Carba-Sugar Analogs of Glucosamine-6-Phosphate: New Activators for the glmS Riboswitch


Abstract: Riboswitches are 5'-untranslated mRNA regions mostly found in bacteria. They are promising drug targets to overcome emerging bacterial resistance against commonly used antibiotics. The glmS riboswitch is unique among the family of riboswitches as it is a ribozyme that undergoes self-cleavage upon binding to glucosamine-6-phosphate (GlcN6P). Previously, we showed that carba glucosamine-6-phosphate (carba-GlcN6P) induces self-cleavage of the riboswitch with a potency similar to that of GlcN6P. Here, we report a synthetic approach to a new class of carba-GlcN6P derivatives with an alkoxyl substituent in the carba position. Key features of the synthesis are a ring closing metathesis followed by a hydroboration. The strategy gives access to libraries of carba-GlcN6P derivatives. Ribozyme cleavage assays unraveled new activators for the glmS riboswitch from Listeria monocytogenes and Clostridium difficile.

Introduction

Antibiotic-resistant bacteria are expected to cause around 10 million deaths yearly by 2050.[1] This dire prediction explains the urgent need for the development of new antibiotics. Ideally, these antibiotics use new modes of action unexploited by established drugs, thereby slowing down the development of multidrug-resistant pathogens.[2] Riboswitches are promising drug targets. These 5'-untranslated mRNA regions (5'-UTRs) are found mostly in bacteria where they play an important role in gene regulation.[3] Targeting of these regulatory mechanisms could yield a fatal imbalance in the bacterial ability to control the expression of the corresponding gene.[4]

One riboswitch that is especially interesting in this aspect is the glmS riboswitch discovered in 2004 by Winkler et al.[5] It is found in a large variety of Gram-positive bacteria as well as in a lower frequency in Gram-negative bacteria.[5] It has also been predicted and recently characterized in Clostridium difficile and Listeria monocytogenes.[7] The glmS riboswitch is located in 5'-UTR of the gene encoding for the enzyme glucosamine-6-phosphate-synthase (GlmS) that catalyzes the reaction of glutamine and fructose-6-phosphate (Fru6P) to yield glutamate and glucosamine-6-phosphate (GlcN6P).[3,8] GlcN6P can in return bind to the glmS riboswitch and catalyze its self-cleavage leading to the recruitment of RNase J1 and subsequent degradation of the downstream coding RNA.[9]

Artificial activation of the glmS riboswitch would impede the bacterial ability to produce GlcN6P which is needed for the biosynthesis of the bacterial cell wall. The design of artificial GlcN6P mimics is a possibility to inhibit bacterial growth by using a new and unexploited mode of action and, therefore, a promising contribution to the fight against antibiotic resistances.[4,6,10] Previous studies revealed the structural prerequisites of GlcN6P mimics to actively induce the self-cleavage reaction of the glmS riboswitch (Figure 1).[5,10a,b] It has been demonstrated that the cyclic structure, the equatorial amino group in the 2-position, as well as the equatorial hydroxy group in the 4-position are required. Removal of these functionalities or – in the case of the 4-hydroxy group – inversion of the

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Figure 1. Structural requirements for GlcN6P derivatives acting as activators of the glmS riboswitch. Red: The cyclic structure and the equatorial substituents in the 2- and 4-positions are essential. Green: Variation of the ring oxygen is tolerated. 1: Unsubstituted carba-GlcN6P with similar activity to GlcN6P. 2. 3. Substituted carba-GlcN6P derivatives (c.f. text).

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stereochemistry leads to a lack of activation. The axial hydroxy group in position 1 greatly improves the efficacy of the activator, and a preferential binding of the alpha anomer of GlcN6P to the riboswitch has been demonstrated. The 1-deoxy derivative of GlcN6P still activates the glmS riboswitch though with a 70-fold lower activity for the rate constant $k_{obs}$ of the self-cleavage reaction. The least critical position is the 3-position; here an inversion of the stereochemistry decreases $k_{obs}$ by a factor of 3.5. In addition, phosphorylation of the 6-OH group is required for efficient self-cleavage of the riboswitch. These experiments demonstrate that the design space for the exploration of new artificial activators of the glmS riboswitch is very limited.

Substitution of the ring oxygen with carbon is, however, tolerated. As we could show, carba-sugar 1 (carba-GlcN6P) has a similar potency to GlcN6P in modulating riboswitch activity. Furthermore, it was demonstrated that carba-GlcN treatment confers antibacterial activity. Carba-GlcN is taken up by the bacterial PTS and subsequently phosphorylated yielding carba-GlcN6P. In this study it was shown that gene regulation through the glmS riboswitch occurs in carba-GlcN-treated bacteria. An X-ray structure of the glmS riboswitch in complex with GlcN6P (PDB ID: 2Z75) suggests that an equatorially oriented substituent in the carba position might be tolerated or even fill a potential binding pocket and thereby improve the activity of the mimic. Carba-GlcN6P derivatives with substituents in the carba position, however, are not known except for derivative 2 with an axially oriented fluorine substituent. Here, we present synthetic access to the new class of substances 3 with equatorial substituents in the carba position. We introduce an equatorial hydroxy group in the carba position that at a late stage of the synthesis can be alkylated with various groups thereby giving access to compound libraries for the study of structure-activity relationships. Three compounds were tested in vitro for their activity to initiate the self-cleavage reaction of the newly characterized glmS riboswitches of C. difficile and L. monocytogenes. Among these compounds, the variant with a methoxy substituent at the carba position turned out to have the most promising properties.

The classical synthetic approach towards carba-glucosamine uses the Ferrier rearrangement as a key step for the construction of the carbocycle followed by introduction of C-6 by olefination chemistry (Scheme 1, first line). This synthetic route was also used by us and others for the preparation of carba-GlcN(6P) and could be modified to introduce a fluorine substituent in the 5a position (Scheme 1, second line). Initially, we tried to extend this strategy to carba-sugars with an hydroxy or alkoxy substituent in the 5a position. These attempts, however, suffered from multiple drawbacks, including low yields. Furthermore, the desired substituent had to be introduced at an early stage of the synthesis rendering the preparation of a variety of compounds inefficient. In the synthesis of valienamine starting from glucose, the Kim group and later also the Jung group demonstrated the power of ring closing metathesis (RCM) for the construction of a tetrabenzylated cyclohexenol derivative (Scheme 1, third line) sharing some structural features with the anticipated carba-GlcN6P derivative 3. Although it was questionable.

Scheme 1. Synthetic routes to carba-sugars.
whether this route could be transferred to aminosugars, we envisioned the application of RCM as a suitable approach to the unsaturated core of carba-glucosamine. The double bond would then be accessible for late-stage introduction of alkoxy substituents in the 5α-position by hydroboration and subsequent alkylation (Scheme 1 fourth line). In the following, we describe the successful realization of this approach.

Results and Discussion

Synthesis

Retrosynthetically, the carba-GlcN6P derivatives 31, 32, and 33 presented in this work were obtained through phosphorylation, protecting group manipulation, and derivatization of the common key intermediate 17 (Scheme 2). Key intermediate 17 was accessible by hydroboration of the unsaturated carba-sugar derivative 15 that served as a suitable substrate for an anti-Markovnikov introduction of a hydroxy group.[19] Cyclohexene 15 was obtained by RCM of acyclic diene 12 which was synthesized from 1,5-diol 7. Diol 7 was envisioned to be accessible from commercially available N-acetylglucosamine (GlcNAc) in several steps including a reduction of the hemiacetal functionality.

We started our synthesis with the preparation of pentabenzylated D-glucosamine (GlcN) 6 (Scheme 3). Initially, we intended to obtain 6 by the cleavage of the acetal function of perbenzylated GlcN 8, which was obtained from GlcN·HCl according to Ye and co-workers[16a] in one step. However, it turned out that acetal cleavage of 8 required harsh conditions that resulted in complete decomposition of the compound. The hindered acetal cleavage is readily explained by the presence of the positively charged ammonium group in the 2-position under the acidic conditions that prevents further protonation of the acetal. A similar observation has been made by Yamamoto et al. during the cleavage of the methyl glycoside of GlcN.[20]

Therefore, a new route to lactol 6 was established. GlcNAc was converted to the allyl glycoside by Fischer glycosidation and subsequently the acetamide was cleaved with Ba(OH)2 to give allyl glycoside 4 as reported.[21] Crude product 4 was perbenzyliated with NaH and BnBr to provide compound 5 in 78% yield over three steps. The allyl group of 5 was removed in two steps by IrI-catalyzed rearrangement to the vinyl glycoside[22] followed by hydrolysis under mildly acidic conditions to give the desired pentabenzylated GlcN 6 in a yield of 73%. Compared to the literature-known route to 6 in 12 steps from GlcN·HCl,[23] our access represents a significant improvement. Reduction of lactol 6 with LiAlH4 gave the corresponding 1,5-diol 7 in 95%.

For the conversion of diol 7 into diene 12, the primary alcohol of 7 was selectively protected with TBDPSCI to give silyl ether 9 in 88% yield (Scheme 4). Swern oxidation of the secondary alcohol of 9 to the corresponding ketone and subsequent Wittig reaction yielded olefin 10 in 80% yield over 2 steps. A following treatment of 10 with TBAF recovered the primary alcohol 11 in a yield of 83%. The primary alcohol 11 was oxidized under Swern conditions to the aldehyde followed by addition of vinylMgBr to give diene 12 as a mixture of
diastereomers in a ratio of 1.2:1 according to HPLC analysis in a combined yield of 87% yield over two steps. The subsequent closing metathesis (RCM) with second-generation Grubbs catalyst[24] in toluene led to the unsaturated carba-sugars 13 and 14 in a combined yield of 82% preserving the ratio of diastereomers (13/14 = 1.2:1). It is known that amines are challenging substrates for the RCM due to their ability to coordinate to metal-alkylidene complexes leading to decomposition of the catalyst.[25] Several attempts have been made to decelerate or even overcome the decomposition by decreasing the nucleophilicity of the amines through addition of organic acids or conversion of the amines to carbamates or amides, etc.[26] However, for the reaction of diene 12, addition of camphor sulfonic acid did not improve yields. In our case, best results were obtained by portion-wise addition of the catalyst at equal time intervals over 2.5 h in toluene.[27] As the stereochemistry at C1 of key intermediate 17 corresponds to that of the minor RCM product 14, we developed an efficient way to convert 13 into 14 by Ley Griffith oxidation followed by a Luche reduction in 87% over 2 steps.

To convert cyclohexene 14 into key intermediate 17, 14 was benzylated to produce 15 in almost quantitative yield (Scheme 5A). Subsequently, a hydroboration was performed to introduce the hydroxy group at the carba-position, yielding the diastereomeric cyclohexane derivatives 16 and 17 in a yield of 64%. As expected, 16 was the major product of this reaction resulting from a syn addition of the borane to the double bond from the opposite side of the two benzylxoy substituents in the allylic positions. The stereochemistry at C5 and C-5a, however, was easily inverted in a three-step sequence consisting of Ley Griffith oxidation to 18, selective triethylamine-induced isomerization of the 5-position, and CBS reduction of ketone 19 to give the desired product 17 after a single purification at the end of the sequence in a yield of 74% beside minor amounts of the 5a epimer with axial OH group (ratio of diastereoisomers 50:1). With this procedure, key intermediate 17 was readily available for further derivatization and phosphorylation.

To yield new potential activators of the glmS riboswitch, key intermediate 17 was treated with NaH and MeI to obtain methyl ether 20 in a yield of 66% (Scheme 6). With allyl bromide instead, allyl ether 21 was obtained in a yield of 84%. 20, 21, and alcohol 17 were hydrogenated to remove the benzyl protecting groups and, in case of 21, convert the allyl group into a propyl group in excellent yields. To achieve selective phosphorylation of the 6-hydroxy group, we first reacted the TFA salts of 22, 23, and 24 with Cbz-Cl leading to the carbamates 25, 26, and 27. Previously, the regioselective phosphorylation of primary hydroxy groups of nucleosides with phosphoryl chloride in the presence of water and pyridine has been reported.[28] We could show, however, that the application

Scheme 4. Synthesis of cyclohexene 14. DMAP = 4-dimethylaminopyridine; NMO = N-methylmorpholine N-oxide; TBAF = tetrabutylammonium fluoride; TPAP = tetrapropylammonium peruthenate.

Scheme 5. Synthesis of key intermediate 17. BMS = borane dimethylsulfide complex.
of this elegant method to hexoses with unprotected OH groups in the 4- and 6-position is not possible due to the formation of cyclic phosphates.\(^{[11]}\) Therefore, we investigated alternative methods for regioselective phosphorylation. Whereas the application of the phosphoramidite \((\text{BnO})_2\text{PN(Pr)}_2\) and subsequent oxidation by meta-chloroperoxybenzoic acid\(^{[29]}\) resulted in only low yields, the phosphorus(V) species CIPO(OPh)_2 in pyridine\(^{[30]}\) successfully delivered the diphenyl phosphoric acid esters 28, 29, and 30. The attachment of the phosphate group to the 6-position was confirmed by a significant downfield shift of the resonances of the protons in the 6-position and by observation of indicative \(J_{\text{H},P}\) and \(J_{\text{P},\text{H}}\) coupling constants in a \(1^\text{H},3^1\text{P}\) HMBC spectrum (see the Supporting Information). The final deprotection was carried out by firstly removing the Cbz group by catalytic hydrogenation with Pd-charcoal followed by final deprotection was carried out by firstly removing the Cbz group by catalytic hydrogenation with Pd-charcoal followed by subsequent oxidation of indicative 3,31 of the resonances of the protons in the 6-position and by observation of indicative 3. The attachment of the phosphate group to the 6-position was confirmed by a significant downfield shift of the resonances of the protons in the 6-position and by observation of indicative \(J_{\text{H},P}\) and \(J_{\text{P},\text{H}}\) coupling constants in a \(1^\text{H},3^1\text{P}\) HMBC spectrum (see the Supporting Information). The final deprotection was carried out by firstly removing the Cbz group by catalytic hydrogenation with Pd-charcoal followed by final deprotection was carried out by firstly removing the Cbz group by catalytic hydrogenation with Pd-charcoal followed by subsequent oxidation of indicative 3,31 of the resonances of the protons in the 6-position and by observation of indicative 3.

**Biological activity**

We next investigated the glmS ribozyme-activating properties of 31, 32, and 33 and of the corresponding non-phosphorylated carba-GlcN6P derivatives 22, 23, and 24. We employed the glmS ribozymes from \(L.\ monocytogenes\) and \(C.\ difficile\)\(^{[22]}\) and the previously described metabolite-induced cleavage assay.\(^{[11]}\) The potential of the six compounds to induce ribozyme cleavage was tested at a concentration of 0.2 mM. These experiments revealed that hydroxy compound 31 and methoxy compound 32 are able to activate the glmS ribozyme from \(L.\ monocytogenes\), whereas propoxy compound 33 is not (Figure 2a). As expected, the non-phosphorylated derivatives 22, 23, and 24 did not activate the glmS ribozyme (Figure 2b). Activation of the glmS ribozyme from \(C.\ difficile\) by 31 and 32 was found to be less pronounced, whereas 33, and 22–24 did not activate ribozyme self-cleavage (Figures 2c,d). Next, we determined concentration-dependent activation of both glmS ribozymes by 31 and 32 (Figure 3). These experiments revealed an EC_{50} value of 0.080 mM for 32 for activation of the glmS ribozyme from \(L.\ monocytogenes\) whereas the value for the glmS ribozyme from \(C.\ difficile\) and those observed with 31 were significantly higher. Based on these results, we determined the time-dependent cleavage of the glmS ribozyme from \(L.\ monocytogenes\) induced by the most potent compound (32) and determined a rate constant \(k_{\text{obs}}\) of 0.041 (± 0.01) min\(^{-1}\) at a concentration of 32 of 0.2 mM (Figure 4).

**Conclusion**

In conclusion, we have developed a synthetic access to a new class of carba-sugars with an alkoxy substituent in the carba position. Such compounds were not accessible by the previously published routes to carba-glucosamine derivatives. Ring closing metathesis followed by hydroboration of the double bond formed at a late stage of the synthesis gave access to key intermediate 17. This strategy allows access to a variety of carba-GlcN6P derivatives with a substituent in the carba position, we demonstrated this by the synthesis of, for example, hydroxy compound 31, methoxy compound 32, and propoxy compound 33. Ribozyme cleavage assays revealed that carba-GlcN6P derivatives with substituents in the carba position are able to activate the glmS ribozyme from \(L.\ monocytogenes\) and \(C.\ difficile\). Methoxy-substituted carba-sugar 32 turned out to be the most active compound within this series. The observation that the larger methoxy substituent of 32 confers higher activity of the carba-sugar than the smaller hydroxy group of 31 indicates that space is not a limiting factor for riboswitch activation. Although the propoxy substituent of 33 diminishes activity, it is quite possible that other substituents are beneficial for riboswitch activation possibly through intercalation between RNA nucleobases. The newly developed synthetic route opens the way for broad investigation in this direction, which we are currently undertaking.

**Experimental Section**

**General methods:** Anhydrous reactions were carried out under nitrogen or argon by using the Schlenk technique. Commercially available chemicals were used without further purification. Technical solvents were distilled prior to use. Thin-layer chromatography (TLC) was performed using silica-coated aluminum sheets (TLC Silica gel 60 F254) from Merck. Detection was carried out by excitation of the fluorescence at 254 nm or by dipping in one of

Figure 3. Determination of EC$_{50}$ values for glmS riboswitch cleavage. Cleavage of the glmS riboswitch from *L. monocytogenes* induced by a) 32 and b) 31. Cleavage of the glmS riboswitch from *C. difficile* induced by c) 32 and d) 31. N = 1 in duplicate.
the following staining solutions and subsequent gentle heating. Anisaldehyde reagent: ethanol (135 mL), conc. H2SO4 (5 mL), 4-anisaldehyde (3.7 mL), glacial acetic acid (1.5 mL); vanillin reagent: ethanol (250 mL), conc. H2SO4 (2.5 mL), vanillin (6 g); potassium anisaldehyde (3.7 mL), glacial acetic acid (1.5 mL); vanillin reagent: (3 equiv.) and benzyl chloroformate (1.7 equiv.) are added at room temperature. In the following resolution steps, the 1H,13C HMBC, 1H,31P HMBC & NOESY). As an analysis program, NMR spectra were recorded on an Avance III 400 or Avance III 600 spectrometer from Bruker. Measurements were performed at 4 °C. The resulting suspension is stirred for 3 h at 70 °C. TLC analysis (CHCl3/MeOH 8:2) showed complete conversion of the starting material. Solid NaHCO3 was added to neutralize the solution to pH 7, and the suspension was filtered through celite and washed several times with MeOH. The solvent was removed under reduced pressure. The residue was used in the next step without further purification. To a stirred solution of the crude product in H2O (350 mL) was added Ba(OH)2·8H2O (150 g, 0.48 mol). The solution was heated to reflux and stirred for 16 h. After cooling to room temperature, the precipitate was filtered off and the filtrate was carefully treated with dry ice to remove the barium salts. The solvent was removed by freeze drying. Crude product 4 was obtained as a colorless powder and was used in the next step without further purification. To a suspension of the crude product 4 in anhydrous DMF (340 mL) was added NaNH (18.0 g, 60 % dispersion in mineral oil, 0.45 mmol) at 0 °C. The resulting suspension was stirred for 30 min at 0 °C. Then, benzyl bromide (38.7 mL, 0.33 mol) was added dropwise over 30 min at 0 °C. The solution was allowed to warm to room temperature and stirred for 1 h. The mixture was cooled to 0 °C, another portion of NaNH (18.0 g, 60 % dispersion in mineral oil, 0.45 mmol) was added, and the suspension stirred for 30 min. at 0 °C. Benzyl bromide (38.7 mL, 0.33 mol) was added dropwise over 30 min at 0 °C. The solution was allowed to warm to room temperature and stirred for 1 h. The mixture was reduced under reduced pressure, and the crude product is purified as given in the individual procedures.

**General procedure C: phosphorylation.** To a stirred solution of the Cbz-protected GlcN derivative in pyridine (10.0 mL mmol⁻¹), CIPPOH(O)H (1.1 equiv.) is added at 40 °C in one portion upon the color of the solution turns from colorless to yellowish. The reaction mixture is stirred at room temperature overnight and quenched with water (5.0 mL/1.0 mmol). The solvent is removed under reduced pressure and toluene is evaporated from the residue. The crude product is purified as given in the individual procedures.

**General procedure D: hydrogenation.** The diphenyl phosphoric acid ester is dissolved in MeOH (8.0 mL mmol⁻¹) and 10 % Pd C catalyst (water wet, 10 % w/w of phosphoric acid ester) is added. The resulting suspension is stirred under hydrogen atmosphere (1 atm.) for 1 h. The catalyst is removed by centrifugation, and the supernatant is concentrated under reduced pressure. The residue is dissolved in MeOH (8.0 mL mmol⁻¹), 1 M HClaq (1 equiv.) is added, and the reaction mixture is stirred for 2 h. Then, PT02 (10 % w/w of phosphoric acid ester) is added and the suspension stirred under hydrogen atmosphere (1 atm) for 2 d. The catalyst is removed by filtration and the solvent is removed under reduced pressure.

** Allyl 3,4,6-tri-O-benzyl-2-( dibenzyllmino)-2-deoxy-D-glucopyranoside (5):** Allyl glycoside 4 was synthesized from GlcNAc by a combination of two known procedures[29] for Fischer glycosidation and subsequent cleavage of the acetamide with slight modifications as follows. AcCl (15 mL, 77 mmol) was added dropwise to allylic alcohol (250 mL) under argon at 0 °C over a period of 5 min. At room temperature, GlcNAC (30 g, 135.6 mmol) was added, and the reaction mixture was stirred for 3 h. TLC analysis (CHCl3/MeOH 8:2) showed complete conversion of the starting material. Solid NaHCO3 was added to neutralize the solution to pH 7, and the suspension was filtered through celite and washed several times with MeOH. The solvent was removed under reduced pressure. The residue was used in the next step without further purification. To a stirred solution of the crude product in H2O (350 mL) was added Ba(OH)2·8H2O (150 g, 0.48 mol). The solution was heated to reflux and stirred for 16 h. After cooling to room temperature, the precipitate was filtered off and the filtrate was carefully treated with dry ice to remove the barium salts. The solvent was removed by freeze drying. Crude product 4 was obtained as a colorless powder and was used in the next step without further purification. To a suspension of the crude product 4 in anhydrous DMF (340 mL) was added NaNH (18.0 g, 60 % dispersion in mineral oil, 0.45 mmol) at 0 °C. The resulting suspension was stirred for 30 min at 0 °C. Then, benzyl bromide (38.7 mL, 0.33 mol) was added dropwise over 30 min at 0 °C. The solution was allowed to warm to room temperature and stirred for 1 h. The mixture was cooled to 0 °C, another portion of NaNH (18.0 g, 60 % dispersion in mineral oil, 0.45 mmol) was added, and the suspension stirred for 30 min. at 0 °C. Benzyl bromide (38.7 mL, 0.33 mol) was added dropwise over 30 min at 0 °C. The solution was allowed to warm to room temperature and stirred for 1 h. The mixture was reduced under reduced pressure, and the crude product is purified as given in the individual procedures.

**General procedure A: hydrogenation.** The benzylated carba-sugar derivative is dissolved in MeOH (6.7 mL mmol⁻¹) and 10 % Pd C catalyst (water wet, 35 % w/w of carba-sugar) is added. After addition of TFA (10 equiv.) the reaction is placed in a laboratory autoclave and stirred under 15 bar hydrogen pressure until HPLC monitoring shows complete consumption of the starting material. Then, the catalyst is removed by filtration over a plug of celite followed by filtration through a regenerated cellulose syringe filter. The solvent is removed under reduced pressure to give the target compound as a colorless TFA salt.

**General procedure B: Cbz protection of C-2 amine.** To a stirred solution of the GlcN derivative in water (6.7 mL mmol⁻¹), NaHCO3 (3 equiv.) and benzyl chloroformate (1.7 equiv.) are added at room temperature. The resulting solution is stirred overnight. The solvent is reduced under reduced pressure, and the crude product is purified as given in the individual procedures.

**Figure 4.** Time-dependent cleavage of the glmS riboswitch from L. monocytogenes induced by 32 at a concentration of 0.2 mM (blue). In red: cleavage of the riboswitch after 90 min in the absence of a metabolite. N = 1 in duplicate.
β-isomer: $R$ 0.34 (petroleum ether/ EthOAc 1:1); $^1$H NMR (400 MHz, CDCl$_3$): δ 7.43–7.11 (m, 25H, arenes), 6.09 (m, 1H, CH$_2$CH), 5.43 (dd, J 12.2, 17.2 Hz, 1H, CH$_2$CH$_2$), 5.33 (dd, J 10.4, 1.4 Hz, 1H, CH$_2$CH), 5.04 (d, J 11.2 Hz, O-CH$_2$Ph), 4.89 (d, J 1.1 Hz, O-CH$_2$Ph), 4.75 (m, J 10.8 Hz, 1H, O-CH$_2$Ph), 4.64 (d, J 7.7 Hz, 1H, H-1), 4.60–4.44 (m, 4H, O-CH$_2$Ph), O-CH$_2$Ph, CH$_2$CH$(')$CH$_2$), 4.10 (m, 1H, CH$_2$CH$_2$CH$_2$), 4.03 (d, J 13.8 Hz, 2H, N-CH$_2$Ph), 3.81 (dd, J 9.9 Hz, 8.4 Hz, 1H, H-3), 3.73 (d, J 10.7 Hz, 2.2 Hz, 1H, H-6), 3.66 (dd, J 10.7, 5.1 Hz, 1H, H-6), 3.58 (s, J 9.8 Hz, 8.4 Hz, 1H, H-4), 3.45 (d, J 9.8 Hz, 5.3 Hz, 2.2 Hz, 1H, H-5), 3.03 (ppm (d, J 9.9 Hz, 7.9 Hz, 1H, H-2)), $^1$C NMR (CDCl$_3$, 101 MHz): δ 139.9, 139.1, 138.2, 138.1 (5×C$_{quart.}$ arenes), 134.3 (CH$_2$CH), 128.9, 128.4, 128.3, 128.1, 127.89, 127.76, 127.6, 127.3, 126.8 (arenes), 117.2 (CH$_2$), 101.2 (C-1), 81.3 (C-3), 74.9 (C-5), 74.8, 74.3, 73.5 (3×O-CH$_2$Ph), 69.7, 69.3 (6-C, CH$_2$CH$_2$), 63.5 (C-2), 55.1 ppm (2×N-CH$_2$Ph); HRMS (ESI) m/z calc for C$_{32}$H$_{26}$NO$_7$: 670.3527 [M + H]$^+$ found: 670.3514.

3,4,6-Tri-O-benzyl-2-(dibenzylamino)-2-deoxy-p-glucopyranose (6): Method A. [Ir(Me$_2$Ph)$_2$(CH$_2$)$_2$I]PF$_6$ (44 mg, 0.05 mmol) was suspended in anhydrous THF (2 mL) under argon. The suspension was evacuated, diluted with H$_2$ and stirred for 10 min at room temperature. After evacuation of excess H$_2$, compound 5 (1.2 g, 1.75 mmol) dissolved in anhydrous THF (2.5 mL) was added dropwise over 15 min under argon atmosphere. The solution was stirred for further 10 min. TLC analysis (petroleum ether/EtOAc 1:1) showed complete conversion of the starting material. The solvent was removed under reduced pressure and the residue was purified by FC (petroleum ether/EtOAc 1:1) using a short column. The purified residue was dissolved in acetone/H$_2$O (1 N, 22 mL) and the solution was stirred for 1 h at 75°C. TLC analysis (petroleum ether/EtOAc 3:1) showed complete conversion of the starting material into a slower moving compound. The solution was quenched with H$_2$O (10 mL) and diluted with EtOAc (20 mL). The aqueous layer was extracted once more with EtOAc (20 mL) and the combined organic layers were washed with brine (5 mL), dried over MgSO$_4$ and concentrated under reduced pressure. The residue was purified by FC (petroleum ether/EtOAc 1:1 to 3:1) to give 6 (800 mg, 1.30 mmol, 73%) as a colorless syrup as a mixture of anomers (α/β 9:1).

Method B. Compound 5 (40.0 g, 59.7 mmol) was dissolved in degassed dry MeOH (320 mL), 1,3-Dimethylbarbituric acid (23.3 g, 149.3 mmol) was added. Pd(PPh$_3$)$_3$ (3.5 g, 3 mmol) was added and the mixture heated to reflux overnight. The solvent was removed under reduced pressure and the residue dissolved in EtOAc (50 mL) and washed with saturated NaHCO$_3$ (3×150 mL) and 100% (100 mL). The organic phase was concentrated under reduced pressure and purified by FC (petroleum ether/EtOAc 10:1—3:1) to give 6 (26.5 g, 42.1 mmol, 70%) as a colorless syrup as a mixture of anomers (α/β 9:1). $R$ 0.6 (petroleum ether/EtOAc 3:1), vanillin; $^1$H NMR (CDCl$_3$, 400 MHz): δ 7.51–7.14 (m, 25H, arenes), 5.25 (m, 0.34H, H-1), 5.07 (m, 2H, O-CH$_2$Ph), 4.79 (m, 1H, O-CH$_2$Ph), 4.71 (d, d J 7.9 Hz, 0.65H, H-1), 4.57 (m, 3H, O-CH$_2$Ph, O-CH$_2$Ph), 4.31 (dd, J 10.8 Hz, 8.6 Hz, 0.34H, H-3), 4.11 (m, 1H, O-CH$_2$Ph, H-5), 3.97 (dd, J 10.3 Hz, 8.5 Hz, 0.66H, H-3), 3.89 (m, 2.69H, N-Mn), 3.73 (m, J 2.35Hz, 4.24 Hz, H-16), 3.56 (br, s, 1H, OCH$_2$Ph), 3.48 (m, 1H, H-5), 3.02 (m, 1H, H-2), 2.87 (br, s, 1H, OCH$_2$Ph), 2.75 ppm (dd, J 10.3 Hz, 7.9 Hz, 1H, H-2); $^1$C NMR (CDCl$_3$, 101 MHz): δ 140.9, 139.4, 139.5, 138.7, 138.1, 138.0, 137.9, 137.7 (10×C$_{quart.}$ arenes), 129.2, 128.7, 128.5, 128.54, 128.49, 128.45, 128.42, 128.17, 128.76, 128.70, 127.75, 127.72, 127.70, 127.5, 127.4, 127.3, 126.8 (arenes), 95.5 (C-1), 95.1 (C-1), 81.3 (C-3), 80.9 (C-4), 80.3 (C-6), 79.6 (C-4), 74.9 (C-5), 74.80, 74.75, 74.2, 73.7, 73.6, 73.5 (6×O-CH$_2$Ph), 70.3 (C-5), 69.0, 68.7 (2×C-6), 64.3 (C-2), 60.8 (C-2), 55.9, 55.3 ppm (4×O-CH$_2$Ph); HRMS (ESI) m/z calc for C$_{40}$H$_{35}$NO$_{13}$: 630.3214 [M + H]$^+$ found: 630.3198.

(2R,3R,4S,5R,1,3,4-Tris(benzoxyl)-6-(tert-butyldiphenylsilyloxy)-5-dibenzylamino)hexan-2-ol (9): To a stirred solution of 7 (26.6 g, 42.1 mmol) in anhydrous EtOAc (200 mL), tert-butyldiphenylsilyl chloride (12.7 mL, 48.4 mmol), triethylamine (7.6 mL, 54.7 mmol) and N,N-dimethylaminopyridine (205 mg, 1.7 mmol) were added at room temperature under nitrogen. After stirring for 20 h, TLC analysis (petroleum ether/EtOAc 1:1) showed complete conversion of the starting material. The reaction was stopped with H$_2$O (100 mL) and the aqueous layer was extracted with CH$_2$Cl$_2$ (3×150 mL). The combined organic layers were washed with H$_2$O and brine, dried over MgSO$_4$ and concentrated under reduced pressure. The residue was purified by FC (petroleum ether/EtOAc 2:1) to give 8 (32.4 g, 37.2 mmol, 88%) as a colorless syrup, R 0.29 (petroleum ether/EtOAc 8:1), vanillin; $^1$H NMR (CDCl$_3$, 400 MHz): δ 7.71 (m, 4H, arenes), 7.53–7.19 (m, 31H, arenes), 4.81 (dd, J 11.6 Hz, J 2.3 Hz, O-CH$_2$Ph), 4.68 (s, 2H, O-CH$_2$Ph), 4.56 (dd, J 11.6 Hz, J 2.3 Hz, O-CH$_2$Ph), 4.56 (dd, J 11.6 Hz, J 2.3 Hz, O-CH$_2$Ph).
O-CH_{2}Ph), 3.97 (m, 7H, N-CH_{2}Ph, H-1a/b, H-6a/b, H-3), 3.54 (d, 2H, CH_{2}Ph), 3.15 (m, 1H, H-2), 1.11 ppm (s, 9H, 3×CH_{3}); 13C NMR (CDCl_{3}, 101 MHz): δ 140.2, 139.0, 138.8, 138.4 (5×C quar.), C-127.3, 127.0, 126.6 (arenes), 116.0 (C CH_{2}), 82.0 (C-4), 80.5 (C-3), 74.4, 73.0 (3×O-CH_{2}Ph), 70.9 (C_{7}H_{3}), 70.3 (C_{9}H_{5}), 59.2, 59.2, 55.8 (3×CH_{2}), 27.1 (3×CH_{3}), 19.1 ppm (C quar., SiPh); HRMS (ESI) m/z calc 2,494.0548 [M+H]^{+}; found: 2,494.0548; HPLC: t_{R} 8.90 min (Europerso 100 C_{18}, 16 mm, 9.0 mL/min), 80–100% MeCN in H_{2}O in 20 min).

(25,S,3R,4R)-N,N'-Dibenzy-l-3,4-bis(benzoxyl)-5-(benzoxyl) methyl-1-[(tert-butylidiphenylsilyloxy)hex-5-en-2-amine (10): To a stirred solution of oxalyl chloride (3.1 g, 47.8 mmol) in CH_{2}Cl_{2} (200 mL) was added dimethyl sulfoxide (10.8 mL, 151.7 mmol) at room temperature; afterwards the reaction was stopped by the addition of saturated NH_{4}Cl (31.9 mmol) carefully at 0 °C. The cooling bath was removed, and the reaction mixture was allowed to warm up to room temperature and stirred for 30 min. The reaction was stopped by addition of H_{2}O (200 mL) and the aqueous layer was extracted with CH_{2}Cl_{2} (3×200 mL). The organic layer was washed with brine (100 mL), dried over MgSO_{4}, and concentrated under reduced pressure. The residue was purified by FC (petroleum ether/ EtOAc 7:1) to give 10 (43.9 mg, 50.6 mmol, 80%) as a colorless oil. R_{f} 0.34 (petroleum ether/EtOAc 8:1), vanillin; ^{1}H NMR (CDCl_{3}, 400 MHz): δ 7.68 (m, 4H, arenes), 7.47-7.12 (m, 31H, arenes), 5.22 (m, 1H, C CH_{2}), 4.87 (d, J 1.8 Hz, 1H, C CH_{2}), 4.71 (d, 1H, J 11.6 Hz, O-CH_{2}Ph), 4.55 (d, 1H, J 11.9 Hz, O-CH_{2}Ph), 4.43 (m, 4H, O-CH_{2}Ph, O-CH_{2}Ph, H-4), 4.27 (d, 1H, J 11.9 Hz, O-CH_{2}Ph), 3.97 (m, 7H, N-CH_{2}Ph, N-CH_{2}Ph, H-8a, H-8b), 3.54 (d, 2H, J 13.6 Hz, N-CH_{2}Ph), 2.65 (15H, H-2), 1.11 ppm (s, 9H, 3×CH_{3}); ^{13}C NMR (CDCl_{3}, 101 MHz): δ 142.2, 140.5, 139.2, 138.5, 138.4 (5×C quar., C quar.), 135.8, 135.7 (arenes, SiPh), 133.53, 133.50 (2×C quar., SiPh), 129.74, 129.67, 129.4, 129.3, 128.43, 128.40, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.55, 127.49, 127.47, 127.3, 127.0, 126.6 (arenes), 116.0 (C CH_{2}), 82.0 (C-4), 80.5 (C-3), 74.5, 72.4, 70.6 (3×O-CH_{2}Ph), 69.5 (C-6), 60.4 (C-4), 60.2 (C-2), 55.8 (2×N-CH_{2}Ph), 27.1 (3×CH_{2}), 19.2 ppm (C quar., SiPh); HRMS (ESI) m/z calc for C_{42}H_{45}NO_{4}Si_{5} 864.4599 [M+H]^{+}; found: 864.4581; HPLC: t_{R} 9.20 min (Europerso 100 C_{18}, 16 mm, 9.0 mL/min), 80–100% MeCN in H_{2}O in 20 min).

Research Article
under reduced pressure, and the crude enone was dissolved in anhydrous DCM (100 mL). TPAP (370 mg, 1.1 mmol) was added to the reaction mixture. To a stirred solution of TPAP was added a stream of N₂. Afterwards, another portion of the reaction mixture was cooled to room temperature, and the solvent was removed under reduced pressure. The obtained charcoal black residue was purified by column chromatography (petroleum ether/EtOAc 10:1) to give a brown oil as a separable mixture of diastereomers. 13 R: 0.35 (1 isomer, petroleum ether/EtOAc 6:1), vanillin; 1H NMR (CDCl₃, 400 MHz): δ 7.47–7.25 (m, 2H, arenes), 5.87 (m, 1H, H-5a), 5.18 (d, 1H, 11.5 Hz, H-2); 13C NMR (CDCl₃, 101 MHz): 139.8 (2×C quard.), 138.8, 138.3, 138.1 (2×C quard. O-CH₂Ph), 135.0 (C quard. C-5), 129.3, 129.2, 128.5, 128.4, 128.7, 127.6, 127.3, 126.7 (arenes), 83.0 (C-4), 81.3 (C-8), 73.8, 72.3 (3×O-CH₂Ph), 70.3 (C-6), 66.7 (C-1), 65.4–55.1 ppm (2×N-CH₂Ph); HRMS (ESI) calcd for C₄₂H₄₃NO₄: 626.3265 [M + H]⁺; found: 626.3251. 

Conversion of 13 to 14: Compound 13 (6.5 g, 10.4 mmol) was dissolved in anhydrous DCN (100 mL). TPAP (370 mg, 1.1 mmol) and NMO (2.4 g, 20.8 mmol) were added, and the solution was stirred for 1 h at room temperature. The reaction mixture was filtered over a short plug of silica which was washed with petroleum ether/EtOAc 1:1 (100 mL). The solvent was removed under reduced pressure, and the crude enone was dissolved in a 1:1 mixture of anhydrous THF and anhydrous MeOH (200 mL). The reaction mixture was cooled to 20°C, and cerium chloride heptahydrate (4.7 g, 12.5 mmol) was added. The solution was stirred for 30 min after which NaBH₄ (500 mg, 15.6 mmol) was slowly added. The reaction mixture was stirred for 1 h, and the reaction stopped by the addition of saturated NH₄Cl (75 mL). The mixture was extracted with EtOAc (3×75 mL), and the combined organic phases were washed with brine (75 mL), dried over MgSO₄, and concentrated under reduced pressure. The crude product was purified by FC (petroleum ether/EtOAc 10:1–6:1) to afford 14 (5.7 g, 9.1 mmol, 87%).
Chemistry—A European Journal

Research Article
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Conversion of 16 to 17: Compound 16 (200 mg, 0.27 mmol) was dissolved in anhydrous DCM (5 mL). TRAP (10 mg, 27 μL) and NMO (634 mg, 0.55 mmol) were added, and the solution was stirred for 1 h at room temperature. The reaction mixture was filtered over a short plug of silica which was washed with petroleum ether/EtOAc (10:1) to yield a colorless oil. Conversion of 16 to 17 was confirmed by HPLC (petroleum ether/EtOAc 11:1) and characterization.

19: 1H NMR (500 MHz, CDCl3): δ 7.46–7.07 (m, 30H, arenes), 4.92 (d, 1H, O-CH''H'''Ph), 4.50 (d, 1H, O-CH''H'''Ph), 4.25 (m, 4H, 2×N-CH2-Ph), 3.93 (m, 2H, H-6a, H-6b), 3.82 (dd, J 11.0 Hz, 8.7 Hz, 1H, H-4), 3.54 (s, 3H, CH3), 3.28 (dd, J 11.3, 19 Hz, H-5a), 2.87 (dd, J 11.0, 1.9 Hz, H-2), 2.46 ppm (m, 1H, H-5); 13C NMR (151 MHz, CDCl3): δ 140.9, 139.8, 139.0, 138.9, 138.6 (Cquat. arenes), 128.53, 128.51, 128.44, 128.42, 128.40, 128.3, 128.13, 128.03, 127.95, 127.8, 127.7, 127.5, 127.1, 126.7 (arenes), 80.9 (C-3, C-4), 79.9 (C-7), 77.4 (C1), 75.3, 74.4, 73.3 (4×O-CH2Ph), 65.0 (C-6), 59.2 (C-2), 57.9 (CH3), 56.1 (2×N-CH2-Ph), 43.6 ppm (C-5); HRMS (ESI) m/z calc for C32H24NO2: 477.1694 [M+H]+; found: 477.1709.

11.50 ppm (C-4), 11.1 ppm (C-3), 74.2, 73.9, 72.3, 70.4 (O-CH1Ph), 71.7 (C-3A), 62.7 (C-6), 57.7 (C-2), 54.9 (2×N-CH3Ph), 42.8 ppm (C-5); HRMS (ESI) m/z calc for C19H22NO3: 343.1840 [M+]+; found: 343.1824.

4.93 ppm (ddd, 1H, C49H51NO5: 734.3840 [M+H]+; found: 734.3853.

The product was purified by HPLC to give the free base as a colorless solid that can be directly used in biological assays. \(^1\)H NMR (600 MHz, D2O): \(\delta\) 7.68 (C-7), 73.8 (C-3), 69.9 (C-4), 66.6 (C-1), 56.8 (C-6), 56.4 (CH3), 53.1 (C-2), 43.2 ppm (C-5); HPLC: \(t_r\) 7.9 min (Phenomenex Luna 5 μm HILIC 200 Å, 250×21.2 mm, 20–30% MeCN in 15 min TEAB buffer pH 7.0 over 15 min, 10.0 mL/min \(^1\), ELSD); HRMS (ESI) \(m/z\) calcd for C18H27NO7: 392.1680 [M + H] \(^+\); found: 392.1674.

Benzy1 ((1R,2R,3R,4S,5S,6R)-4-[(diphenylophosphoryl)oxy]methyl)-2,3,5,6-tetrahydroxycyclohexylcarbamate (28): Compound 25 (53 mg, 162 μmol) was treated according to general procedure C. The crude product was purified by FC (DCM/MeOH 10:1) to give 26 as a colorless syrup (46.2 mg, 82.4 μmol, 51%). \(^1\)H NMR (600 MHz, CDOD): \(\delta\) 7.46–7.22 (m, 15H, arenes), 5.12 (s, 2H, CH2Ph), 4.72 (dd, \(J\) 9.6 Hz, 4.9 Hz, 2.0 Hz, 1H, H-6), 4.64 (dd, \(J\) 9.5 Hz, 4.9 Hz, 2.0 Hz, 1H, H-6), 3.93 (s, 1H, H-1), 3.56 (2H, H-3, H-5a), 3.36 (2H, H-2, H-4), 1.99 ppm (1H, 1H-5); \(^13\)C NMR (151 MHz, CDOD): \(\delta\) 158.7 (C-3), 153.6 (C-6), 142.4, 129.0, 128.6, 128.2, 128.1, 127.9, 127.6, 127.5, 118.7, 110.2, 108.0 (C-4 or C-5a), 75.9 (C-4), 69.5 (C-5a), 43.0 (C-3), 22.3 (OCH2CH2), 9.9 ppm (C-1); HPLC: \(t_r\) 9.1 min (KINETEX C18 5 μm 100 Å, 250×21.2 mm, 25% MeCN in H2O in 20 min, 10.0 mL/min \(^1\), UV); HRMS (ESI) \(m/z\) calcd for C18H27NO7: 392.1680 [M+H] \(^+\); found: 392.1674.

Benzy1 ((1R,2R,3R,4S,5S,6R)-4-[(diphenylophosphoryl)oxy]methyl)-2,3,5,6-tetrahydroxycyclohexylcarbamate (29): Compound 26 (60 mg, 176 μmol) was treated according to general procedure C. The crude product was purified by FC (DCM/MeOH 10:1) to give 27 as a colorless syrup (64 mg, 110 μmol, 64%). \(^1\)H NMR (400 MHz, CDOD): \(\delta\) 7.42–7.21 (m, 15H, arenes), 5.57 (m, 1H, NH), 5.16 (m, 2H, CH2Ph), 4.69 (dd, \(J\) 10.4 Hz, 8.9 Hz, 1.8 Hz, 1H, H-6), 4.45 (m, 1H, H-6), 4.20 (m, 1H, H-1), 3.65 (dd, \(J\) 9.8 Hz, 1.8 Hz, 1H, H-3), 3.43 (m, 1H, H-2), 3.30 (3H, CH3), 3.26 (m, 1H, H-4), 3.08 (m, 1H, H-5a), 2.55 (brs, 3H, 3X OH), 1.94 ppm (1H, 1H-5); \(^13\)C NMR (101 MHz, CDOD): \(\delta\) 156.9 (C O), 150.5, 150.4, 150.3 (Cquart.), 136.3, 130.0, 129.9, 128.6, 128.2, 128.1, 127.9, 125.6, 124.3, 124.2, 121.7, 121.6, 121.0, 120.1, 119.9, 116.7 (C-4), 66.9 (CH2Ph), 61.8 (C-6), 57.8 (CH6), 48.6 ppm (C-5); HPLC: \(t_r\) 9.1 min (KINETEX C18 5 μm 100 Å, 250×21.2 mm, 25–60% MeCN in H2O in 20 min, 10.0 mL/min \(^1\), UV); HRMS (ESI) \(m/z\) calcd for C30H36NO10P 624.1969 [M+H] \(^+\); found: 624.1949.

Benzy1 ((1R,2R,3R,4S,5S,6R)-4-[(diphenylophosphoryl)oxy]methyl)-2,3,5,6-tetrahydroxycyclohexylcarbamate (30): Compound 27 (20.7 mg, 56.0 μmol) was treated according to general procedure C. The crude product was purified by FC (DCM/MeOH 10:1) to give 28 as a colorless syrup (26 mg, 37.6 μmol, 67%). \(^1\)H NMR (400 MHz, CDOD): \(\delta\) 7.44–7.25 (m, 15H, arenes), 5.12 (2H, CH2Ph), 4.78 (m, 1H, H-6a), 4.59 (m, 1H, H-6b), 3.67 (m, 1H, O-CH2CH2), 3.61 (m, 1H, H-3), 3.42 (4H, H-4, H-5a, O-CH2CH2), 1.91 (m, 1H, H-5), 1.64 (m, 2H, CH2CH2), 0.99 ppm (t, \(J\) 7.4 Hz, 3H, CH3); \(^13\)C NMR (CDCl3, 101 MHz): \(\delta\) 157.3 (C O), 136.9 (Cquart.), 128.1, 127.6, 127.5 (arenes), 75.9 (C-4 or C-5a), 72.5 (C-3), 71.2 (C-4 or C-5a), 70.7 (C-3), 68.7 (CH2Ph), 58.0 (C-6), 55.0 (C-2), 43.4 (C-5), 22.8 (CH2CH2), 9.6 ppm (CH); HRMS (ESI) \(m/z\) calcd for C30H36NO10P: 624.1969 [M + Na] \(^+\); found: 624.1969.

**Chemistry—A European Journal**

Research Article

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procedure D to give the title compound as HCl salt 31·HCl (2.9 mg, 9.4 μmol, quant) as a colorless solid. 1H NMR (400 MHz, D2O): δ 4.23 (m, 2H, H-6a, H-1 or H-3), 4.13 (m, 1H, H-6b), 3.82 (m, 1H, H-4 or H-5a), 3.76 (m, 1H, H-1 or H-3), 3.55 (dd, J 10.0 Hz, 10.0 Hz, 1H, H-4), 3.46 (m, 4H, OCH3, H-5a), 3.23 (m, 13H, OCH2CH2CH3), 0.95 ppm (t, J 7.3 Hz, 3H, OCH3), 22.3 ppm (1C, C-6), 1.95 (m, 1H, H-5), 1.64 (m, 2H, OCH2CH2CH3), 0.95 ppm (t, J 7.4 Hz); 13C NMR (101 MHz, D2O): δ 75.8 (C-7), 70.3 (C-3), 63.9 (C-4), 64.8 (C-1), 58.89 (d, JCP 7.5 Hz, 1 C, C-5), 57.0 (OCH3, 53.3 (C-2), 46.7 (6×CH3), 42.96 (d, JCP 7.5 Hz, 1 C, C-5), 8.2 ppm (6×CH3);31P NMR (162 MHz, D2O): δ 3.0 ppm (3P, 1H); HPLC: λmax 5 min (Phenomenex Luna 5 μm HILIC 200 Å, 250×21.2 mm, 40% isocratic. MeCN in 15 mM TEAB buffer pH 6.99, 10.0 mL min⁻¹, ELSD); HRMS (ESI) m/z calcd for C10H22NO8P: 314.1010 [M-H]-; found: 286.0638.

Preparation of glmS ribozyme RNA: PCR amplification of C. difficile and L. monocytogenes glmS riboswitch encoding DNA sequences was accomplished by GoTag DNA Polymerase (Promega) using 5' primers which incorporate the T7 promoter (annealing temperature for C. difficile DNA: 58°C, for L. monocytogenes DNA: 61°C). PCR products were purified on Nucleospin columns according to the manufacturer’s instructions (Macherey & Nagel) and subsequently transcribed using T7 RNA polymerase at 37°C for 30 min. The labeled ribozymes were desalted with G25 columns (GE Healthcare) that had been equilibrated with DEPC-treated water and again purified by polyacrylamide gel electrophoresis.

Metabolite-dependent glmS riboswitch cleavage assay: The 32P-labeled glmS RNA from C. difficile or L. monocytogenes was incubated at 37°C with GlcN. GlcNP6, compounds 22, 23, 24, 31, 32, 33 or without GlcN derivative in cleavage buffer (10 mM MgCl2, 50 mM HEPES, 200 mM KCl, pH 7.5). The potential cleavage reaction was terminated by the addition of PAGE loading buffer (45 mM HEPES, 45 mM boric acid, 4 M urea, 10% sucrose, 0.05% SDS, 100 mM EDTA) followed by separation on a 17% denaturing polyacrylamide gel for analysis. Cleavage products were detected by autoradiography on a phosphorimagery FLA-3000 (Fujiﬁlm) and individual cleavage per sample was assessed using AIDA (Eylsa-Raytest) and Prism (GraphPad) software. To determine the rate constants kobs of ribozyme cleavage, trace amounts of 32P-labeled ribozyme-RNA were incubated at 37°C in cleavage buffer with indicated concentrations of carba-sugar 31 or 32. Aliquots were withdrawn from the reaction pool at various time points and the reaction was quenched by the addition of PAGE loading buffer. The cleavage products were separated by denaturing PAGE and kobs were determined by plotting the fraction cleaved as a function of time. Curves were fitted according to pseudo-first order association kinetics.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: carba-sugars · carbohydrate mimics · glmS riboswitch · ribozymes

Throughout the manuscript, we use the numbering of the parent GlcN derivative for all carba-sugars. The carbaposition is numbered 5a (cf. Scheme 1, first line).