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Thiourea-based spacers in potent divalent inhibitors of *Pseudomonas aeruginosa* virulence lectin LecA†

Aliaksei V. Pukin, Arwin J. Brouwer, Leonie Koomen, H. C. Quarles van Ufford, Johan Kemmink, Nico J. de Mol and Roland J. Pieters*

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A new divalent highly potent inhibitor of the *Pseudomonas aeruginosa* lectin and virulence factor LecA was prepared. It contains two thiourea linkages which were found to be in the *Z,Z* isomeric form. This brings the spacer into an elongated conformation required to bridge the two binding sites, which results in the chelating binding mode responsible for the high potency.

Introduction

Multivalent inhibitors have been shown to be far more potent than their monovalent counterparts, in many cases.[1,2] The multivalency strategy of inhibitor design has been particularly successful in the inhibition of protein–carbohydrate interactions, where valencies are relatively high and monovalent binding potencies are relatively low.[3] In many cases multivalent inhibitor designs have been reported that have flexible arms and topologies that allow strong binding of lectins with multiple binding sites.[4] While these were great advancements, selectivity in the inhibition of target proteins, a major goal for medicinal applications, will more likely be achieved by well-defined multivalent glycoligands that contain rigidified spacers. We previously explored the creation of well-defined glycoligands by choosing the simplest type of multivalent lectin, a divalent lectin.[5] For this purpose we choose LecA. LecA is a virulence factor of the problematic pathogen *Pseudomonas aeruginosa*, and is involved in adhesion, invasion and biofilm formation,[6,7] and a popular target for the development of multivalent ligands,[8,9] that are increasingly successful in the inhibition of infection[10,11] and biofilm formation.[12] LecA is a tetramer with one galactose binding site per subunit, however there are two binding sites that are much closer together (*ca.* 26 Å) than the other combinations, so it is effectively a divalent lectin for our purposes. Creating a well-defined divalent system proved possible by using a spacer consisting of a direct fusion of glucose-triazole units (see **1**, Fig. 1)

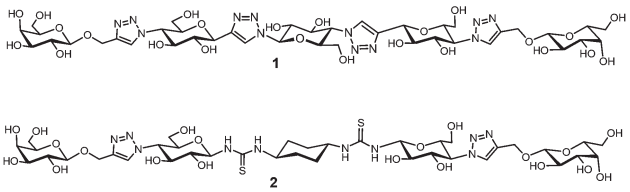

Fig. 1 Structures of divalent LecA ligands with different rigid spacers.

without unnecessary rotatable bonds.[13] The units in the design can rotate, but the overall shape does not deviate far from linearity. Furthermore, the carbohydrate parts ensure good solubility in water, a necessary feature for biomedical applications. Binding and inhibition data with **1** showed a clear preference for this compound over both longer and shorter versions by several orders of magnitude. This phenomenon was interpreted as an indication that **1** was binding in a chelating fashion[14] by both galactoside moieties at either end of the molecule. Molecular modelling confirmed the likelihood of this scenario. More recently, the chelation binding mode was confirmed by X-ray crystallography of the complex, which showed that the inhibitor indeed spanned the two binding sites.[15] Remarkably the entire spacer was crystallographically visible which was not previously observed for multivalent carbohydrates. Interestingly, besides the suitable fit of the molecule, additional interactions were observed between the protein and the spacer, mostly by water bridged hydrogen bonds. These protein–spacer interactions, may contribute significantly to the compound affinity and may provide another level of compound optimization and specificity increase.[16] While in the previous work it was quite clear that **1** in many ways was a well optimized compound for LecA, it was not clear which structural features are needed to achieve the effective chelation. For this reason we explored the use of simpler

Department of Medicinal Chemistry & Chemical Biology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands. E-mail: R.J.Pieters@uu.nl

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linkages between the glucose units, *i.e.* thiourea moieties. Thiourea moieties can be more easily installed, have the potential for preferred conformations, but are also known to exhibit undesirable conformations. We here describe the synthesis of a novel simplified spacer containing two thiourea moieties and its elaboration into divalent LecA ligand **2**. This ligand has the same number of atoms separating the two galactose (sub)-ligands than as **1** but two of the cyclic triazole linkages are replaced by the non-cyclic thioureas. Furthermore, whereas the central carbohydrate unit of **1** makes the molecule pseudo-symmetric, **2** is fully symmetric. NMR analyses were performed in order to gain insight into the preferred conformation of the spacer due to the isomeric possibilities (*Z*,*Z vs.* *Z*,*E*) of the thiourea groups of **2**. Furthermore, docking of the desired and undesired isomeric forms was performed. Finally the dissociation constant of **2** to LecA was determined.

Results

Synthesis

The synthesis of **2** was performed according to Scheme 1 and started with the previously described compound **3**.[17] This protected azidosugar was converted to the β anomeric isothiocyanate **4** *via* the anomeric iodide. Two of the isocyanates were simultaneously coupled to the *trans*-1,4-diaminocyclohexane **7**, to provide **5** containing the two newly installed thiourea linkages. The two galactose ligands were attached *via* a double CuAAC reaction with the propargyl sugar **8** to yield **6**. Final deprotection was achieved with NaOMe in MeOH to provide the divalent LecA ligand **2**.

NMR

Thiourea moieties are attractive linkages that are easily installed from amines in high yield. As such they have seen a great deal of use in various chemical contexts and have also been previously explored by us for carbohydrate conjugation to multivalent scaffolds.[18] One relevant issue with respect to the configuration of the coupled product is whether the coupled thiourea is present as the *Z*,*Z* or the *E*,*Z* isomer, or as a mixture (Fig. 2).[19] In order for both galactose ligands of **2** to reach their binding pockets of LecA, the thiourea moieties of the spacers must be of the *Z*,*Z* isomer type, leading to the most extended conformation of **2**. There are literature reports for the *Z*,*Z* preference of substituted sugar thioureas groups linked to the anomeric carbon of a sugar.[14] More practically, an NOE signal between the two thiourea NH resonances was reported to be a strong indication for the presence of the *Z*,*Z* isomer.[20] For **5**, indeed an NOE signal was observed between the two thiourea NH hydrogens, thus providing support for the presence of the *Z*,*Z* isomeric form. An additional strong NOE was seen between NHa and H2 of the glucose is also consistent with this model. Additional conformational information can be obtained from the coupling constant between the glucose C(1)–H (*i.e.* H1) with its nearest thiourea NH (*i.e.* Ha). The observed relatively large ^3J coupling constant of the 8.4 Hz for **5** is indicative of an anti-orientation as drawn. This is also in agreement with a previous report.[14] Combining the mentioned *Z*,*Z* and the anti preferences leads to a well-defined spacer with the most extended conformation possible. Interestingly, although the thiourea NH next to the sugars (Ha) shows a sharp signal with the mentioned ^3J coupling constant of the 8.4 Hz, the thiourea NH of **5** neighbouring the cyclohexyl ring (Hb) shows a broad signal without discernible coupling constants. Variable temperature NMR showed no major changes to the sharp Ha signal at higher temperature (55 °C), which appears to be in slow exchange and indicates a single conformation of the linked glucose. The broad Hb signal sharpens somewhat at 55 °C. At lower temperatures, down to −60 °C, the signal disappears, but we did not reach an appearance of a split signal due to different conformations. It seems that the conformation of the cyclohexyl part is somewhat less well defined than that of the glucose, since at the room temperature NMR spectrum it appears in intermediate exchange. Unlike in compound **5**, in compound **6** the NHa signal is broad, but the appearance of the signal of H1 as an apparent triplet is consistent with larger coupling constants between NHa and H1 as before and similarly the NOE between NHa and H2 was observed, thus supporting a similar anti-conformation for these thiourea linked spacers.

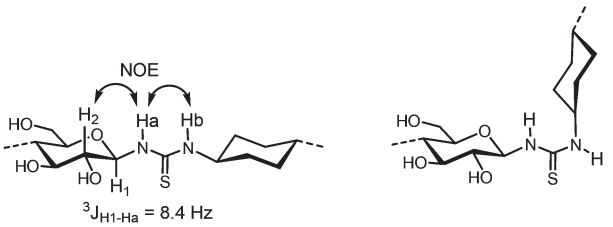
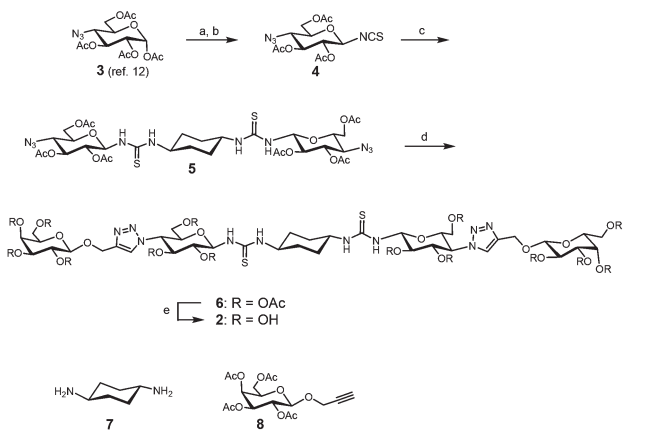


Fig. 2 Two different possible isomeric forms of the thiourea linkage: left: *Z*,*Z* and right *Z*,*E*.



Scheme 1 Reagents and conditions. (a) HMDS, I_2, CH_2Cl_2; (b) NBu_4SCN, CH_3CN, 45%; (c) **7**, CH_2Cl_2, 84%; (d) **8**, $CuSO_4{\cdot}H_2O$, sodium ascorbate, DMF, H_2O, 54%; (e) NaOMe, MeOH, 42% after prep. HPLC.

Modeling

Based on these conformational indications, compound **2** was docked into the LecA protein and subsequently a molecular dynamics simulation was performed, as previously reported for **1**.[11] The obtained structure is shown in Fig. 3. The structure clearly shows a good fit and that chelation is very likely. The structure of **2** in which one of the thiourea groups was present as a *Z*,*E* isomer was also explored. For this structure simultaneous binding of the terminal galactoside ligands was not possible due to the nearly 90° angle that this brings to the spacer (not shown).

LecA binding

The binding affinities of the two divalent structures **1** and **2** for LecA were determined by ITC as previously reported.[13] The observed K_d's were very similar. The K_d for **1** was 29 (±6) nM, which was in close agreement with a previous determination. The number for the thiourea containing compound **2** was 30 (±11) nM.

Discussion and conclusions

Despite the fact that the synthesis of thiourea-based **2** contained steps with a moderate yield, it has some major advantages over the synthesis of triazole-based **1**. The synthesis is much shorter, as it takes only 7 steps from commercial starting materials to make the bis-azide spacer **5** compared to 15 steps to make the corresponding spacer for the synthesis of **1**. The main question to be answered in this work is whether or not, a thiourea moiety can replace the triazole units in a rigid well-defined spacer. Based on the above results it is clear that the replacement was allowed and resulted in similar affinities for both compounds. Considering **1** was the result of considerable optimization and exhibited an 800-fold binding potency increase in comparison to a relevant monovalent ligand,[13] achieving the same potency with **2** is a remarkable result. This enhancement is certainly largely due to the chelation type of binding that these compounds are capable off. Besides that, in the X-ray structure of **1** bound to lecA[15] additional spacer–protein interactions were observed, mostly water bridged hydrogen bonds. It is not clear to what extent these contribute to the binding energy. The protein–spacer interactions for **1** were to all three glucose units of the spacer, *i.e.* including the central one. These are not possible for **2** since it contains a cyclohexyl group at that position. On the other hand, **2** contains two thiourea moieties that are highly capable of forming hydrogen bonds *via* both the sulphur as an acceptor and the two NH groups as donors. This in contrast to the triazole function of **1** containing only hydrogen bond acceptors, although the CH has been implicated as a H-bond acceptor.[21] Overall the effects seem to balance each other out, as no major differences in affinity were seen between **1** and **2**. The high potency of **2** is a clear indication that its spacer is present in an extended conformation. Modeling showed that chelation is not possible if one of the thioureas is present as a *Z*,*E* isomer. NMR observations and literature precedence supports the notion that both thioureas are present in the *Z*,*Z* isomeric form. This observation, makes this type of spacer an attractive candidate for use in other systems, taking advantage of the synthetic ease of its formation and desirable conformational properties. For medicinal applications it should be noted that thioureas are present in quite of number of approved drugs[22,23] and those under development,[24–27] usually for their antiviral or antibiotic effects, but selected structures have also been reported to exhibit toxicity.[28,29]

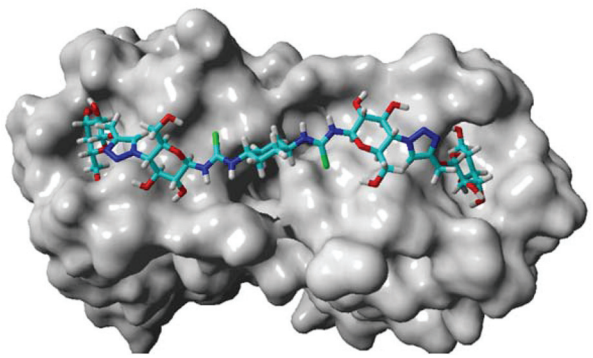


Fig. 3 A model of compound **2** in complex with LecA based on the X-ray structure of LecA with galactose (PDB ID: 1OKO). The positions of protein-bound sugar moieties are identical compared to the model we previously published of compound **1** with LecA.

Experimental

Reagents and general methods

All reagents were obtained from commercial sources and used without further purification. Peptide grade and HPLC grade solvents were purchased from Actu-All (Oss, The Netherlands). The petroleum ether used was petroleum ether 40–60 °C. Solvents were evaporated under reduced pressure at 40 °C. Reactions were carried out at ambient temperature unless stated otherwise. Microwave reactions were performed in a Biotage Initiator (300 W) reactor. Reactions in solution were monitored by TLC analysis using Merck pre-coated silica gel 60 F-254 (0.25 mm) plates. Spots were visualised by UV light and by heating plates after dipping in a ninhydrine solution or in a cerium molybdate solution (Hanessian's stain). Column chromatography was performed on Siliaflash P60 (40–63 μm) from Silicycle (Canada). ^{1}H NMR data was acquired on an Agilent 400 MHz spectrometer in $CDCl_3$ or D_2O as solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (0.00 ppm) or to the solvent residual signal of D_2O (4.79 ppm). Coupling constants (J) are reported in Hertz (Hz). Splitting patterns are designated as singlet (s), doublet (d), triplet (t), multiplet (m), and broad (b). ^{13}C NMR data was acquired on an Agilent 400 MHz spectrometer at 100 MHz in $CDCl_3$ or D_2O as solvent. Most of the ^{13}C NMR spectra were recorded using the attached proton test (apt) pulse sequence. Chemical shifts (δ) are reported in parts per million (ppm)

relative to the solvent residual signal, $CDCl_3$ (77.0 ppm). 2D NMR data (HSQC, COSY, TOCSY, HMBC, NOESY) were acquired on an Agilent 400 MHz spectrometer. Melting points were measured on a Büchi melting point apparatus and are uncorrected. Analytical HPLC was accomplished on a Shimadzu-10Avp (Class VP) with a UV-detector operating at 214 and 254 nm by using a Dr Maisch ReproSil-Pur 120 C18-AQ column (5 μm, 250 × 4.60 mm) at a flow rate of 1 mL min^{-1} using a standard protocol: 100% buffer A for 5 min, followed by a linear gradient of buffer B (0–100% in 45 min), 100% buffer B (5 min), a linear gradient to 100% buffer A (in 5 min), and finally isocratic buffer A (5 min). The mobile phase was H_2O/CH_3CN/TFA (95 : 5 : 0.1, v/v/v, buffer A) and H_2O/CH_3CN/TFA (5 : 95 : 0.1, v/v/v, buffer B). Preparative HPLC was accomplished on an Applied Biosystems model 450 setup with a UV-detector operating at 214 nm by using a Dr Maisch ReproSil-Pur C18-AQ column (10 μm, 250 × 22 mm) at a flow rate of 12 mL min^{-1} using a standard protocol: 100% buffer A for 5 min, followed by a linear gradient of buffer B (0–100% in 120 min), 100% buffer B (5 min), a linear gradient to 100% buffer A (in 5 min), and finally isocratic buffer A (5 min), using the same buffers as described for analytical HPLC. High-resolution electrospray ionization (HRMS ESI) mass spectra were measured on a Bruker micrOTOF-Q II in positive mode and calibrated with ESI tuning mix from Agilent Technologies.

1-Isothiocyanate-2,3,6-tri-*O*-acetyl-4-azido-β-D-galactopyranose (4).[30] To a solution of **3** (3.73 g, 10.0 mmol) in dry dichloromethane (20 mL) hexamethyldisilane (1.9 mL, 9.8 mmol) and iodine (2.58 g, 10.0 mmol) were added. After stirring for 1 h at rt, sodium thiosulfate pentahydrate (10 g, mmol) was added and the mixture was stirred for 30 minutes, after which it was filtered and the filtrate was concentrated *in vacuo*. To the crude iodide dry acetonitrile (250 mL), 4 Å molsieves (16 g) and tetrabutylammonium thiocyanate (5.93 g, 20.0 mmol) were added. The mixture was stirred for 24 h at reflux and cooled to rt. After filtration and concentration of the filtrate *in vacuo*, column chromatography was performed (2 step gradient; step 1: 10% EtOAc in petroleum ether; step 2: 25%) to yield isothiocyanate **4** as a white solid (1.56 g, 4.2 mmol, 45%). R_f = 0.70 (EtOAc : petroleum ether, 1 : 1).

^{1}H NMR (400 MHz, $CDCl_3$): δ 2.11, 2.12, 2.14 (3s, 9H, 3× OAc), 3.50 (ddd, 1H, $J_{5,6b}$ = 2.1 Hz, $J_{5,6q}$ = 4.6 Hz, J_{gem} = 10.4 Hz, H-5), 3.70 (t, 1H, J = 10.1 Hz, H-4), 4.25 (dd, 1H, J_{5-6a} = 4.6 Hz, J_{gem} = 12.4 Hz, H-6a), 4.42 (dd, 1H, J_{5-6b} = 2.1 Hz, J_{gem} = 12.4 Hz, H-6b), 5.01 (m, 2H, H-1, H-2), 5.18 (m, 1H, H-3). ^{13}C NMR (400 MHz, $CDCl_3$): δ 20.5, 20.5, 20.7 (CH_3), 59.5 (C-4), 62.3 (CH_2), 72.1 (C-2), 73.4 (C-3), 74.2 (C-5), 83.4 (C-1), 144.3 (NCS), 169.2, 169.7, 170.3 (C═O). HRMS: calcd for $C_{13}H_{16}N_4NaO_7S^+$ $[M + Na]^+$ 395.0632, found 395.0657.

Protected thiourea spacer 5. To a solution of isothiocyanate **4** (893 mg, 2.40 mmol) in dry dichloromethane (15 mL) *trans*-1,4-diaminocylohexane (114 mg, 1.00 mmol) was added. After stirring for 2 h at rt, the solvent was evaporated *in vacuo* and the residue was purified using column chromatography (petroleum ether : ethyl acetate, 1 : 1). Precipitation from dichloromethane/petroleum ether afforded **5** as a white solid (734 mg, 0.84 mmol, 84%). mp = 201 °C.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.29 (m, 4H, CH_2CH_2), 2.06, 2.12, 2.13 (3s, 18H, 6× OAc), 2.20 (m, 4H, CH_2CH_2), 3.67 (m, 2H, 2× H-5), 3.82 (bt, 2H, 2× H-4), 4.05 (bs, 2H, 2× CH (cyclohexyl)), 4.20 (bd, 2H, 2× H-6a), 4.62 (bs, 2H, 2× H-6b), 4.96 (t, 2H, J = 9.3 Hz, 2× H-2), 5.34 (t, 2H, J = 9.3 Hz, 2× H-3), 5.63 (t, 2H, J = 8.8 Hz, 2× H-1), 6.52 (d, 2H, J = 7.7 Hz, 2× C1-NH), 6.92 (bs, 2H, 2× $CH_2CHN\underline{H}$). ^{13}C NMR (400 MHz, $CDCl_3$): δ 20.5, 20.7, 21.3 (CH_3), 30.7, 31.0 (CH_2-CH_2), 53.0 (C-5), 60.4 (CH (cyclohexyl)), 62.6 (C-6), 70.9 (C-2), 74.5 (C-3), 82.4 (C-1, C-5), 169.6, 171.1, 171.7 (C═O), 182.6 (C═S). HRMS: calcd for $C_{32}H_{47}N_{10}O_{14}S_2^+$ $[M + H]^+$ 859.2709, found 859.2732.

Protected thiourea inhibitor 6. Copper(II) sulfate pentahydrate (126 mg, 0.58 mmol) was dissolved in water (1.0 mL) and added to a solution of **5** (500 mg, 0.58 mmol) and prop-2-yn-1-yloxy-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose (600 mg, 1.55 mmol) in DMF (8 mL), and stirred for 10 min at rt under N_2 gas atmosphere. A solution of L-sodium ascorbate (191 mg, 0.97 mmol) in water (1.0 mL) was added and the mixture was stirred 18 h at rt. After addition of dichloromethane, the organic phase was washed with EDTA (1.0 M), $NaHCO_3$ (sat.), water and brine, and dried over sodium sulfate. Column chromatography (dichloromethane/ethyl acetate, 1/4) yielded **6** as a white solid (514 mg, 0.32 mmol, 54%). R_f = 0.67 (EtOAc).

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.29 (m, 4H, CH_2CH_2), 1.92, 1.98, 2.01, 2.05, 2.11, 2.11, 2.17 (7s, 42H, 14× OAc), 2.10 (m, 4H, CH_2CH_2), 3.99, 4.06 (2m, 6H, 2× H-5 (Gal), 2× H-6 (Glc), 2× CH (cyclohexyl)), 4.13 (dd, 2H, $J_{5,6a}$ = 7.1 Hz, J_{gem} = 11.3 Hz, 2× H-6a (Gal)), 4.28 (dd, 2H, $J_{5,6b}$ = 5.8 Hz, J_{gem} = 11.3 Hz, 2× H-6b (Gal)), 4.51 (bd, 4H, 2× H-1 (Gal), 2× H-5 (Glc)), 4.82 (d, 2H, J_{gem} = 12.5 Hz, 2× OCH^a (triazole)), 4.89 (bt, 2H, H-5 (Glc)), 4.97 (d, 2H, J_{gem} = 12.5 Hz, 2× OCH^b (triazole)), 5.05 (dd, 2H, $J_{2,3}$ = 10.4 Hz, 2× H-3 (Gal)), 5.14 (t, 2H, J = 9.3 Hz, 2× H-2 (Glc)), 5.21 (dd, 2H, $J_{3,4}$ = 8.0 Hz, $J_{2,3}$ = 10.4 Hz, 2× H-2 (Gal)), 5.42 (d, 2H, $J_{1,2}$ = 3.3 Hz, 2× H-4 (Gal)), 5.80 (t, 2H, J = 9.8 Hz, H-3 (Glc)), 6.01 (t, 2H, J = 8.8 Hz, 2× H-1 (Glc)), 6.58 (m, 4H, 4× NH), 7.77 (s, 2H, 2× CH (triazole)).

^{13}C NMR (400 MHz, $CDCl_3$): δ 20.1, 20.6, 20.7, 20.8, 20.8 (CH_3), 29.7, 30.2, 30.8, 31.4 (CH_2 (cyclohexyl)), 52.7 (CH (cyclohexyl)), 60.6 (C-4 (Glc)), 61.3 (C-6 (Gal)), 61.8 (OCH_2 (triazole)), 62.0 (C-6 (Glc)), 67.2 (C-4 (Gal)), 68.7 (C-2 (Gal)), 70.7 (C-5 (Gal)), 70.8 (C-3 (Gal)), 71.0 (C-2 (Glc)), 72.5 (C-3 (Glc)), 73.6 (C-5 (Glc)), 82.9 (C-1 (Glc)), 99.3 (C-1 (Gal)), 123.3 (C═$\underline{C}$H (triazole)), 143.9 ($\underline{C}$═CH (triazole)), 169.1, 169.5, 170.1, 170.2, 170.8 (C═O), 182.7 (C═S). HRMS: calcd for $C_{66}H_{91}N_{10}O_{20}S_2^+$ $[M + H]^+$ 1631.5135, found 1631.5164.

Thiourea inhibitor 2. To a solution of **6** (668 mg, 0.41 mmol) in methanol (20 mL) and water (8 mL) an aqueous sodium hydroxide solution (1.0 M, 100 μL) was added. Additional sodium hydroxide solution was added after 3 h (100 μL), after 5 h (200 μL) and after 2 days (1.7 mL). After stirring for 3 days in total at rt, dowex-H^+ was added until the pH was neutral (indicator paper). After stirring for 15 minutes the mixture was filtered and the filtrate was concentrated *in vacuo*. Purification by preparative HPLC afforded inhibitor **2** as a

clear colorless film (184 mg, 0.17 mmol, 42%). R_f = 0.53 (EtOAc).

^{1}H NMR(400 MHz, D_2O): δ 1.47 (bd, 4H, CH_2CH_2), 2.11 (bd, 4H, CH_2CH_2), 3.27 (dd, 2H, 2× H-6a (Glc)), 3.53–3.86 (m, 14H, 2× [H-2, H-3, H-5, H-6 (Gal), H-2, H-6b (Glc)], 3.94 (bd, 2H, 2× H-4 (Gal)), 4.24 (m, 6H, 2× [CH (cyclohexyl), H-3, H-5 (Glc), 2× H-5 (Glc)), 4.53 (d, 2H, J = 7.8 Hz, 2× H-1 (Gal)), 4.68 (bt, 2H, H-4 (Glc)), 4.99 (dd, 4H, J_{gem} = 12.7 Hz, 2× OCH_2 (triazole)), 5.66, 5.90 (2× bs, 2H, 2× H-1 (Glc)), 8.23 (s, 2H, 2× CH (triazole)).

^{13}C NMR (400 MHz, D_2O): δ 30.0 (CH_2 (cyclohexyl)), 53.1 (CH (cyclohexyl)), 59.7 (C-6 (Glc)), 60.9 (C-6 (Gal)), 61.8 (OCH_2 (triazole)), 68.5 (C-4 (Gal)), 70.6, 72.2, 72.6, 74.1, 75.3 (C-2, C-3, C-5 (Gal), C-2, C-3, C-5 (Glc)), 83.4 (C-1 (Glc)), 102.0 (C-1 ((Gal)), 125.4 (C=CH (triazole)), 143.9 (C=CH (triazole)). HRMS: calcd for $C_{38}H_{62}N_{10}NaO_{20}S_2^+$ $[M + Na]^+$ 1065.3475, found 1065.3420.

Modeling

A model of the complex of compound **2** with LecA was constructed based on our predicted model of compound **1** with LecA, which has been validated by X-ray crystallography.[15] The modeling software package Yasara was employed to convert the two triazole linkers flanking the central sugar moiety of compound **1** in our previous model into thiourea linkers. After adjustment of the force field parameters, the molecule was subjected to an energy minimization in which the bound galactose units and the protein were kept in fixed position. The *Z*,*Z* isomeric forms of the thiourea linkers were enforced using dihedral angle restraints.

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