Synthesis of a heparinoid pentasaccharide containing L-guluronic acid instead of L-iduronic acid with preserved anticoagulant activity

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Abstract: L-iduronic acid is a key constituent of heparin and heparan sulfate polysaccharides due to its unique conformational plasticity, which facilitates the binding of polysaccharides to proteins. At the same time, this is the synthetically most challenging unit of heparinoid oligosaccharides, therefore, there is a high demand for its replacement with a more easily accessible sugar unit. In the case of idraparinux, an excellent anticoagulant heparinoid pentasaccharide, we demonstrated that L-iduronic acid can be replaced by an easier-to-produce L-sugar while maintaining its essential biological activity. From the inexpensive D-mannose, through a highly functionalized phenylthio mannoside, L-gulose donor was prepared by C-5 epimerization in 10 steps with excellent yield. This unit was incorporated into the pentasaccharide by α-selective glycosylation and oxidized to L-guluronic acid. The complete synthesis

required only 36 steps, with 21 steps for the longest linear route. The guluronate-containing pentasaccharide inhibited coagulation factor Xa by 50% relative to the parent compound, representing an excellent anticoagulant activity. To the best of our knowledge, this is the first biologically active heparinoid anticoagulant which contains a different sugar unit instead of L-iduronic acid.

Introduction

Heparin (H) and heparan sulfate (HS) are hexuronic acid-containing highly sulfated linear glycosaminoglycans that are ubiquitous in mammalian tissues and play a role in the regulation of embryogenesis, angiogenesis, neural development, blood coagulation, and inflammation.[1-3] In addition, H and HS are also implicated in a number of pathological processes such as cancer, neurodegenerative diseases, as well as bacterial and viral infections, including SARS-CoV-2.[4,5] Heparinoid oligosaccharides have significant therapeutic potential against a wide variety of diseases, generating a huge demand for their efficient chemical synthesis.[6-9]

The biological effects of H and HS stem from their interactions with various proteins, in which the remarkable conformational plasticity of the L-iduronic acid (IdoA) residues often plays a key role in promoting better binding to proteins.[10-12] However, IdoA and its non-oxidized precursor, L-idose are rare sugars not available from natural sources, and their chemical synthesis in a form suitable for glycosylation is time-consuming and inefficient, which poses a serious problem in the production of heparinoid oligosaccharides.[13] This challenge could be overcome by replacing the L-iduronic acid residue with a building unit that has similar conformational flexibility but is more readily available.

The best known and most thoroughly studied function of H/HS polysaccharides is the anticoagulant activity of heparin.[14] Allosteric binding of heparin to the serine protease inhibitor antithrombin (AT) through a specific pentasaccharide sequence, termed **DEFGH**, initiates a conformational change in antithrombin which then inhibits the key coagulation enzymes, thrombin and factor Xa. Synthetic analogues of the AT-binding pentasaccharide domain of heparin such as the approved anticoagulant drug fondaparinux (**1**)[15] and its non-glycosaminoglycan analogue idraparinux (**4**)[16] are also able to activate antithrombin and exert anticoagulant effect via selectively inhibiting factor Xa (**Figure 1**).

During the development of fondaparinux and idraparinux, it has been investigated how replacing the L-iduronic acid unit with different D- and L-sugars affects anticoagulant activity. Replacement of the IdoA residue in **1** by readily available, inexpensive D-sugars such as D-glucuronic acid (**2**) or D-xylose of high conformational flexibility (**3**) led to inactive

compounds.[17] Among the conformationally locked analogues of idraparinux (**4**), compound **5** in which unit **G** was fixed in the $^{1}C_{4}$ chair form, hardly inhibited factor Xa, while compound **6**, which contained a unit **G** fixed in the $^{2}S_{O}$ skew-boat conformation, showed an excellent anti-Xa activity (3.5% versus 88% activity compared to that of the parent compound **4**).[18] These results provided direct evidence for the crucial role of the $^{2}S_{O}$ conformation of L-iduronic acid in the activation of antithrombin by heparin. Recently, we replaced the iduronate residue of idraparinux by a 6-deoxy-L-talopyranose (6d-L-Tal), which is the most easily available L-hexose epimer of L-idose, in the hope that 6d-L-Tal, by incorporating into the highly sulfated heparin sequence **7**, can adopt the bioactive $^{2}S_{O}$ conformation.[19] This simplification of the structure led to almost complete loss of anti-Xa activity, probably due to the lack of the essential carboxylic moiety of unit **G** and the reduced abundance of the bioactive $^{2}S_{O}$ conformer in the conformational equilibrium.

Based on the accumulated results shown in **Figure 1**, especially the high activity of **6**, we hypothesized that the L-iduronic acid residue of the anticoagulant pentasaccharides **1** or **4** could be replaced with L-guluronic acid (L-GulA), a C-2 epimer of L-IdoA, without loss of anti-Xa activity. However, such a replacement is of practical significance only if the guluronic acid building block could be efficiently produced.

We have recently developed an expeditious synthesis of L-idosyl glycosyl donors from properly functionalized α-1-thio-D-glucosides by C-5 epimerization using the elimination-hydroboration-oxidation method.[20] With this idosyl donor, we have prepared idraparinux (**4**) in the most efficient and shortest way published to date.[21] We contemplated that using the commercially available and inexpensive D-mannose, the C-2 epimer of glucose, as a starting material, the L-gulose donor can be efficiently prepared to produce the idraparinux analog pentasaccharide **8**, in which L-guluronic acid (GulA) replaces L-iduronic acid. Importantly, the proposed C-5 epimerization method requires an α-configured thioglycoside starting material that is more readily available from mannose than glucose, which is likely to make L-gulose donor production more efficient compared to L-idose donor production.

Here we present the synthesis of pentasaccharide **8** and answer the question whether the L-guluronic acid unit has the conformational plasticity required for biological activity and thus can be a good substitute for L-iduronic acid in heparinoid oligosaccharides.

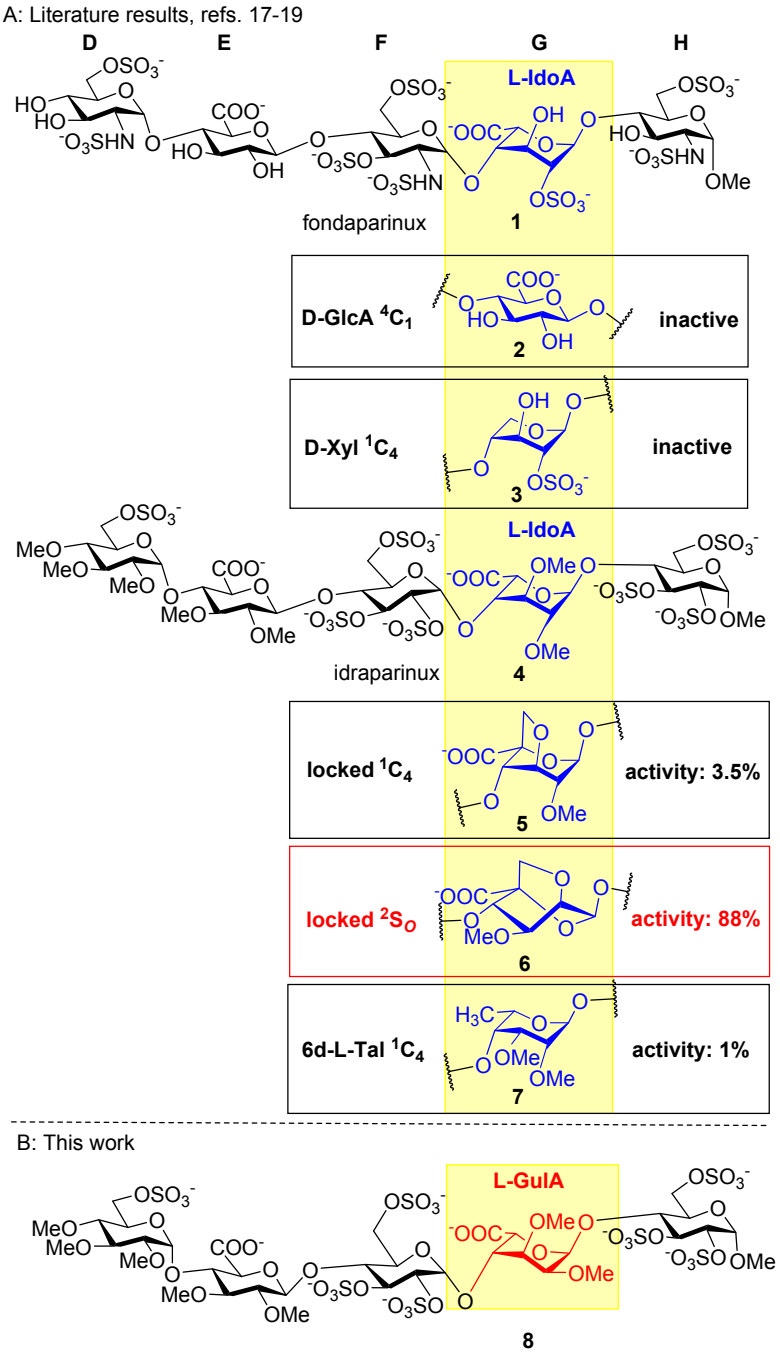


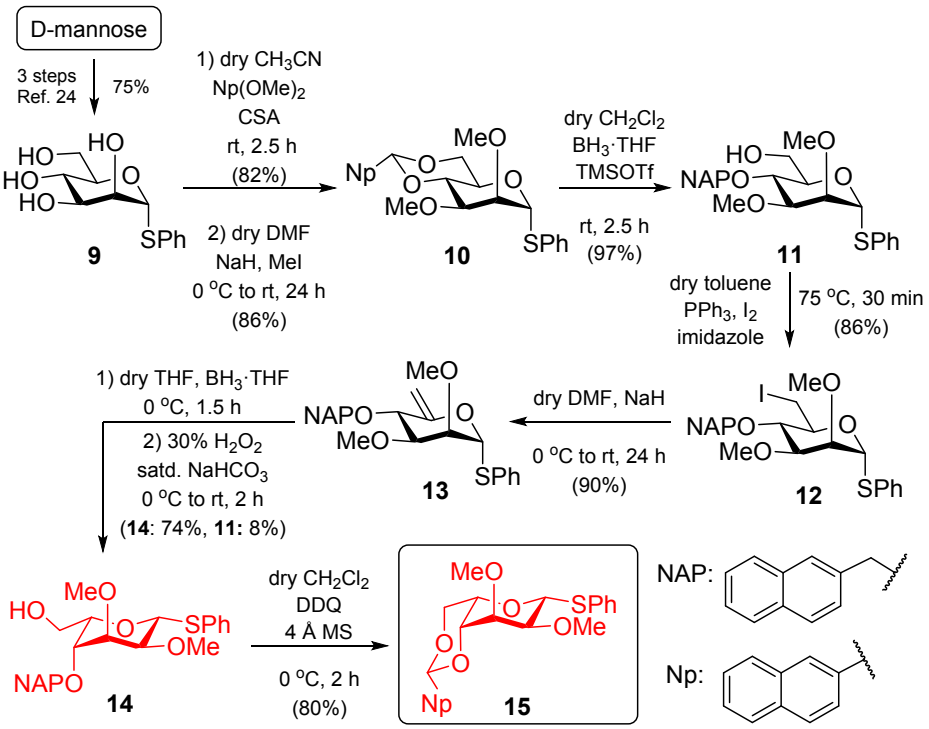
Figure 1. A: Effect of replacing the L-iduronic acid unit of the anticoagulant pentasaccharides fondaparinux (**1**) and idraparinux (**4**) on the factor Xa inhibitory activity; **B**: The designed idraparinux-analogue pentasaccharide containing L-GulA instead of L-IdoA

Results and Discussion

2.1. Synthesis of the L-guluronic acid containing pentasaccharide

The L-guluronic acid unit (unit **G**) was designed to be incorporated into the pentasaccharide as its non-oxidized precursor, similarly to our previous idraparinux syntheses.[21,22] A suitable L-

gulose donor **15** was synthesized from inexpensive D-mannose, as shown in **Scheme 1.** First, the α-phenylthio derivative **9** was prepared in three steps including acetylation, treatment with thiophenol in the presence of $BF_3 \cdot Et_2O$, and Zemplén deacetylation.[23] The thio aglycone of **9** makes the molecule suitable for glycosylation, and the α anomeric configuration is key to providing high L-selectivity in the C-5 epimerization reaction.[20,24,25] Compound **9** was converted to the corresponding 4,6-*O*-(2-naphthyl)methylene acetal,[26] then the methyl ether functionalities were introduced to positions 2 and 3 to produce **10**.



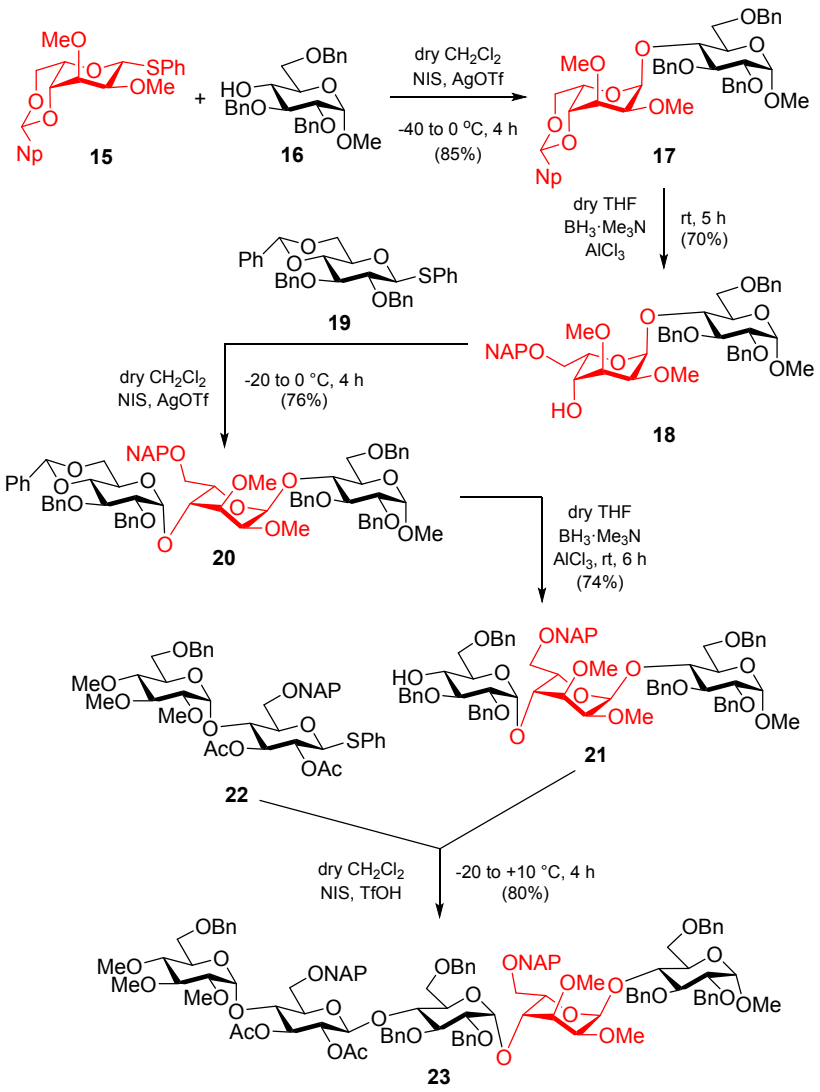
Scheme 1. Synthesis of the L-guluronic acid precursor building block **15**. (Overall yield 25% over ten steps from D-mannose)

The primary hydroxyl group of **10** was liberated with full regioselectivity in a reductive ring-opening reaction with $BH_3 \cdot THF$ complex in the presence of catalytic trimethylsilyl trifluoromethanesulfonate (TMSOTf).[27] The resulting **11** was treated with iodine and triphenylphosphine to yield **12**, dehydroiodination of which with NaH in DMF provided the 5,6-unsaturated derivative **13** with excellent yield.[28] The key C-5 epimerization was performed by hydroboration using $BH_3 \cdot THF$ complex and subsequent oxidation with H_2O_2 to provide the required L-*gulo* configured **14** with 74% yield.[20] The reaction proceeded with high chemo- and stereoselectivity, oxidation of the thio aglycone was not observed and regeneration of the D-mannoside derivative **11** was negligible (8%). The 6-OH group of the L-gulopyranosyl

derivative was protected in an oxidative ring closure[29] using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in dry CH_2Cl_2 to yield the 4,6-*O*-(2-naphthyl)methylene acetal derivative **15**. This synthesis of L-guloside donor **15** from D-mannose required 10 steps and was performed in 25% overall yield. For comparison, an L-idoside donor with the same substitution pattern has previously been produced in 14% yield from D-glucose in 12 steps,[21] where the longer synthesis route was due to the non-trivial production of 1,2-*cis*-α thioglucoside by the thiol-ene coupling reaction.[30]

With donor **15** in our hands, we started to assemble the pentasaccharide by synthesizing the **GH** disaccharide **17** (Scheme 2). Replacement of L-iduronic acid with a L-*gulo* configured unit required the formation of a 1,2-*cis*-α interglycosidic bond in the **GH** disaccharide instead of the original 1,2-*trans*-α bond (compare **8** with **1** or **4**). Although stereoselective 1,2-*cis* glycosylation is a difficult task, a 4,6-*O*-benzylidene acetal group at the donor has been shown to be a good strategy to promote 1,2-*cis*-α glycosidation in the D-*gluco*- and galactopyranose series.[31-33] In addition, we have recently demonstrated a strong stereocontrolling effect of the 4,6-*O*-naphthylmethylene acetal group of an L-idoside donor, which ensures the stereoselective formation of α-L-idosidic bond in the absence of a C-2-participating group.[20,21] Thus, significant α-selectivity was expected for glycosylation of acceptor **16**[34] with donor **15** containing a 4,6-acetal protecting group. The reaction was promoted using *N*-iodosuccinimide (NIS) and silver trifluoromethanesulfonate (AgOTf) and indeed, high α-stereoselectivity was observed to give the desired 1,2-cis-α-linked **GH** disaccharide (**17**) in 85% yield.

Compound **17** was subjected to a reductive acetal-opening reaction using $BH_3{\cdot}Me_3N/AlCl_3$ reagent combination in THF to liberate the 4-OH group. The resulting disaccharide acceptor **18** was glycosylated with monosaccharide donor **19**[35] upon NIS/AgOTf promotion to provide the L-gulopyranosyl-containing **FGH** trisaccharide **20**, with the required α-interglycosidic linkage, in 76% yield.

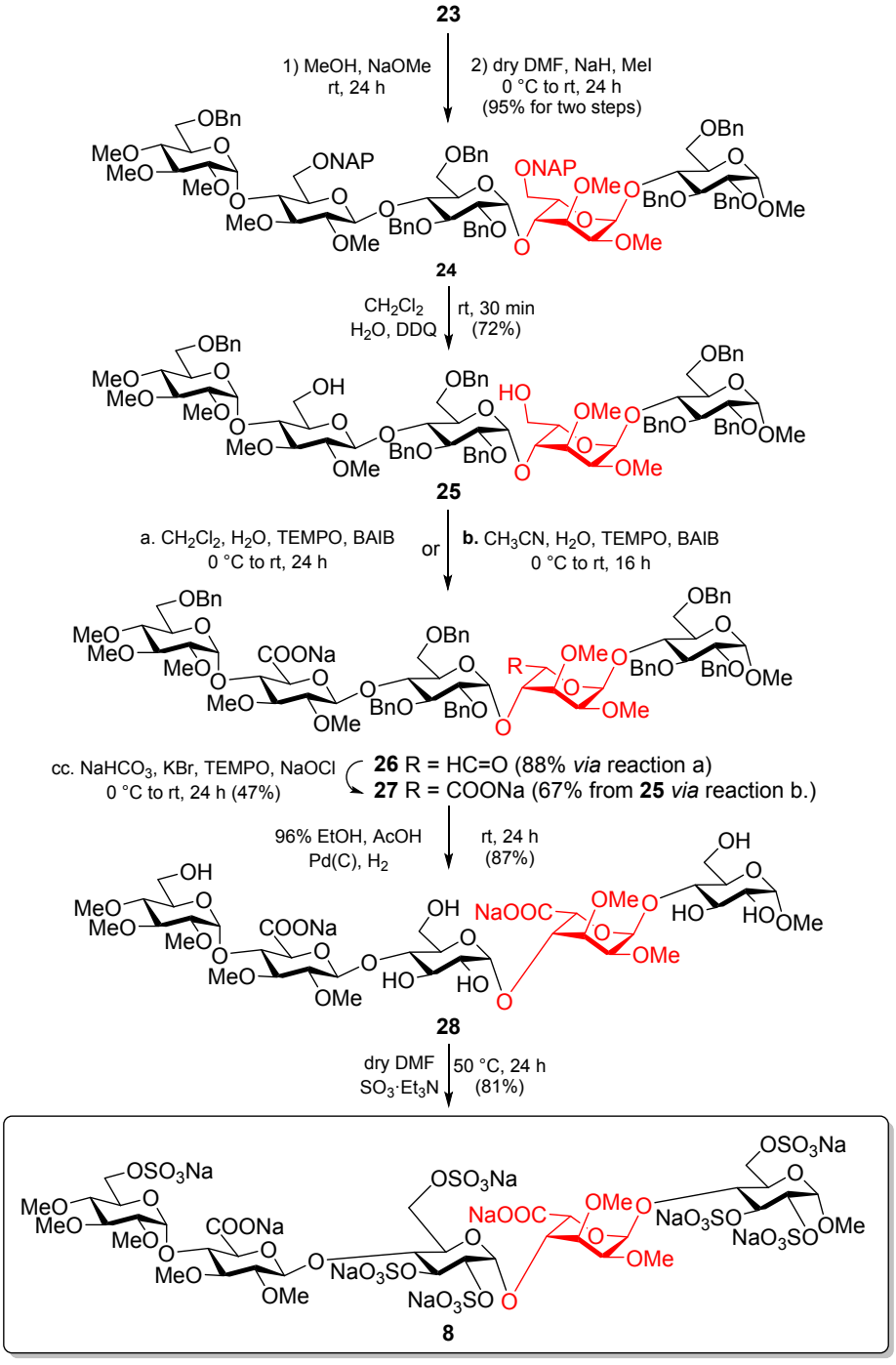


Scheme 2. Glycosylation with L-gulose donor **15** with full α-selectivity and construction of the L-gulose-containing **DEFGH** pentasaccharide by [2+3] block synthesis.

The 4,6-acetal ring of **20** was opened in a reductive manner using $BH_3{\cdot}Me_3N$ and $AlCl_3$ in THF to result in the **FGH** trisaccharide acceptor **21** in high regioselectivity with 74% yield. The pentasaccharide skeleton was assembled by glycosylation of the **FGH** trisaccharide acceptor **21** with **DE** disaccharide donor **22**,[22] using NIS/trifluoromethanesulfonic acid (TfOH) activator system in dry CH_2Cl_2. The [2+3] block synthesis provided the protected pentasaccharide **23** with excellent yield and complete stereoselectivity.

Final transformations at the pentasaccharide level involved the introduction of methyl ether functions to units **E**, **F** and **H** and the oxidation of the uronic acid precursor units **E** and **G** (**Scheme 3**). First, the acetyl protecting groups were removed from positions 2 and 3 of unit **E** under Zemplén conditions, and the liberated hydroxyl groups were methylated using NaH and

MeI reagents to yield pentasaccharide **24**. Subsequently, the (2-naphthyl)methyl-ether (NAP) groups were removed under oxidative conditions using DDQ in a mixture of CH_2Cl_2 and H_2O releasing the primary hydroxyls of units **E** and **G** (**25**).



Scheme 3. Transformations at the pentasaccharide level to the final product **8**.

Oxidation of precursor units **E** and **G** to uronic acids was first attempted with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and (diacetoxyiodo)benzene (BAIB) in CH_2Cl_2 and H_2O. However, only oxidation of unit **E** went to completion, while oxidation of the primary

hydroxyl group of unit **G** (L-gulopyranosyl unit) stopped at the aldehyde level to yield **26**, probably due to steric congestion and solubility problems. This derivative was completely oxidized in saturated $NaHCO_3$ solution using KBr, TEMPO and sodium hypochlorite solution (NaOCl) to give the desired compound **27** with moderate yield. Performing the TEMPO- and BAIB-mediated oxidation of **25** in a more polar medium, a 3:1 mixture of acetonitrile and water, both hydroxyl groups were successfully converted to carboxyl groups in a single step to give the required compound **27** containing both D-glucuronate and L-guluronate units. Finally, the benzyl groups were removed by catalytic hydrogenation to give heptaol **28**, the hydroxyl groups of which were sulfated in dry DMF at 50 °C using $SO_3 \cdot Et_3N$ complex. Thus, the planned idraparinux analogue pentasaccharide **8** containing L-guluronic acid instead of L-iduronic acid was obtained in good yield.

The complete and unambiguous ^1H and ^{13}C NMR resonance assignment of pentasaccharide **8** was achieved with the aid of classical (^1H-^1H COSY, TOCSY and ^1H-^{13}C HSQC, HMBC) and advanced (^1H-^1H EASY ROESY,[36] ^1H-^{13}C HSQC-CLIP-COSY[37] and NORD HSQC-TOCSY[38]) 2D NMR experiments. As the ring conformation of unit **G** has been suggested as a major determinant of biological activities, we planned to study the conformation of the L-guluronic acid unit of **8**. In the ^1H NMR spectra line broadening was observed for the resonance signals of unit **G**, which refers to an equilibrium between different conformational states of the sugar ring.[39,40] The line broadening also hindered the measurement of ^1H-^1H scalar coupling constants, therefore a more detailed conformational analysis of unit **G** became unfeasible. Moreover, the NOE cross-peaks in the ROESY spectrum were not indicative either for a particular ring conformation. Thus, at this point we can confirm the conformational plasticity of the L-guluronic acid unit of pentasaccharide **8**, which feature can be important for the appropriate binding to AT protein, and so for its biological activity.

2.2. Anticoagulant activity and biocompatibility of the L-guluronic acid-containing pentasaccharide

Table 1. Anti-Xa activity of fondaparinux, idraparinux, and the L-guluronic acid-containing pentasaccharide **8**

	Anti-Xa activity (μg/mL)
fondaparinux (**1**)	1
idraparinux (**4**)	1.36
L-guluronic acid-containing pentasaccharide (**8**)	0.67

The factor Xa inhibitory activity of the L-guluronic acid-containing derivative **8** and reference compound **4** was determined *in vitro* by a functional chromogenic anti-Xa assay calibrated against fondaparinux, using pooled normal human plasma (**Table 1**).[41,42] The data show that the new pentasaccharide retains 50% of the factor Xa inhibitory activity compared to the parent compound idraparinux (**4**), implying that compound **8** has an excellent anticoagulant effect, almost equivalent to the marketed anticoagulant drug fondaparinux (**1**). This is the first evidence that L-iduronic acid is not an irreplaceable structural element of heparinoid anticoagulants, antithrombin can be effectively activated by a pentasaccharide containing another conformationally flexible pyranuronic acid as a G unit. At the same time, the reduced activity of compound **8** compared to idraparinux highlights that, in addition to conformational plasticity, other factors also play a role in the biological effect, which is in line with our recent results.[43]
In order to find out whether the L-IdoA L-GulA exchange alters the toxicity profile of the pentasaccharide, and to ascertain the biocompatibility of the GulA-containing derivative, the potential cytotoxic effect of compounds **4** and **8** was tested on HaCaT keratinocyte cell line and WM35 melanoma cells using MTT assay. Applied up to 100 μM, none of the compounds decreased the viability of the cell lines within 72 hours indicating that the compounds are not toxic for the investigated cell lines (Supplementary **Figure S3**).

Conclusions

Anticoagulant therapeutics, including heparin derivatives, are the cornerstones of the treatment and prevention of coagulation disorders such as deep vein thrombosis and pulmonary embolism, including thromboprophylaxis in modern surgery. Pentasaccharides offer an alternative anticoagulant strategy in patients suffering from heparin induced thrombocytopenia, a life-threatening side-effect of unfractionated heparin and, to a lesser extent, low molecular weight heparin therapy. Selective inhibiton of FXa makes pentasaccharides a highly effective anticoagulants and at the same time safer than unfractionated heparin. Therefore, the efficient production of heparinoid anticoagulant pentasaccharides is of paramount importance.
We presented here an exceptionally short 36-step synthesis of a heparinoid anticoagulant pentasaccharide (**8**) from commercially available inexpensive starting materials, D-glucose, D-mannose and methyl α-D-glucopyranoside. The short and efficient synthesis was due to the replacement of L-iduronic acid, which plays a key role in anticoagulant activity but is extremely difficult to synthesize, with L-guluronic acid, another L-sugar that has been efficiently produced.

The 4,6-*O*-naphthylmethylene-protected L-gulose thioglycoside building block **15** was prepared from D-mannose in 10 steps, the key step being C-5 epimerization based on elimination-hydroboration-oxidation on a thiomannoside. Importantly, this synthesis was twice as efficient as the synthesis of the analogous L-idose building block from D-glucose.[21] In addition, this gulosyl thioglycoside has been shown to be an excellent α-selective glycosyl donor during incorporation into the pentasaccharide.

NMR experiments confirmed conformational flexibility of the L-guluronic acid unit in the newly synthesized pentasaccharide, which is known to be necessary for biological activity. According to the i*n vitro* heparin assay pentasaccharide **8** retained its antithrombin-activating effect to a significant extent. Thus, it is the first proof that AT can be activated by a heparinoid pentasaccharide in which the key L-iduronic acid is replaced by a pyranuronic acid which is similarly flexible conformationally but different in configuration.

Besides the interactions with the coagulation system, heparin and heparan sulfate interact with a number of other proteins, the therapeutic exploitation of which is highly desirable. In this context, the efficient synthesis of heparinoids and their mimetics represents a key requirement. Our present work also demonstrates that the replacement of L-iduronic acid with L-guluronic acid might be a suitable strategy for the efficient production of bioactive mimetics of heparin and heparan sulfate.

Supporting Information. (Experimental procedures, determination of factor Xa inhibitory activity and cytotoxicity of pentasaccharides **4** and **8**, copy of NMR spectra, copy of HRMS spectra.)

Data availability: The datasets used and/or analysed during the current study available from the corresponding author (A.B.) on reasonable request**.**

Author contributions

Conceptualization, M. H. and A. B.; investigation, F. D., E. L., M. R., B. I. T., I. T., Zs. B., K. K. E, A. B. and M. H.; writing—original draft preparation, M. H., B. I. T., K. K. E., I. T., Zs. B., and A. B.; writing—review and editing, M. H., B. I. T., I. T., Zs. B., K. K. E. and A. B.; supervision M. H. and A. B.; funding acquisition, M. H., K. K. E. and A. B. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The author declares no conflict of interest.

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42. Anti-Xa activity of fondaparinux and compounds **4** and **8** is expressed as µg/mL according to the recommendation of the manufacturer, however this functional assay is rather reflecting to the potency of the FXa inhibitory feature of the investigated pentasaccharides (STA Fondaparinux calibrator, Stago, Asnieres, France).