inhibitor. If a 1:1 complex is formed in solution, a simple statistical effect can be invoked if no positive

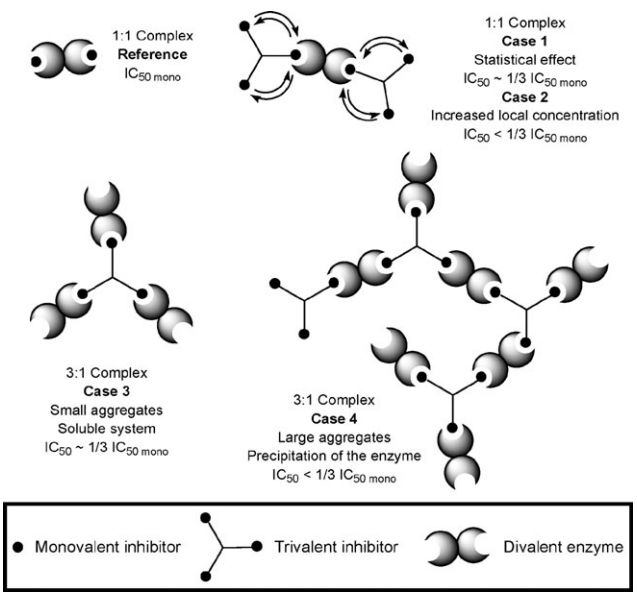


Fig. 1 Possible modes of binding for a mono- and trivalent inhibitors with a dimeric enzyme.

effect is observed and the IC_{50} value observed will be similar to 1/3 IC_{50mono} (Fig. 1, Case 1). Nevertheless, the IC_{50} measured can be improved with a lower value in comparison to 1/3 IC_{50mo} (Fig. 1, Case 2). If each binding site of the enzyme is occupied by a ligand of the multivalent inhibitor, the formation of 3:1 complexes would afford aggregates of proteins. If the size of the aggregates remains small enough to maintain a good solubility of the complex, the IC_{50} observed will be similar to 1/3 IC_{50mono} (Fig. 1, Case 3). Nevertheless, if the size of the multivalent inhibitor-enzyme clusters becomes large enough to cause their precipitation, the quantity of enzyme present in the solution will diminish and therefore the IC_{50} value measured will be lower than 1/3 IC_{50mono} (Fig. 1, Case 4). In this case, the IC_{50} value measured will be a "virtual" value because of the lower quantity of the enzyme available in solution.

Results and discussion

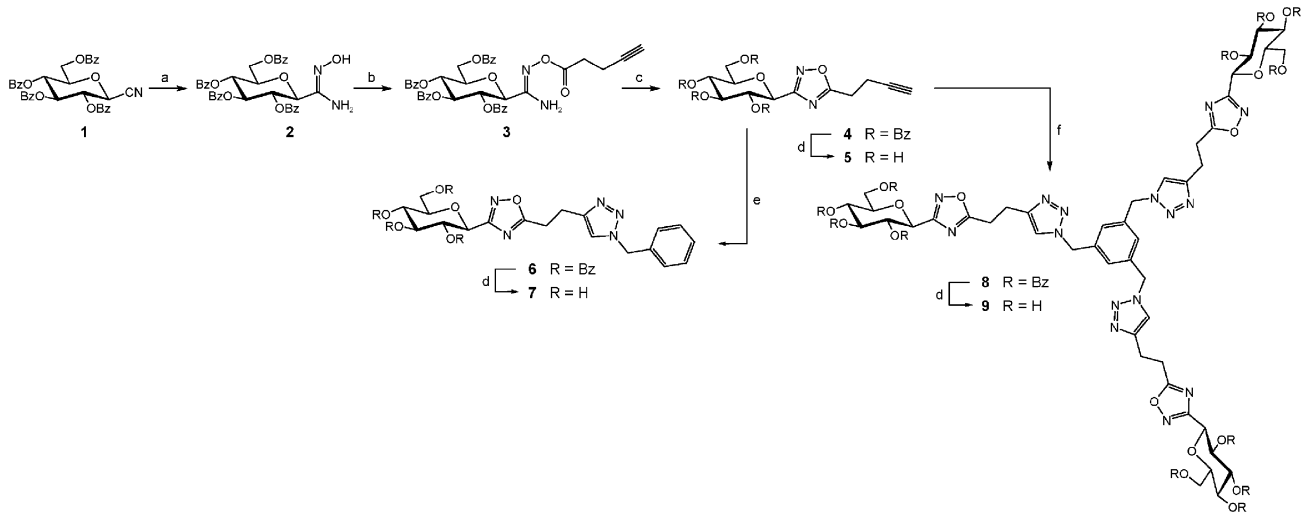
Synthesis of trivalent inhibitors

We have recently reported the preparation of 3-*C*-glycosyl-5-aryl-1,2,4-oxadiazoles which displayed good inhibition towards GP.[10b] In this context, we synthesized an alkyne-terminated 3-*C*-glycosylated 1,2,4-oxadiazole which could then be involved in a 1,3-dipolar cycloaddition with a tris-azido-functionalized derivative to obtain a multivalent GP inhibitor candidate. A more condensed trimeric inhibitor was also prepared by direct coupling of an amidoxime with a tris-acyl chloride and subsequent dehydrative cyclization to the corresponding trivalent *C*-glycosylated oxadiazole. These inhibitors were designed in order to determine the influence of a spacer arm between the core and the pharmocophore ligands on the inhibition of the enzyme.

Synthesis of the trivalent inhibitor with a spacer arm

The perbenzoylated glucosyl cyanide **1**[19] was reacted with hydroxylamine hydrochloride in pyridine to afford the desired amidoxime **2** (Scheme 1). In our previous work,[10b] the crude product obtained was rather difficult to purify by silica gel column chromatography. The amidoxime **2** could be obtained pure without chromatography simply by diluting the crude product in ethyl acetate and then washing the organic layer with 1 M aqueous HCl to remove pyridine and excess of hydroxylamine, followed by saturated aqueous $NaHCO_3$ and brine. This simple chromatography-free purification process afforded the expected amidoxime **2** in 99% yield and high purity. The formation of the *O*-acyl-amidoxime **3** was achieved with 4-pentynoic acid in the presence of EDCI/HOBt as coupling agents.

We previously observed that reactions times lasting from a few hours to a few days were required for the thermal cyclodehydration of *O*-acyl-amidoximes. In order to optimize both time and yield, we performed this reaction under TBAF catalysis[20] and/or microwaves activation[21] (Table 1). We observed that the use of TBAF catalysis at room temperature provided the cyclic oxadiazole **4** within 1 day (entry 1) while thermal activation combined with TBAF catalysis drastically shortened the reaction time to 10 minutes (entry 2). No



Scheme 1 *Reagents and conditions*: (a) $NH_2OH{\cdot}HCl$, C_5H_5N, 50 °C, 5 h, 99%; (b) $HC{\equiv}C(CH_2)_2CO_2H$, EDCI, HOBt, CH_2Cl_2/DMF (9:1), −8 °C then r.t., 16 h, 67%; (c) PhMe, TBAF 10 mol%, μW (150 °C, 200 W, 5 min), 97%; (d) NaOMe, MeOH then Amberlite IR-120 (H^+ form); (e) $PhCH_2N_3$, CuI, Et_3N, μW (110 °C, 150 W, 15 min), 88%; (f) $C_6H_3(CH_2N_3)_3$, CuI, Et_3N, μW (110 °C, 150 W, 15 min), 98%.

Table 1 Cyclodehydration of *O*-acyl-amidoxime **3** to the 1,2,4-oxadiazole **4** in toluene

Entry	Catalyst	*T*/°C	Microwave condition	Time	Yield (%)
1	10% TBAF	25	None	24 h	99
2	10% TBAF	110	None	10 min	97
3	None	150	100 W	1 h	No reaction
4	None	175	200 W	2 h	Decomposition
5	10% TBAF	150	200 W	5 min	97
6	10% TBAF	150	200 W	30 min	66

reaction or decomposition of the starting material was observed when applying microwaves activation without TBAF catalysis (entries 3 and 4). Nevertheless, the association of TBAF catalysis and microwaves activation performed on a short timescale (entry 5 and 6) provided a result comparable to that observed under conventional heating (entry 2). These results underline the beneficial influence of TBAF catalysis.

The alkyne-terminated oxadiazole **4** was then engaged in a Huisgen's Cu(I)-catalyzed 1,3-dipolar cycloaddition[22] reaction under microwaves activation with benzyl azide to afford the desired 1,4-disubstituted 1,2,3-triazole **6** in excellent yield. Debenzoylation of compounds **4** and **6** afforded two hydroxylated GP inhibitor candidates **5** and **7**. Similarly, the reaction of 1,3,5-tris(azidomethyl)benzene[23] with the alkyne derivative **4** under microwaves activation and Cu(I) catalysis afforded the cycloadduct **8**. The saponification of the benzoate esters provided the fully hydroxylated macromolecule **9**.

Synthesis of the trivalent inhibitor without spacer arm

We next prepared a more condensed trifunctional macromolecule were the *C*-glucosyl-oxadiazole moiety was directly attached to a benzene ring (Scheme 2). Condensation of amidoxime **2** with 1,3,5-benzenetricarbonyl trichloride afforded the corresponding triester **10** in 73% yield. At first, compound **10** was subjected to cyclodehydration under thermal conditions (reflux in 1,4-dioxane). The product obtained was not the expected tris-oxadiazole **13** but the bis-oxadiazole **11** with one unreacted *O*-acyl amidoxime moiety as evidenced by mass spectrometry (m/z = 2035.4 $[M + H]^+$). Interestingly, molecular ions could be observed neither for compound **13** nor the mono-oxadiazole intermediate. Saponification of the ester groups of **11** resulted in the concomitant cleavage of the *O*-acyl amidoxime function and afforded the benzoic acid derivative **12** whose structure was clearly demonstrated by mass spectrometry (m/z = 581 $[M - H]^-$) and NMR analyses. The triple thermal cyclodehydration of **10** was then performed under microwaves activation and TBAF catalysis for 40 minutes. The tris-oxadiazole derivative **13** was isolated in 72% yield as the only product of the reaction highlighting again the positive influence of TBAF catalysis and microwaves activation for this cyclodehydration process. Deprotection under Zemplén conditions afforded the expected hydroxylated trivalent GP inhibitor candidate **14**.

Inhibition of glycogen phosphorylase

The inhibition of GP was determined, as previously reported,[10b] for the three monovalent *C*-glycosylated oxadiazoles (**5**, **7** and **15**[10b]) and the two trivalent derivatives **9** and **14** (Fig. 2, Table 2). The enzymatic assays were performed at two concentrations and most molecules displayed poor inhibition properties at a concentration of 625 μM and moderate to good inhibition at higher concentration (2.5 mM). In addition, K_i values could be estimated only for trivalent derivatives **9** and **14** (see ESI†).

The alkyne-terminated *C*-glycosylated oxadiazole derivative **5** displayed no inhibition at 625 μM and poor activity at

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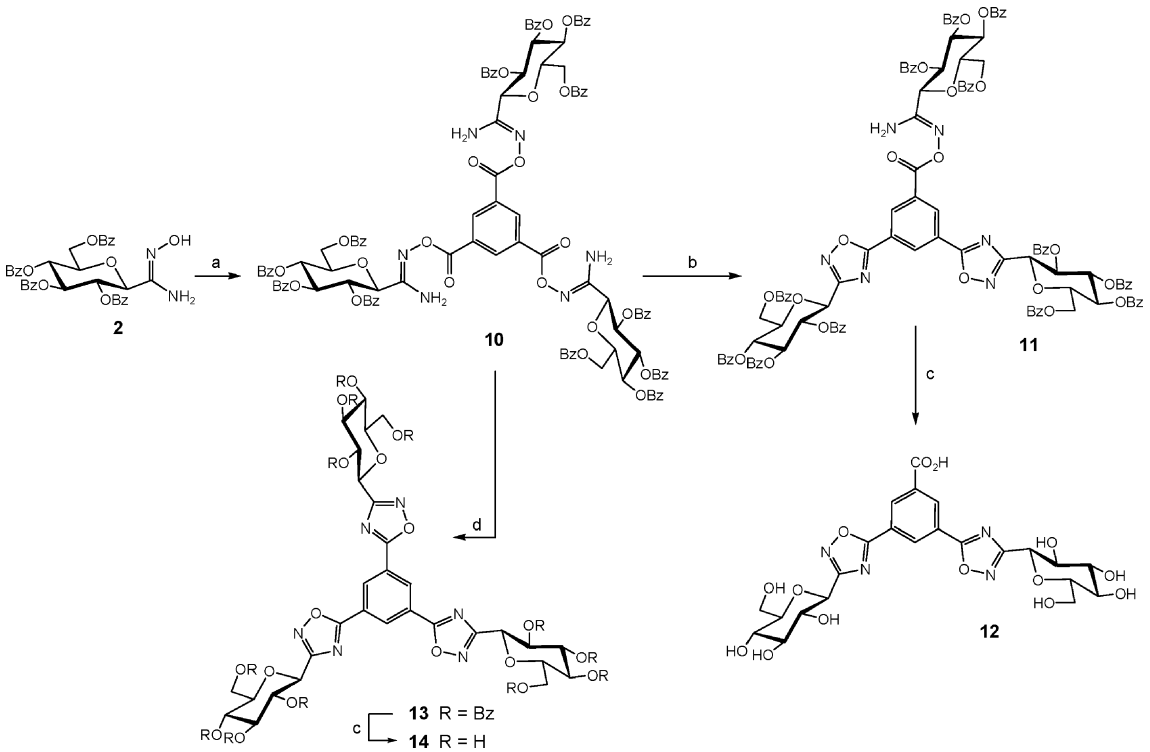

Scheme 2 *Reagents and conditions*: (a) $C_6H_3(COCl)_3$, 1,4-dioxane, r.t., 24 h, 73%; (b) 1,4-dioxane, 100 °C, 4 days; (c) NaOMe, MeOH then Amberlite IR-120 (H^+ form); (d) PhMe, TBAF 30 mol%, μW (150 °C, 200 W, 40 min), 72%.

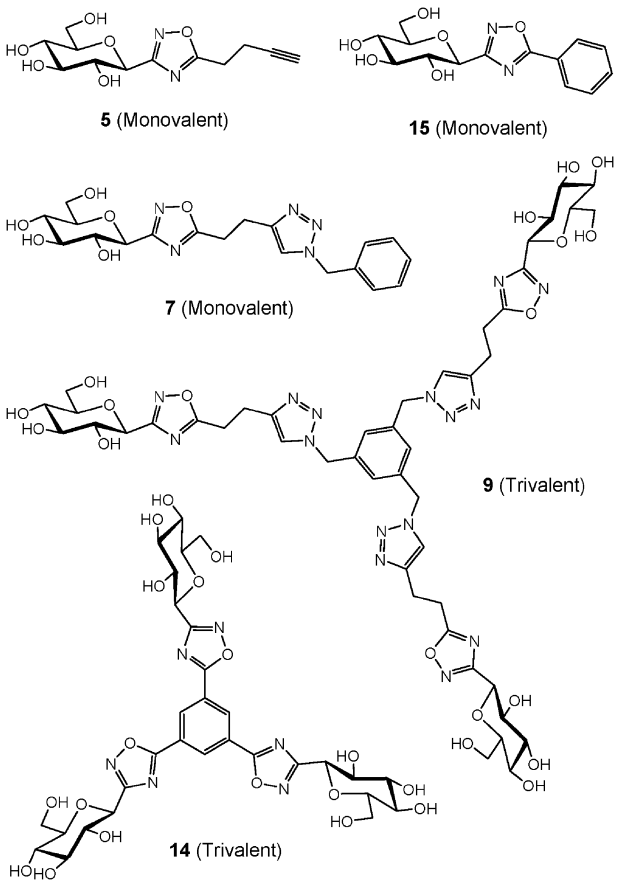

Fig. 2 Structure of monovalent and trivalent GP inhibitors tested.

Table 2 Inhibition of GP observed for monovalent and trivalent inhibitors at two concentrations

		Inhibition (%)		
Inhibitor	Valency	At 625 μM	At 2.5 mM	K_i/μM
5	1	0	22 ± 4	n.d.[a]
7	1	0	0	n.d.[a]
15	1	10	n.d.[a]	n.d.[a]
9	3	30 ± 5	56 ± 5	480 ± 45[b]
14	3	35 ± 5	62 ± 5	535 ± 50[b]

[a] n.d. = not determined. [b] Estimated.

2.5 mM. The inhibition properties disappeared completely when a spacer arm was added such as in the structure of **7**. Interestingly, the trivalent analogue **9** of the non-active derivative **7** was now inhibiting GP with values of 30% at 625 μM and 56% at 2.5 mM. The valency of the molecule is therefore responsible for an increase in inhibition from 0 to 56% when comparing **7** and **9** at 2.5 mM. The *C*-glycosylated oxadiazole derivative **15** bearing a phenyl group on the 5-position of the oxadiazole ring displayed 10% inhibition at 625 μM.[10b] The inhibition was again increased to 35% at 625 μM for trivalent analogue **14**. We anticipated that the distance between the core and the carbohydrate moiety would influence for the binding to the enzyme. Nevertheless, this was not the case based on the inhibition measured for **9** and **14**.

The increase of valency from monovalent to trivalent species is responsible for an increase in inhibition of GP. The inhibition per residue for trivalent molecules is always similar the

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corresponding monovalent analogue in accordance with the statistical effect model proposed (Fig. 1, Case 1). The 3:1 complex non soluble mode of binding (Fig. 1, Case 4) can be ruled out for these trivalent inhibitors of GP to the dimeric enzyme since no precipitate was observed under the concentrations of trivalent inhibitors and enzyme used for the inhibition studies. The inhibition observed for the trivalent species was never better than 1/3 of the inhibition (in %) observed for the corresponding monovalent molecules. In conclusion, two modes of binding are possible with either 1:1 or 3:1 complexes (Fig. 1, Case 1 or Case 3, respectively) resulting in an observed inhibition close to 1/3 of the inhibition for the parent monovalent inhibitor.

In the present study, the expected binding sites of these glucose-based multivalent inhibitors are the catalytic site of GP homodimer which are separated from each other by a long distance and pointing into opposite directions.[8d] The structure of the trivalent inhibitors tested did not permit such an intramolecular interaction with both catalytic sites on the same GP dimer, but rather an interaction with two independent GP dimers. In comparison, the bis(5-chloroindole-2-carboxamide) derivative is binding simultaneously at each indole binding site near the interface between the monomeric units of the GP dimer.[17] The linker is composed of 12 atoms between two indole aromatic units which are therefore available for interacting with the binding site of each monomer of GP.

The designed trivalent molecules could also bind to the enzyme on a different site. The large aromatic appendage present in the aglycon, composed of oxadiazole, phenyl and triazole rings, might interact with the surface of the protein through hydrophobic interactions. The stability of the inhibitor-protein complex would therefore be lower than complex involving an internal binding site such as the catalytic site. The observed inhibitions would therefore be weak as currently observed in the present study. Nevertheless, we do not possess any experimental data confirming or denying such a mode of interaction.

Conclusions

In conclusion, we have designed two kinds of multivalent inhibitors of GP based on the acylation of an amidoxime intermediate followed by thermal dehydrative cyclization to the corresponding oxadiazole. The introduction of an alkyne residue at the 5-position of the oxadiazole ring allowed the coupling to a trivalent azido-functionalized benzene ring leading to an extended trivalent inhibitor candidate. The enzyme inhibition assays revealed poor to moderate inhibitory effect of these analogues. But, more important was the fact that multivalent inhibitors were always superior to their monovalent counterparts. This study provides one of the few examples of multivalent inhibition for an enzyme, even though the inhibitions observed remain modest.

Experimental

General methods

Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with silica gel 60 F_{254} (Merck). TLC plates were inspected by UV light (λ = 254 nm) and developed by treatment with a mixture of 10% H_2SO_4 in EtOH/H_2O (1:1 v/v) followed by heating. Silica gel column chromatography was performed with Geduran® silica gel Si 60 (40–63 μm) purchased from Merck (Darmstadt, Germany). Reactions under microwave activation were performed on a CEM Discover system. HRMS (LSIMS) mass spectra were recorded in the positive mode using a Thermo Finnigan Mat 95 XL spectrometer. MS (ESI) mass spectra were recorded in the positive mode using a Thermo Finnigan LCQ spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 23 °C using Brüker Advance DRX300 or DRX500 spectrometers with the residual solvent as the internal standard. The following abbreviations are used to explain the observed multiplicities: s, singlet; d, doublet; dd, doublet of doublet; ddd, doublet of doublet of doublet; t, triplet; td, triplet of doublet; q, quadruplet; m, multiplet; br, broad; p, pseudo. Structure elucidation was deduced from 1D and 2D NMR spectroscopy which allowed, in most cases, complete signal assignments based on COSY, HSQC, and HMBC correlations. NMR solvents were purchased from Euriso-Top (Saint Aubin, France). Atom numbering of the molecules is presented in the ESI.†

Syntheses

1,3,5-Tris(azidomethyl)benzene. A solution of 1,3,5-tris(bromomethyl)benzene (3.07 g, 8.6 mmol) and sodium azide (3.36 g, 51.6 mmol) in DMF (100 mL) was stirred at 65 °C for 24 hours. The solution was cooled to room temperature then poured into water (400 mL). The aqueous layer was extracted with Et_2O (3 × 250 mL). The combined organic layers were washed with water (2 × 400 mL) and brine (300 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated with **extreme care** (*water-bath at room temperature, reduced pressure and Plexiglas shield*) to afford 1,3,5-tris(azidomethyl)benzene (2.05 g, 98%) as a colorless oil. R_f = 0.83 (PE/EtOAc, 8:2). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 4.39 (s, 6H, CH_2N_3), 7.25 (s, 3H, H-ar).

***C*-(2,3,4,6-Tetra-*O*-benzoyl-β-D-glucopyranosyl)-formamidoxime (2).** A solution of 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl cyanide **1** (3.00 g, 4.96 mmol) and hydroxylamine hydrochloride (0.86 g, 12.4 mmol) in pyridine (10 mL) was stirred at 50 °C for 5 hours. The mixture was diluted with EtOAc (250 mL) and washed with 100 mL portions of water, 1 M HCl, saturated $NaHCO_3$, water and brine successively. The organic layer was dried ($MgSO_4$), filtered and evaporated to obtain the pure amidoxime **2** (3.24 g, 99%) as a white foam. R_f = 0.48 (PE/EtOAc, 1:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.21 (ddd, 1H, J = 9.7 Hz, J = 5.1 Hz, J = 2.7 Hz, H-5), 4.31 (d, 1H, J = 9.8 Hz, H-1), 4.47 (dd, 1H, J = 5.1 Hz, J = 12.4 Hz, H-6a), 4.62 (dd, 1H, J = 2.7 Hz, J = 12.4 Hz, H-6b), 4.76 (bs, 2H, NH_2), 5.69 (t, 1H, J = 9.8 Hz, H-2), 5.73 (t, 1H, J = 9.8 Hz, H-4), 5.96 (t, 1H, J = 9.8 Hz, H-3), 7.24–7.43 (m, 10H, H-ar), 7.47–7.57 (m, 2H, H-ar), 7.81–8.04 (m, 8H, H-ar).

***O*-(Pent-4′-ynoyl)-3-*C*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-formamidoxime (3).** A solution of 4-pentynoic acid (27 mg, 0.27 mmol) in CH_2Cl_2/DMF (4 mL, 9:1) was cooled to −8 °C before addition of 1-hydroxybenzotriazole (HOBt)

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(36.5 mg, 0.27 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (52 mg, 0.27 mmol) and amidoxime **2** (146 mg, 0.23 mmol). The mixture was kept at −8 °C for 30 minutes then stirred at room temperature for 16 hours. The solvents were evaporated off and the crude product was purified by flash silica gel column chromatography (PE then PE/EtOAc, 1:1) to afford the *O*-acylamidoxime **3** (110 mg, 67%) as a white foam. R_f = 0.53 (PE/EtOAc, 1:1). $[\alpha]_D$ = −2.9 (c = 1.00/CH_2Cl_2). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.94 (t, 1H, J = 2.5 Hz, H-5′), 2.40–2.63 (m, 4H, H-2′ H-3′), 4.31 (ddd, 1H, J = 2.7 Hz, J = 5.1 Hz, J = 9.8 Hz, H-5), 4.54 (d, 1H, J = 9.8 Hz, H-1), 4.53–4.59 (m, 1H, H-6a), 4.67 (dd, 1H, J = 2.7 Hz, J = 9.5 Hz, H-6b), 5.36 (s, 2H, NH_2), 5.76 (t, 1H, J = 9.8 Hz, H-4), 5.80 (t, 1H, J = 9.8 Hz, H-2), 6.01 (t, 1H, J = 9.8 Hz, H-3), 7.25–7.57 (m, 12H, H-ar), 7.83–8.07 (m, 8H, H-ar). ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3 (C-3′), 32.1 (C-2′), 63.0 (C-6), 69.2 (C-4), 69.3 (C-5′), 70.0 (C-2), 73.7 (C-3), 75.6 (C-1), 76.7 (C-5), 82.5 (C-4′), 128.40, 128.45, 128.5 (3s, 8C, CH-ar), 128.77, 128.82, 129.4 (3s, 4C, C^{IV}-ar), 129.8, 129.9, 130.00, 130.04 (4s, 8C, CH-ar), 133.4, 133.5, 133.7 (3s, 4C, CH-ar), 153.7 (N=CR–NH_2), 165.3, 165.6, 165.7, 166.3 (4s, 4C, *C*OPh), 169.0 (C-1′). ESI-MS (positive mode) m/z: 719.2 $[M + H]^+$, 741.3 $[M + Na]^+$, 786.9 $[M + HCOOH + Na]^+$, 1436.9 $[2M + H]^+$, 1458.9 $[2M + Na]^+$, 1504.5 $[2M + HCOOH + Na]^+$. HR-ESI-MS (positive mode) m/z: calcd. for $C_{40}H_{34}N_2O_{11}$ $[M + H]^+$ 719.2241, found 719.2244.

5-(But-1″-yn-4″-yl)-3-*C*-(2′,3′,4′,6′-tetra-*O*-benzoyl-β-D-glucopyranosyl)-1,2,4-oxadiazole (4). In a CEM Discover 5 mL vial was introduced a solution of *O*-acylamidoxime **3** (369 mg, 0.5 mmol) and TBAF (50 µL, 50 µmol, 1 M in THF) in toluene (5 mL). The reaction vial was heated at 150 °C for 5 min upon microwave irradiation (200 W). The solvent was evaporated and the residue purified by flash silica gel column chromatography (PE then PE/EtOAc, 1:1) to afford the oxadiazole **4** (348 mg, 97%) as a white foam. R_f = 0.65 (PE/EtOAc, 7:3). $[\alpha]_D$ = +8.3 (c = 1.12/CH_2Cl_2). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.84 (t, 1H, J = 2.6 Hz, H-1″), 2.65 (td, 2H, J = 2.6 Hz, J = 7.5 Hz, H-3″), 3.10 (t, 2H, J = 7.5 Hz, H-4″), 4.33 (ddd, 1H, J = 3.4 Hz, J = 5.1 Hz, J = 9.8 Hz, H-5′), 4.53 (dd, 1H, J = 5.2 Hz, J = 12.4 Hz, H-6′a), 4.65 (dd, 1H, J = 3.0 Hz, J = 12.4 Hz, H-6′b), 5.09 (d, 1H, J = 9.5 Hz, H-1′), 5.82 (t, 1H, J = 9.5 Hz, H-4′), 6.00 (m, 2H, H-2′ H-3′), 7.29–7.58 (m, 12 H, H-ar), 7.81–8.02 (m, 8H, H-ar). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.0 (C-3″), 26.2 (C-4″), 63.3 (C-6′), 69.4 (C-4′), 70.2 (C-1″), 70.6 (C-2′), 72.4 (C-1′), 74.1 (C-3′), 77.0 (C-5′), 80.8 (C-2″), 128.3 (s, 2C, CH-ar), 128.4 (s, 4C, CH-ar), 128.5 (s, 2C, CH-ar), 128.7, 128.7, 128.8, 129.5 (4s, 4C, C^{IV}-ar), 129.7, 129.8, 129.8, 129.9 (4s, 8C, CH-ar), 133.2, 133.3, 133.4, 133.5 (4s, 4C, CH-ar), 164.6, 165.2, 165.8, 166.3 (s, 4C, *C*OPh), 166.2 (C-3), 179.0 (C-5). ESI-MS (positive mode) m/z: 701.1 $[M + H]^+$, 723.2 $[M + Na]^+$, 1400.9 $[2M + H]^+$, 1422.9 $[2M + Na]^+$. HR-ESI-MS (positive mode) m/z: calcd. for $C_{40}H_{32}N_2O_{10}Na$ $[M + Na]^+$ 723.1955, found 723.1954.

3-*C*-(β-D-Glucopyranosyl)-5-(but-1″-yn-4″-yl)-1,2,4-oxadiazole (5). A solution of benzoylated oxadiazole **4** (261 mg, 0.37 mmol) and NaOMe (5 mg, 0.09 mmol) in CH_2Cl_2/MeOH (5 mL, 2:3) was stirred at room temperature for 4 hours. The solution was neutralized with a cation exchange resin (Amberlite IR-120, H^+ form) and the resin washed with MeOH (3 × 5 mL). The filtrate was evaporated off and the residue was dissolved in MeOH then precipitated with CH_2Cl_2. The resulting solid was washed with CH_2Cl_2 (2 × 5 mL) and dried under vacuum to afford the hydroxylated oxadiazole **5** (103 mg, 98%) as a white foam. R_f = 0.26 (CH_2Cl_2/MeOH, 9:1). $[\alpha]_D$ = +9.6 (c = 0.51/H_2O). ^{1}H NMR (300 MHz, CD_3OD) δ 2.36 (t, 1H, J = 2.6 Hz, H-1″), 2.73 (td, 2H, J = 2.6 Hz, J = 7.3 Hz, H-3″), 3.18 (t, 2H, J = 7.3 Hz, H-4″), 3.42–3.53 (m, 3H, H-3′ H-4′ H-5′), 3.65–3.73 (m, 2H, H-2′ H-6′a), 3.87 (dd, 1H, J < 1.0 Hz, J = 12.0 Hz, H-6′b), 4.44 (d, 1H, J = 9.7 Hz, H-1′). ^{13}C NMR (75 MHz, CD_3OD) δ 16.6 (C-3″), 27.1 (C-4″), 62.8 (C-6′), 71.2 (C-4′), 71.3 (C-1″), 73.3 (C-2′), 74.8 (C-1′), 79.2 (C-3′), 82.3 (C-2″), 82.6 (C-5′), 169.2 (C-3), 180.5 (C-5). ESI-MS (positive mode) m/z: 285.0 $[M + H]^+$, 307.1 $[M + Na]^+$, 590.9 $[2M + Na]^+$. HR-ESI-MS (positive mode) m/z: calcd. for $C_{12}H_{16}N_2O_6Na$ $[M + Na]^+$ 307.0906, found 307.0907.

5-[2″-(1‴-Benzyl-1‴,2‴,3‴-triazol-4‴-yl)ethyl]-3-*C*-(2′,3′,4′,6′-tetra-*O*-benzoyl-β-D-glucopyranosyl)-1,2,4-oxadiazole (6). In a CEM Discover 5 mL vial was introduced a solution of benzyl azide (110 mg, 0.828 mmol), alkyne **4** (193 mg, 0.276 mmol), copper iodide (26 mg, 0.138 mmol) and DIPEA (240 µL, 1.38 mmol) in toluene (5 mL). The solution was sonicated for 1 min then heated at 110 °C for 15 min upon microwave irradiation (150 W). The solvent was evaporated off and the residue purified by flash silica gel column chromatography (PE/EtOAc, 1:1) to afford the cycloadduct **6** (202 mg, 88%) as a colorless oil. R_f = 0.24 (PE/EtOAc, 1:1). $[\alpha]_D$ = +1.4 (c = 1.02/CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.22 (bs, 2H, H-2″), 3.28 (bs, 2H, H-1″), 4.37 (ddd, 1H, J = 3.0 Hz, J = 5.3 Hz, J = 9.7 Hz, H-5′), 4.56 (dd, 1H, J = 5.3 Hz, J = 12.4 Hz, H-6′a), 4.68 (dd, 1H, J = 3.0 Hz, J = 12.4 Hz, H-6′b), 5.10 (d, 1H, J = 9.7 Hz, H-1′), 5.48 (s, 2H, NCH_2Ph), 5.86 (t, 1H, J = 9.7 Hz, H-4′), 5.97 (t, 1H, J = 9.7 Hz, H-2′), 6.06 (t, 1H, J = 9.7 Hz, H-3′), 7.24–7.56 (m, 18H, H-5‴ H-ar), 7.80–8.03 (m, 8H, H-ar). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.8 (C-2″), 26.6 (C-1″), 53.6 (NCH_2Ph), 62.9 (C-6′), 68.1 (C-4′), 69.8 (C-2′), 71.7 (C-1′), 73.4 (C-3′), 76.5 (C-5′), 122.2 (C-5‴), 127.8, 127.9, 128.0, 128.4, 128.5, 128.9, 129.7, 129.8 (8s, 25C, CH-ar), 133.4, 133.6, 133.8, 133.9 (4s, 4C, C^{IV}-ar), 134.7 (C^{IV}-ar), 145.3 (C-4‴), 164.7, 165.0, 165.6, 165.3 (4s, 4C, *C*OPh), 165.6 (C-3), 180.1 (C-5). ESI-MS (positive mode) m/z: 834.1 $[M + H]^+$, 856.1 $[M + Na]^+$, 1666.4 $[2M + H]^+$. HR-ESI-MS (positive mode) m/z: calcd. for $C_{47}H_{40}N_5O_{10}$ $[M + H]^+$ 834.2775, found 834.2781.

5-[2″-(1‴-Benzyl-1‴,2‴,3‴-triazol-4‴-yl)-ethyl]-3-*C*-(β-D-glucopyranosyl)-1,2,4-oxadiazole (7). A solution of benzoylated cycloadduct **6** (128 mg, 0.153 mmol) and NaOMe (5 mg, 0.09 mmol) in CH_2Cl_2/MeOH (5.5 mL, 10:1) was stirred at room temperature for 4 hours. The solution was neutralized with a cation exchange resin (Amberlite IR-120, H^+ form) and resin washed with MeOH (3 × 5 mL). The filtrate was evaporated off and the residue purified by flash silica gel column chromatography (CH_2Cl_2 then

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CH_2Cl_2/MeOH, 8:2 then EtOAc/MeOH, 8:2) to afford the hydroxylated cycloadduct **7** (63 mg, 98%) as a white foam. R_f = 0.68 (EtOAc/MeOH, 4:1). $[\alpha]_D$ = +5.5 (c = 0.95/MeOH). ^{1}H NMR (300 MHz, CD_3OD) δ 3.18–3.23 (m, 2H, H-2″), 3.24–3.36 (m, 2H, H-1″), 3.44–3.52 (m, 3H, H-3′ H-4′ H-5′), 3.68–3.74 (m, 2H, H-2′ H-6′a), 3.88 (dd, 1H, J < 1.0 Hz, J = 11.4 Hz, H-6′b), 4.44 (d, 1H, J = 9.7 Hz, H-1′), 5.55 (s, 2H, NCH_2Ph), 7.28–7.38 (m, 5H, H-ar), 7.79 (s, 1H, H-5‴). ^{13}C NMR (75 MHz, CD_3OD) δ 23.3 (C-2″), 27.2 (C-1″), 54.9 (NCH_2Ph), 62.8 (C-6′), 71.3, 79.2, 82.6 (3s, 3C, C-3′ C-4′ C-5′), 73.4 (C-2′), 74.9 (C-1′), 123.9 (C-5‴), 129.1, 129.5, 130.0 (3s, 5C, CH-ar), 136.8 (C^{IV}-ar), 146.9 (C-4‴), 169.2 (C-3), 180.9 (C-5). ESI-MS (positive mode) m/z: 418.1 [M + H]$^+$, 440.1 [M + Na]$^+$, 856.6 [2M + Na]$^+$. HR-ESI-MS (positive mode) m/z: calcd. for $C_{19}H_{23}N_5NaO_6$ [M + Na]$^+$ 440.1546, found 440.1549.

1,3,5-Tris-4′-2″-[3‴-*C*-(2⁗,3⁗,4⁗,6⁗-tetra-*O*-benzoyl-β-D-glucopyranosyl)-1‴,2‴,4‴-oxadiazol-5‴-yl]-ethyl-1′,2′,3′-triazol-1′-ylmethylbenzene (8). In a CEM Discover 5 mL vial was introduced a solution of 1,3,5-tris(azidomethyl)benzene (*POTENTIALLY EXPLOSIVE*, 4.9 mg, 20 µmol), alkyne **4** (63 mg, 90 µmol), copper iodide (1.9 mg, 10 µmol) and DIPEA (17 µL, 100 µmol) in toluene (6 mL). The solution was sonicated for 1 min then heated at 110 °C for 15 min upon microwave irradiation (150 W). The solvent was evaporated off and the residue purified by flash silica gel column chromatography (PE/EtOAc, 1:1 then EtOAc) to afford the tris-cycloadduct **8** (46 mg, 98%) as a colorless oil. R_f = 0.67 (EtOAc). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.10–3.32 (m, 12H, H-1″ H-2″), 4.34–4.40 (m, 3H, H-5⁗), 4.51 (dd, 3H, J = 5.1 Hz, J = 12.4 Hz, H-6⁗a), 4.66 (dd, 3H, J = 2.7 Hz, J = 12.4 Hz, H-6⁗b), 5.12 (d, 3H, J = 9.4 Hz, H-1⁗), 5.31 (s, 6H, NC$H_2C_6H_3$), 5.87 (t, 3H, J = 9.4 Hz, H-4⁗), 5.97 (t, 3H, J = 9.4 Hz, H-2⁗), 6.05 (t, 3H, J = 9.4 Hz, H-3⁗), 6.93 (s, 3H, H-2 H-4 H-6), 7.23–7.50 (m, 36H, H-ar), 7.74–8.00 (m, 24H, H-ar). ^{13}C NMR (75 MHz, $CDCl_3$) δ 22.5 (s, 3C, C-1″), 26.6 (s, 3C, C-2″), 53.1 (N$CH_2C_6H_3$), 63.3 (s, 3C, C-6⁗), 69.3 (s, 3C, C-4⁗), 70.7 (s, 3C, C-2⁗), 72.3 (s, 3C, C-1⁗), 74.1 (s, 3C, C-3⁗), 77.0 (s, 3C, C-5⁗), 122.2 (s, 3C, C-5′), 127.0 (s, 3C, C-2, C-4, C-6), 128.39, 128.43, 128.5 (3s, 24C, CH-ar), 128.69, 128.71, 128.8, 129.5 (4s, 12C, C^{IV}-ar), 129.7, 129.78, 129.82, 129.9 (4s, 24C, CH-ar), 133.2, 133.4, 133.6 (3s, 12C, CH-ar), 137.0 (s, 3C, C-1, C-3, C-5), 145.6 (s, 3C, C-4′), 164.9, 165.2, 165.8, 166.2 (4s, 12C, *C*OPh), 166.4 (s, 3C, C-3‴), 180.0 (s, 3C, C-5‴). ESI-MS (positive mode) m/z: 1173.5 [M + 2H]$^{2+}$.

1,3,5-Tris-4′-2″-[3‴-*C*-(β-D-glucopyranosyl)-1‴,2‴,4‴-oxadiazol-5‴-yl]ethyl-1′,2′,3′-triazol-1′-ylmethylbenzene (9). A solution of benzoylated tris-cycloadduct **8** (114 mg, 49 µmol) and NaOMe (5 mg, 92 µmol) in CH_2Cl_2/MeOH (5.5 mL, 10:1) was stirred at room temperature for 6 hours. The solution was neutralized with a cation exchange resin (Amberlite IR-120, H^+ form) and resin washed with MeOH (3 × 5 mL). The filtrate was evaporated off and the residue was dissolved in MeOH then precipitated with PE. The resulting solid was washed with PE (5 × 5 mL), dissolved into pure water and freeze-dried to afford the hydroxylated tris-cycloadduct **9** (45 mg, 84%) as a white foam. ^{1}H NMR (300 MHz, D_2O) δ 3.02–3.19 (m, 6H, H-1″), 3.20–3.30 (m, 6H, H-2″), 3.51–3.86 (m, 18H, H-2⁗ H-3⁗ H-4⁗ H-5⁗ H-6⁗a H-6⁗b), 4.54 (d, 3H, J = 8.8 Hz, H-1⁗), 5.39 (s, 6H, NC$H_2C_6H_3$), 6.98 (s, 3H, H-2 H-4 H-6), 7.73 (s, 3H, H-5′). ^{13}C NMR (125 MHz, D_2O) δ 22.0 (s, 3C, C-1″), 26.3 (s, 3C, C-2″), 53.4 (s, 3C, N$CH_2C_6H_3$), 61.1 (s, 3C, C-6⁗), 72.1 (s, 3C, C-2⁗), 73.2 (s, 3C, C-1⁗), 69.6, 77.1, 80.6 (3s, 9C, C-3⁗ C-4⁗ C-5⁗), 124.2 (s, 3C, C-5′), 127.3 (s, 3C, C-2 C-4 C-6), 137.2 (s, 3C, C-1 C-3 C-5), 146.5 (s, 3C, C-4′), 167.5 (s, 3C, C-3‴), 181.0 (s, 3C, C-5‴). ESI-MS (positive mode) m/z: 1118.2 [M + Na]$^+$. HR-ESI-MS (positive mode) m/z: calcd. for $C_{45}H_{57}N_{15}NaO_{18}$ [M + Na]$^+$ 1118.3904, found 1118.3918.

***N*,*N*′,*N*″-1,3,5-Tris(benzoyloxy)-*C*-(2′,3′,4′,6′-tetra-*O*-benzoyl-β-D-glucopyranosyl)tricarboximidamide (10).** A solution of 1,3,5-benzenetricarbonyl trichloride (102 mg, 0.38 mmol) and amidoxime **2** (794 mg, 1.24 mmol) in 1,4-dioxane (15 mL) was stirred at room temperature for 24 hours. The solvent was then evaporated off and the mixture was diluted with EtOAc (150 mL). The organic layer was washed by 100 mL portions of saturated $NaHCO_3$, water and brine successively. The organic layer was dried ($MgSO_4$), filtered and evaporated. The crude product was purified by flash silica gel column chromatography (EtOAc) to afford the *O*-acylamidoxime **10** (571 mg, 73%) as a white foam. R_f = 0.75 (PE/EtOAc, 3:7). $[\alpha]_D$ = −48.6 (c = 1/CH_2Cl_2). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.23–4.33 (m, 3H, H-5′), 4.49–4.57 (m, 6H, H-1′ H-6′a), 4.58–4.65 (m, 3H, H-6′b), 5.46 (bs, 6H, NH_2), 5.70 (t, 3H, J = 9.6 Hz, H-2′), 5.76 (t, 3H, J = 9.6 Hz, H-4′), 5.98 (t, 3H, J = 9.6 Hz, H-3′), 7.22–7.58 (m, 36H, H-ar), 7.80–8.04 (m, 24H, H-ar), 8.52 (s, 3H, H-2 H-4 H-6). ^{13}C NMR (75 MHz, $CDCl_3$) δ 62.9 (C-6′), 69.0 (C-4′), 70.0 (C-2′), 73.5 (C-3′), 75.6 (C-1′), 76.8 (C-5′), 128.3, 128.4, 128.5, 128.7, 129.3, 129.7, 129.8, 129.90, 129.94, 130.2 (10s, 20C, CH-ar), 133.3, 133.4, 133.6, 134.1 (4s, 4C, C-ar), 154.5 (H_2NC═NO), 161.5 (NOC═O), 165.2, 165.58, 165.63, 166.1 (4s, 4C, *C*OPh). LSIMS (positive mode, thioglycerol) m/z: 2071.6 [M + H]$^+$. HR-ESI-MS (positive mode) m/z: calcd. for $C_{114}H_{91}N_6O_{33}$ [M + H]$^+$ 2071.5627, found 2071.5651.

3,5-Bis[3′-*C*-(β-D-glucopyranosyl)-1′,2′,4′-oxadiazol-5′-yl]-benzoic acid (12). A solution of tris-*O*-acylamidoxime **10** (532 mg, 256 µmol) in 1,4-dioxane (12 mL) was stirred at 100 °C for 4 days. The reaction was then cooled to room temperature and the solvent evaporated off. The crude product **11** was used without further purification. A solution of crude **11** (239 mg) and NaOMe (10 mg) in CH_2Cl_2/MeOH (5 mL, 1:1) was stirred at room temperature for 5 hours. The solvent was evaporated off and the crude mixture was purified by flash reverse-phase silica gel chromatography (H_2O then H_2O/MeOH 7:3) to afford the benzoic acid derivative **12** (95 mg, 58% over two steps) as a white foam. R_f = 0.20 (EtOAc/MeOH 1:1). $[\alpha]_D$ = +7.1 (c = 0.41/MeOH). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.62–3.76 (m, 3H, H-3″, H-4″, H-5″), 3.81–3.89 (m, 2H, H-2″, H-6″a), 3.99 (m, 1H, H-6″b), 4.75 (m, 1H, H-1″), 8.77 (s, 2H, H-ar), 8.87 (s, 1H, H-ar). ^{13}C NMR (75 MHz, $CDCl_3$) δ 61.2 (C-6″), 69.7, 77.1, 80.8 (3s, 3C, C-3″, C-4″, C-5″), 72.1 (C-2″), 73.4 (C-1″), 124.7 (s, 2C, C^{IV}-ar), 126.9 (C^{IV}-ar), 129.8 (CH-ar), 133.0 (s, 2C, CH-ar),

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139.5 (CO_2H), 168.4 (C-3′), 175.8 (C-5′). ESI-MS (negative mode) m/z: 581 $[M - H]^-$. HR-ESI-MS (negative mode) m/z: calcd for $C_{23}H_{25}N_4O_{14}$ $[M - H]^-$ 581.1367, found 581.1369.

1,3,5-Tris[3′-*C*-(2″,3″,4″,6″-tetra-*O*-benzoyl-β-D-glucopyranosyl)-1′,2′,4′-oxadiazol-5′-yl]benzene (13). In a CEM Discover 5 mL vial was introduced a solution of *O*-acylamidoxime **10** (454 mg, 0.22 mmol) and TBAF (70 µL, 70 µmol, 1 M in THF) in toluene (5 mL). The reaction vial was heated at 150 °C for 40 min upon microwave irradiation (200 W). The solvent was evaporated and the residue purified by flash silica gel column chromatography (PE then PE/EtOAc, 1:1) to afford the trivalent oxadiazole **13** (317 mg, 72%) as a white foam. R_f = 0.79 (PE/EtOAc, 1:1). $[\alpha]_D$ = −35.1 (c = 1.00/CH_2Cl_2). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.41 (ddd, 3H, J = 3.1 Hz, J = 5.1 Hz, J = 9.5 Hz, H-5″), 4.59 (dd, 3H, J = 5.2 Hz, J = 12.5 Hz, H-6″a), 4.71 (dd, 3H, J = 2.9 Hz, J = 12.5 Hz, H-6″b), 5.22 (d, 3H, J = 9.5 Hz, H-1″), 5.91 (t, 3H, J = 9.7 Hz, H-4″), 6.04–6.15 (m, 6H, H-2″ H-3″), 7.26–7.52 (m, 36H, H-ar), 7.82–8.02 (m, 24H, H-ar), 8.96 (s, 3H, H-2). ^{13}C NMR (75 MHz, $CDCl_3$) δ 63.3 (s, 3C, C-6″), 69.4 (s, 3C, C-4″), 70.8 (s, 3C, C-2″), 72.6 (s, 3C, C-1″), 74.1 (s, 3C, C-3″), 77.4 (s, 3C, C-5″), 126.2 (s, 3C, C-1 C-3 C-5), 128.5, 128.5, 128.6 (3s, 24C, CH-ar), 129.9, 130.0 (2s, 24C, CH-ar), 131.3 (s, 3C, C-2 C-4 C-6), 133.3, 133.4, 133.6, 133.6 (4s, 12C, CH-ar), 164.9, 165.2, 165.9, 166.3 (4s, 12C, *C*OPh), 167.6 (s, 3C, C-3′), 174.1 (s, 3C, C-5′). ESI-MS (positive mode) m/z: 2039.6 $[M + Na]^+$.

1,3,5-Tris[3′-*C*-β-D-glucopyranosyl)-1′,2′,4′-oxadiazol-5′-yl]-benzene (14). A solution of benzoylated tris-oxadiazole **13** (296 mg, 0.15 mmol) and NaOMe (15 mg, 0.3 mmol) in CH_2Cl_2/MeOH (8 mL, 3:5) was stirred at room temperature for 3 hours. The solution was neutralized with a cation exchange resin (Amberlite IR-120, H^+ form) and the resin washed with MeOH (3 × 5 mL). The filtrate was evaporated off and the residue was dissolved in MeOH then precipitated with CH_2Cl_2. The resulting solid was washed with CH_2Cl_2 (2 × 5 mL) and dried under vacuum to afford the hydroxylated tris-oxadiazole **14** (114 mg, 99%) as a white foam. R_f = 0.63 (CH_2Cl_2/MeOH, 9:1). $[\alpha]_D$ = +10.6 (c = 1.00/H_2O). ^{1}H NMR (300 MHz, D_2O) δ 3.61–3.84 (m, 15H, H-2″ H-3″ H-4″ H-5″ H-6″a), 3.97 (d, 3H, J = 11.9 Hz, H-6″b), 4.67 (d, 3H, J = 9.4 Hz, H-1″), 8.74 (s, 3H, H-2 H-4 H-6). ^{13}C NMR (75 MHz, D_2O) δ 59.0 (s, 3C, C-6″), 71.0 (s, 3C, C-1″), 67.4, 69.9, 74.9, 78.6 (4s, 12C, C-2″ C-3″ C-4″ C-5″), 123.5 (s, 3C, C-1 C-3 C-5), 129.2 (s, 3C, C-2 C-4 C-6), 166.5 (s, 3C, C-3′), 172.0 (s, 3C, C-5′). ESI-MS (negative mode) m/z: 812.9 $[M + HCO_2^-]^-$. ESI-MS (positive mode) m/z: 791.7 $[M + Na]^+$. HR-ESI-MS (positive mode) m/z: calcd. for $C_{30}H_{36}N_6NaO_{18}$ $[M + Na]^+$ 791.1984, found 791.1981.

Glycogen phosphorylase inhibition measurements

Glycogen phosphorylase *b* was prepared from rabbit skeletal muscle according to the method of Fischer and Krebs,[24] using dithiothreitol instead of L-cysteine, and recrystallized at least three times before use. Kinetic experiments were performed in the direction of glycogen synthesis as described previously.[25] Kinetic data for the inhibition of rabbit skeletal muscle glycogen phosphorylase were collected using different concentrations of α-D-glucose-1-phosphate (2–20 mM), constant concentrations of glycogen (1% w/v) and AMP (1 mM), and various concentrations of inhibitors. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and diluted in the assay buffer (50 mM triethanolamine, 1 mM EDTA and 1 mM dithiothreitol) so that the DMSO concentration in the assay should be lower than 5%. The enzymatic activities were presented in the form of double-reciprocal plots (Lineweaver–Burk) applying a nonlinear data analysis program. The inhibitor constants (K_i) were determined by Dixon plots, by replotting the slopes from the Lineweaver–Burk plots against the inhibitor concentrations.[26,27] The means of standard errors for all calculated kinetic parameters averaged to less than 10%. K_i values for compounds **9** and **14** were also estimated and found to be 490 ± 45 µM and 535 ± 50 µM, respectively. The poor solubility of inhibitors **5**, **7** and **15** limited the concentrations used in the kinetic studies. The inhibition of glycogen phosphorylase was therefore determined at 625 µM and 2.5 mM concentrations of these inhibitors and given in Table 2.

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