Carbohydrate-based peptidomimetics targeting neuropilin-1: synthesis, molecular docking study and in vitro biological activities

To cite this version:

HAL Id: hal-02356229
https://hal.archives-ouvertes.fr/hal-02356229
Submitted on 12 Nov 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Carbohydrate-based peptidomimetics targeting neuropilin-1: synthesis, molecular docking study and in vitro biological activities

Richard Mylène\textsuperscript{a,b}, Chateau Alicia\textsuperscript{c,d}, Jelsch Christian\textsuperscript{e,f}, Didierjean Claude\textsuperscript{e,f}, Manival Xavier\textsuperscript{g,h}, Charron Christophe\textsuperscript{g,h}, Maigret Bernard\textsuperscript{i}, Barberi-Heyob Murie\textsuperscript{c,d}, Chapleur Yves\textsuperscript{a,b}, Boura Cédric\textsuperscript{c,d}, Pellegrini-Moise Nadia\textsuperscript{a,b}\textsuperscript{*}

\textsuperscript{a}: SRSMC UMR 7565, Université de Lorraine, BP 70239, F-54506 Vandœuvre-lès-Nancy, France
\textsuperscript{b}: SRSMC UMR 7565, CNRS, BP 70239, F-54506 Vandœuvre-lès-Nancy, France
\textsuperscript{c}: CRAN UMR 7039, Université de Lorraine, BP 70239, F-54506 Vandœuvre-lès-Nancy, France
\textsuperscript{d}: CRAN UMR 7039, CNRS, BP 70239, F-54506 Vandœuvre-lès-Nancy, France
\textsuperscript{e}: CRM2 UMR 7036, Université de Lorraine, BP 70239, F-54506 Vandœuvre-lès-Nancy, France
\textsuperscript{f}: CRM2 UMR 7036, CNRS, BP 70239, F-54506 Vandœuvre-lès-Nancy, France
\textsuperscript{g}: IMoPA UMR 7365, Université de Lorraine, BP 70239, F-54506 Vandœuvre-lès-Nancy, France
\textsuperscript{h}: IMoPA UMR 7365, CNRS, BP 70239, F-54506 Vandœuvre-lès-Nancy, France
\textsuperscript{i}: UMR 7503 LORIA, BP 70239, 54506 Vandoeuvre-lès-Nancy, France

\textbf{ARTICLE INFO}

Neuropilin-1 (NRP-1), a transmembrane glycoprotein acting as a co-receptor of VEGF-A, is expressed by cancer and angiogenic endothelial cells and is involved in the angiogenesis process. Taking advantage of functionalities and stereodiversities of sugar derivatives, the design and the synthesis of carbohydrate based peptidomimetics are here described. One of these compounds (56) demonstrated inhibition of VEGF-A\textsubscript{165} binding to NRP-1 (IC\textsubscript{50} = 39 µM) and specificity for NRP-1 over VEGF-R2. Biological evaluations were performed on human umbilical vein endothelial cells (HUVECs) through activation of downstream proteins (AKT and ERK phosphorylation), viability/proliferation assays and in vitro measurements of anti-angiogenic abilities.

\textbf{Keywords:}
Neuropilin-1; VEGF; Carbohydrate scaffolds; Peptidomimetics; Molecular modeling

\textbf{1. Introduction}

Angiogenesis or formation of new blood vessels requires the binding of signaling molecules, such as vascular endothelial growth factor (VEGF), which is one of the most specific and important growth factors involved in this process.\textsuperscript{1,2} VEGF-A\textsubscript{165} mediates its biological effects through receptors located on the endothelial cells, \textit{i.e.} VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR). VEGF-A\textsubscript{165} is overexpressed by a wide variety of human tumors and this overexpression has been correlated with invasion and metastasis.\textsuperscript{3}

Interestingly, Neuropilin-1 (NRP-1), a receptor protein firstly described in neuronal guidance,\textsuperscript{4} is involved in a wide range of physiological and pathological processes including angiogenesis.\textsuperscript{5} This transmembrane protein was found to be a co-receptor of VEGF-A\textsubscript{165} acting together with VEGFR-2 via its NRP-1 b1/b2 domain, resulting in increased affinity of VEGF-A\textsubscript{165} for the extracellular domain of VEGFR-2.\textsuperscript{4} VEGF-A\textsubscript{165} can also contribute to VEGFR-2/NRP-1 complex formation via its binding through two different sites for both NRP-1 and VEGFR-2.\textsuperscript{7}

Moreover, independently of VEGFR-2, NRP-1 alone might transduce functional signaling as a result of VEGF binding.\textsuperscript{8} NRP-1 is expressed on endothelial cells promoting tumor angiogenesis\textsuperscript{6,8} and on tumor cells and has thus been identified as a potential target for anti-angiogenic therapies.\textsuperscript{10,11} This receptor could also be a mediator of choice for cancer imaging. Few attempts to use NRP-1 in cancer imaging via a [99mTc]-labelled peptide,\textsuperscript{12} multifunctional nanoparticles engineered with gadolinium chelates as MRI contrast agents,\textsuperscript{13} and fluorescent-labeled peptides are reported in the literature.\textsuperscript{14} More recently, NRP-1 targeting peptides were conjugated onto the surface of lipid microbubbles for molecular imaging of tumor angiogenesis.\textsuperscript{15}

Targeting NRP-1 with small molecules mimicking VEGF-A\textsubscript{165} is not very advanced, because of difficulty to mimic protein-protein interactions. Nevertheless, several peptides have been reported to modulate VEGF-A\textsubscript{165}/NRP-1 binding. The first crystal structure of the b1 domain of the human NRP-1 was determined by Lee et al.\textsuperscript{16} The role of the loops at b1 domain of human NRP-
1 as a target binding site for ligand interaction has also been highlighted by Vander Kooi et al. with their study of NRP-1 binding with tuftsin (TKPR), which is very similar to the VEGF-A165 C-terminus (DKPRR). The C-terminal arginine of tuftsin contributes to the majority of interactions with NRP-1 and this was confirmed by a molecular modeling approach developed by Haspel et al. The interaction of NRP-1-b1 domain with VEGF-A165 was recently elucidated by Parker et al. Jia et al. have discovered a specific bicyclic peptide EG3287 antagonist of VEGF-A165 binding to NRP-1. Heptapeptide ATWLLPR (A7R), selected by screening a phage display library was described as an effective antagonist and can be considered as a potent inhibitor of tumor angiogenesis. NRP-1 targeting photodynamic therapy (PDT) using A7R as NRP-1 ligand associated with a porphyrin-like sensitizer has since been developed. New peptides structurally related to VEGF-A165 exon-7 and -8 domains were recently designed and synthesized.

However, peptidic compounds could suffer from poor bioavailability and instability and the design of new organic molecules such as peptidomimetic derivatives seems an attractive alternative. In connection with our ongoing program on the design of sugar-based peptidomimetics, we described a few years ago the design, synthesis and in vitro biological evaluation of sugar-based peptidomimetics targeting NRP-1. Interesting compounds were obtained and one hit compound was identified (compound 1, Chart 1a). Simultaneously, a paper by Jarvis et al. described compound EG00229 as small molecule inhibitor of the NRP-1/VEGF-A165 binding and this molecule has recently shown in vivo activity towards cancer models (Chart 1a). More recently, a fully non-peptidic compound comprising phenylbenzimidazole and benzoxadiazole moieties linked by a stable carbonylthiourea spacer was identified. New antagonists structurally-related to this compound were synthesized and evaluated toward VEGF-A165/NRP-1 binding modulation. Several drug-like compounds containing a common chlorobenzyloxy alklyoxy halogenobenzyl amine scaffold were identified by virtual screening for their efficient binding to NRP-1.

The objective of the present work was the molecular design and synthesis of new NRP-1 ligands inspired by compound 1. The use of carbohydrates as scaffolds to construct bioactive compounds and peptidomimetics is a well-accepted concept since the pioneering works related to peptidomimetics of somatostatin. We wanted to take advantage of functionalities available in monoprotected diaminoalkanes (n = 2, 3 and 4 for Scheme 1A and 1B). The knownexo-glycal 2 was converted to C-glycoside 4 by stereoselective reduction of the double bond. Subsequent saponification of 4 followed by coupling with tryptamine led to amide 6. Alcohol 7 was prepared by a multistep sequence including the selective removal of the exocyclic isopropylidene acetal under acidic conditions, NaOEt mediated oxidative cleavage and reduction of the resulting aldehyde. The alcohol 7 was then converted in the corresponding tosylate 8 in excellent yield. In the same way,exo-glycal 3 was converted in C-glycoside 18. After changing acetate protecting group at O7 for a tert-butyldimethylsilyl ether, subsequent saponification of 20 led to the key acid 21 in good yield. Amide 22 was prepared by coupling with tryptamine. Removal of the tert-butyldimethylsilyl group was achieved by TBAF and the resulting alcohol was converted in tosylate 24 in 87% yield.

The synthetic pathway for the first series required the preparation of the key tosylates 8 and 24 (Scheme 1A and 1B). The knownexo-glycal 2 was converted to C-glycoside 4 by stereoselective reduction of the double bond. Subsequent saponification of 4 followed by coupling with tryptamine led to amide 6. Alcohol 7 was prepared by a multistep sequence including the selective removal of the exocyclic isopropylidene acetal under acidic conditions, NaOEt mediated oxidative cleavage and reduction of the resulting aldehyde. The alcohol 7 was then converted in the corresponding tosylate 8 in excellent yield. In the same way,exo-glycal 3 was converted in C-glycoside 18. After changing acetate protecting group at O7 for a tert-butyldimethylsilyl ether, subsequent saponification of 20 led to the key acid 21 in good yield. Amide 22 was prepared by coupling with tryptamine. Removal of the tert-butyldimethylsilyl group was achieved by TBAF and the resulting alcohol was converted in tosylate 24 in 87% yield.

Refluxing tosylates 8 and 24 with different commercially available monoprotected dianimokanes (n = 2, 3 and 4 for 8 and n = 1 for 24, Scheme 1C) in a sealed flask led to intermediates 9, 10, 11 and 25. Free amines obtained in quantitative yields after removal of benzoylcarbonyl group were treated with N,N'-bis(benzoylcarbonyl)-S-methylisothiourea in DMF. Bis-5-aminopyrimidinone derivatives 12, 13, 14 and 26 were obtained in modest yields, resulting from the reaction of both primary and secondary amines. Finally, removal of protecting groups afforded the expected bis-guanidinoglycosides 15, 16, 17 and 27. Attempts to remove the 4,5-isopropylidene group under different acidic conditions were unsuccessful, the number of nitrogen atoms obviously preventing oxygen protonation required for acetal deprotection.
On the basis of structure 1, we next focused our attention on the replacement of the guanidine function anchored on the secondary amine by a 1,2,3-triazole moiety bearing guanidylated arms (Scheme 2). This synthetic approach required the preparation of two guanidylated alkynes 28 and 29 obtained by a Mitsunobu reaction. The C-glycoside 30 bearing an azido group on C7 was obtained by reaction of sodium azide with tosylate 8. The synthesis of the 1,4-substituted-1,2,3-triazole moiety was next envisioned between azide 30 and alkynes 28 and 29 in a 1/1 H₂O/CH₂Cl₂ mixture, which was the more appropriate solvent in this case.

Compounds 31 and 32 were obtained in good yields. Removal of benzoxycarbonyl protecting groups was achieved by hydrolysis and led to 33 and 34 in nearly quantitative yields. Removal of 4,5-isopropylidene protecting group by aqueous trifluoroacetic acid was performed and fully deprotected derivatives 37 and 38 were then obtained by hydrolysis.

The synthetic plan for the second series implied the introduction of an L-arginine at C1 and the anchoring of a hydrophobic residue at C7. The coupling of C-glycosyl compound 5 with N⁴-nitro-L-arginine methyl ester led to amide 39 (Scheme 3A). The carboxylic acid and guanidinium function of arginine were successively deprotected by classical methods. We next investigated the impact of a more rigid system based on C-glycosylidene derivative obtained from exo-glycal 2 (Scheme 3B). To this end, using a method for methyl ester deprotection which did not affect the anomeric double bond was mandatory. This was achieved by using lithium iodide in pyridine. The resulting vinlylic acid 41 was then coupled with L-arginine methyl ester without protection on its lateral chain. An isomerization of the double bond was observed and two isomers E/Z were obtained in an approximatively 1/1 ratio. This isomerization could obviously be attributed to reversible addition of HOBt on the anomeric carbon of activated exo-glycal, prone to 1,4-additions. At this point, the purification of the isomeric mixture was not possible by normal or reverse phase chromatography. This mixture was thus subsequently treated by lithium iodide in pyridine and reverse phase purification led to isolation of 42, which is the only one obtained as pure material.

Guided by published works highlighting the favorable impact of an hydrophobic group and docking studies pointing out the benefit of a supplementary chemical groups such as a phenyl (see 2.2), an aromatic residue was linked to C7 via a sulfonamide linkage. This required the preparation of the key intermediate tosylates 45 and 51 obtained from 44 and 19 (Scheme 3C). The selective removal of the exocyclic isopropylidene acetal of 4 under acidic conditions led to diol 43 subsequently transformed in alcohol 44. Substitution with sodium azide carried out in DMF led to azido derivatives 46 and 52 in 84 and 97% yields respectively. Catalytic hydrogenation afforded primary amines which were treated with benzene sulfonyl chloride in pyridine to give phenylsulfonylamides 47 and 53 in good yields. We next managed the introduction of L-arginine at C1 and compounds 49 and 55 were obtained in 84% and 71% yields respectively. Finally, fully deprotected derivatives 50 and 56 were obtained in good yields after methyl ester and arginine side chain protection removal.

---

**Scheme 1.** Synthetic route to compounds 8 (A), 24 (B) and 15-17 and 27 (C). Reagents and conditions: (a) H₂, 15 psi, Pd/C, EtOAc, 25°C, 24h, 4 98%, 18 85%; (b) LiOH, THF/H₂O (3/1), 25°C, 18h, 5 98%, 21 65%; (c) tryptamine, EDC, CH₂Cl₂, 25°C, 18h, 6 80%, 22 80%; (d) i: 1N HCl, MeOH, 0°C to 25°C, 8h; ii: NaOH, MeOH, 25°C, 18h, iii: NaBH₄, MeOH, 0°C, 1h, 72% for three steps; (e) TsCl, Et₃N, DMAP, CH₂Cl₂, 25°C, 24h, 8 90%, 24 87%; (f) Na⁺, MeOH, 0°C, 1h, 97%; (g) TDBMSci, imidazole, DMF, 25°C, 24h, quantitative yield; (h) TBAF, THF, 0°C, 18h, 95%; (i) iPrOH, 100 °C, sealed tube, 48h, 9 72%, 10 70%, 11 80%, 25 82%; (j) i: H₂, 30 psi, Pd/C, MeOH, 18h, quantitative yields, ii: N,N′-bis(benzyloxycarbonyl)-S-methylisothiourea, HgCl₂, Et₃N, DMF, 25°C, 14h, 12 25%, 13 25%, 14 35%, 26 30%; (k) H₂, 30 psi, Pd/C, MeOH, 18h, 15 90%, 16 85%, 17 94%, 27 90%.

**Scheme 2.** Synthetic route to compounds 33-34 and 37-38. Reagents and conditions: (a) DEAD, PPh₃, N,N′-bis(benzyloxycarbonyl)guanidine, toluene, 0°C then 25°C, 4h, 28 92%, 29 89%; (b) Na₃PO₄, DMF, 80°C, 2 days, 88%; (c) CuSO₄, sodium ascorbate, 28 or 29, CH₂Cl₂, H₂O, 25°C, 24h, 31 80%, 32 70%; (d) H₂, 30 psi, Pd/C, MeOH, 18h, 33 90%, 34 90%, 37 80%, 38 95%; (e) TFA/H₂O (1/1), 25°C, 10h, 35 85%, 36 53%.
Molecular modeling studies highlighted that presence of a negative or positive charge in para position on the aromatic ring could led to improved affinity (see 2.2). Taking into account preliminary inhibition results (see 2.3), we thus planned to functionailize 56 with substituents able to make additional hydrogen bonds or salt bridges in the receptor binding site. In this regard, an aminomethyl and a hydroxycarboxymethyl groups were added in 55 and 56, respectively, to improve the interaction with L-arginine methyl ester and treatment with lithium hydroxide gave acid 57 in 86% yield for two steps. Finally, fully deprotected derivative 60 was obtained by catalytic hydrogenation of azido group.

Scheme 3. Synthetic route to compounds 40 (A), 42 (B) and 50 and 56 (C). Reagents and conditions: (a) H-L-Arg(NO2)-OMe.2HCl, HATU, DIEA, DMF, 0°C then 25°C, 24h, 50%; (b) i: LiOH, THF/H2O 3/1, 25°C, 18h, ii: H2, 40 psi, Pd/C, MeOH, 18h, 71%; (c) LiI, pyridine, 25°C, 6h, 82%; (d) i: L-Arg-OMe.HCl, HATU, DIEA, DMF, 0°C then 25°C, 18h, ii: LiOH, THF/H2O, 25°C, 8h, 52%, g; (e) 1N HCl, MeOH, 0°C then 25°C, 6h, ii: H2, 15 psi, Pd/C, MeOH, 8h, 58%, f; (f) i: NaIO4, MeOH, 25°C, 18h, ii: NaBH4, MeOH, 0°C, 1h, 93% for two steps; (g) TsCl, Et3N, DMAP, CH2Cl2, 25°C, 18h, 92%; (h) ethynyltrimethylsilane, CuI, PdCl2(PPh3)2, Et3N, 80°C, 2h, 80%; (i) 68, Cu(OAc)2, sodium ascorbate, tBuOH/H2O, 25°C, 18h, 77%.

Schemes 4. Synthetic route to compounds 61 (A), 63 (B) and 69 (C). Reagents and conditions: (a) i: H2, 15 psi, Pd/C, MeOH, 8h, quantitative yield, j; ii: 4-bromomethylphenylsulfonyl chloride, CH2Cl2, Et3N, 25°C, 10h, 51%; (b) Na2S2O4, DMF, 60°C, 15h, quantitative yield; (c) LiOH, THF/H2O, 25°C, 8h, 59 quantitative yield, 66 54% for two steps, 67 67% for two steps; (e) H2, 15 psi, Pd/C, MeOH, 18h, 87%; (f) i: LiOH, THF/H2O, 25°C, 6h, ii: H2, 15 psi, Pd/C, MeOH, 8h, iii: methyl 2-(4-chlorosulfonylphenyl)acetate, pyridine, 25°C, 2h, 30% for three steps; (g) i: H2, 15 psi, Pd/C, MeOH, 8h, quantitative yield, ii: 4-diphenylsulfonyl chloride, pyridine, 25°C, 6h, 82%; (h) ethynyltrimethylsilane, Cuf, PdCl2(PPh3)2, Et3N, 80°C, 2h, 80%; (i) 68, Cu(OAc)2, sodium ascorbate, tBuOH/H2O, 25°C, 18h, 77%.
The synthesis of compound 63 required a slightly modified pathway, notably the saponification of methyl ester 52 before introduction of the sulfonyl chloride. After saponification with lithium hydroxide and catalytic hydrogenolysis of azido ester 52 with Pd/C, sulfonamide 62 was obtained by reaction with methyl 2-(4-chlorosulfonylphenyl)acetate. Coupling with L-arginine methyl ester and removal of both methyl ester functions led to the diacid 63. In connection with ongoing work in our group concerning conjugation of biomolecules with sugar derivatives, compound 56 was coupled with the 2-azidoethyl-6-fluoro-β-D-glucopyranoside 68. Indeed, 2-azidoethyl-6-fluroglycosides are easily prepared and are valuable prosthetic groups for fast and easy labelling of peptides or peptidomimetics by copper(I)-catalyzed azide–alkyne cycloaddition. To this end, derivative 64 was obtained by coupling 4-iodobenzenesulfonyl chloride (Scheme 4C). Sonogashira reaction was then performed with trimethylsilylacetylene. Subsequent removal of methyl ester and trimetysilyl protecting groups was performed and led to alkyne 66 in good yield. As described above, L-arginine methyl ester was introduced on C1 position. The copper(I)-catalyzed azide–alkyne cycloaddition was performed between alkyne 67 and azide 68 and led to fluoro derivative 69 in 77% yield.

2.2. Crystallographic study and molecular modeling

Cocrystallization screenings of NRP-1-b1 fragment were carried out with compound 1 and four others compounds, namely compounds 27, 40, 50 and 56 (Chart 2).

Crystals were obtained in presence of compound 56. They exhibited the same tetragonal crystal packing as those of the free protein fragment but the resolution of the data was substantially improved (1.45 vs. 1.90 Å, see SI Table S1). The structure revealed that the crystallized protein did not form a complex with the synthesized compound. Instead, a bicine molecule was found in the VEGF-A₁₆₅ binding cleft (Figure 1a). The bicine sodium salt was used as pH buffer in the crystallization condition. A sodium atom in octahedral geometry was also found, it is interacting with a bicine hydroxyl-group and five water molecules. The ternary amine and carboxylic group of bicine are hydrogen-bonded to the Tyr353 and Thr349 side-chains, respectively. The bicine molecule also formed interactions with residues of a symmetry related protein molecule (see SI Figures S1, S2). Several methylene hydrogen atoms of bicine form hydrophobic H...Pi and H...H interactions with Tyr297 and Trp301. The Asp320 residue, which forms an important salt bridge with an arginine in the NRP-1/VEGF-A₁₆₅ biological complex and in the complex with EG00229 was in the present structure in interaction with the Na⁺ ion through a water bridge. This NRP-1-b1/bicine complex highlighted the conservation of the anchoring residues of the VEGF-A₁₆₅ binding cleft.
The crystal structures of NRP-1-b1/ligand complexes provided us additional information to guide an in silico optimization of our synthesized ligands. We undertook optimization of structures by molecular modeling and compound 40 was chosen as a starting point. It appeared from preliminary docking calculations that all the obtained poses of the first docked molecule 40 were anchored within the binding site in the same way as the PDB 3I97 EG00229 ligand (Chart 2), namely through a strong ionic interaction involving the guanidinium moiety of the ligand and the protein Asp320. It should be noted that the best pose obtained for the EG00229 compound itself was exactly similar to the pose found in the X-ray structure, therefore validating our docking procedure.26a Depending on poses, several additional favorable H-bond interactions were detected with residues containing hydroxyl groups such as Tyr353, Tyr297, Thr349 and Ser346. It is also noteworthy that these interacting residues are similar to those found in the bicine/NRP1-b1 structure. The best docking poses of 40 and EG00229 were next refined using short 10 ns molecular dynamics runs. The analysis of the different trajectories confirmed the robustness of the ligand guanidine…Asp320 salt bridge which was observed as stable during all simulations (Figure 2a). A supplementary ionic interaction was also established after several ns of the MD simulations stabilizing the position of the arginine terminal-carboxyl of the ligands with the ammonium group of Lys351 (Figure 2b).

Such an interaction between an exposed Lys side chain with the ligand, while in competition with the solvent, is nevertheless observed in many protein/ligand complexes such as the kinases.42 In the case of the X-ray structure complex with EG00229, the presence of an intramolecular hydrogen bond involving the arginine terminal-carboxyl of EG00229 precluded an interaction with Lys351 in the complex. Comparing the behavior of 40 and EG00229 during these MD simulations showed that 40 could present additional stabilizing interactions on the condition to add supplementary chemical groups such as a phenyl moiety at the C7 position. Furthermore, both C6 stereoisomers were considered (scaffolds A and B, Chart 1). Consequently, we checked this hypothesis with both new synthesized compounds 50 (scaffold A) and 56 (scaffold B) which were submitted to the same docking and short MDs protocols. Comparing the interactions found during these MD simulations between 40, 50 and 56 clearly showed that compound 56 should be a better compound, this behavior being mostly due to π-π and π-cation additional interactions coming from the added phenyl ring with Tyr297 or Lys347 side chains (Figure 1b and 1c). These interactions are observed during most of the simulation. According to these simulations, it can also be suggested that expanding the phenyl group with positive or negative charged groups (compounds 61 and 63) or with longer chains such as the one found in 69 could give the possibility to attract the Lys347 or Glu348 or Glu312 charged side chains (Figure 1d).
### 2.3. Biological evaluation

The binding of new derivatives to recombinant NRP-1 protein was determined using a competition assay initially described by Tirand et al.\textsuperscript{43} Binding of compounds to NRP-1 was assessed using biotinylated VEGF-A\textsubscript{165} (bt-VEGF-A\textsubscript{165}), in competition, or not, with an excess of compounds or non labelled VEGF-A\textsubscript{165}. Tuftsin was used as positive control (IC\textsubscript{50} = 25 µM). A preliminary screening with 100 µM inhibitor concentrations was performed. For the most active compounds (inhibition >40% at 100 µM, Table 1), the IC\textsubscript{50} value was determined with concentrations ranging from 10 µM to 500 µM. VEGF might crosslink VEGF-R2 and NRP-1\textsuperscript{26} and it was recently demonstrated that NRP-1 could interact directly with VEGF-R2 without VEGF.\textsuperscript{6b} Thus, it was appropriate to compare binding of new derivatives with NRP-1 and VEGF-R2. The binding of the four best compounds to VEGF-R2 (KDR) were evaluated using a similar competition assay, showing the selectivity for NRP-1 (Table 2).

#### Table 1. Binding inhibition of bt-VEGF-A\textsubscript{165}/NRP-1 (%) in the presence of new compounds for a fixed concentration of 100 µM and IC\textsubscript{50} values.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>Binding inhibition of bt-VEGF-A\textsubscript{165} at 100 µM (%)</th>
<th>IC\textsubscript{50} (µM)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VEGF-A\textsubscript{165}</td>
<td>100± 1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>5 ± 2</td>
<td>nd</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>39 ± 2</td>
<td>nd</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>52 ± 1</td>
<td>120</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>26 ± 3</td>
<td>nd</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>5 ± 3</td>
<td>nd</td>
</tr>
<tr>
<td>7</td>
<td>34</td>
<td>18 ± 2</td>
<td>nd</td>
</tr>
<tr>
<td>8</td>
<td>37</td>
<td>37 ± 1</td>
<td>nd</td>
</tr>
<tr>
<td>9</td>
<td>38</td>
<td>4 ± 7</td>
<td>nd</td>
</tr>
<tr>
<td>10</td>
<td>40</td>
<td>43 ± 2</td>
<td>187 ± 19</td>
</tr>
<tr>
<td>11</td>
<td>42</td>
<td>44 ± 1</td>
<td>181± 22</td>
</tr>
<tr>
<td>12</td>
<td>50</td>
<td>55 ± 2</td>
<td>88 ± 8</td>
</tr>
<tr>
<td>13</td>
<td>56</td>
<td>67 ± 1</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>14</td>
<td>61</td>
<td>40 ± 2</td>
<td>134 ± 2</td>
</tr>
<tr>
<td>15</td>
<td>63</td>
<td>18 ± 1</td>
<td>nd</td>
</tr>
<tr>
<td>16</td>
<td>69</td>
<td>57 ± 1</td>
<td>69 ± 2</td>
</tr>
</tbody>
</table>

\textsuperscript{a}: IC\textsubscript{50} values were measured for all compounds showing inhibition >40% at 100 µM; results were obtained from three independent experiments, each performed using triplicate determinations at each concentration of compound and are presented as the mean +/- SEM; $R^2$ values ranged from 0.9739 to 0.9927; nd, not determined

#### Table 2. Binding inhibition of bt-VEGF-A\textsubscript{165}/KDR (%) in the presence of compounds 50, 56, 61 and 69 for a fixed concentration of 100 µM and IC\textsubscript{50} values.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>Binding inhibition of bt-VEGF-A\textsubscript{165} for 100 µM (%)</th>
<th>IC\textsubscript{50} (µM)\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VEGF-A\textsubscript{165}</td>
<td>96 ± 5</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>5 ± 6</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>15 ± 13</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>4</td>
<td>61</td>
<td>0 ± 13</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
<td>26 ± 7</td>
<td>306 ± 5</td>
</tr>
</tbody>
</table>

\textsuperscript{c}: IC\textsubscript{50} values were measured for compounds 50, 56, 61 and 69, concentrations ranging from 10 µM to 500 µM; results were obtained from three independent experiments, each performed using triplicate determinations at each concentration of compound and are presented as the mean +/- SEM; $R^2$ value for compound 69: 0.9911

In order to determine the biological activity of compounds on endothelial cells, human umbilical vein endothelial cells (HUVECs) expressing VEGF-R1, VEGF-R2 and NRP-1 were used. It was previously demonstrated that the inhibition of VEGF-A\textsubscript{165} binding to NRP-1 had effect on VEGF-A\textsubscript{165} ability to activate VEGFR-2 (KDR).\textsuperscript{20} Compounds...
50, 56, 61 and 69, which have the higher affinities for NRP-1 (Table 1) were selected for their biological evaluation on HUVECs. Binding of compounds to NRP-1 was evaluated by the activation of PI3K/AKT and MAPK downstream signaling pathways, through phosphorylation of AKT, a serine/threonine protein kinase and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), respectively. Both are involved in NRP-1/VEGFR-2 signaling pathways. The cellular binding of the different compounds to NRP-1 led to a mean of 40% decrease of ERK phosphorylation (ratio ranging from 0.61 to 0.68, Figure 3A et 3B) compared to tuftsin (ratio of 0.88), the natural ligand of NRP-1. In contrast, an increase of AKT phosphorylation was observed for each ligand as well as for tuftsin. It should be noted that the most relevant effect was obtained for compound 56 (ratio 2.52), whereas compound 50 showed a lesser effect (ratio 1.56). Effects on HUVECs viability were measured by metabolic assay (WST) (Figure 3C). Modifications observed for signaling pathways ERK1/2 and AKT did not lead to detrimental effect on the viability of endothelial cells whatever the tested concentration.

Figure 3. A: Effect of the different compounds (c = 50 µM) on the activation of downstream proteins of NRP-1 signaling pathways (pERK1/2 and pAKT) evaluated by western blotting. B: Ratio of phosphorylated protein reported to total protein. Optical densities of each band have been quantified, the expression ratio have been normalized with value of non-treated cells (nt) as reference. tuf = cells treated with tuftsin. C: Effect of the different compounds on HUVECs viability measured by a metabolic test (WST) at different concentrations from 12.5 µM to 200 µM. No statistically significant difference observed between the different compounds. n =3.

The anti-angiogenic properties were evaluated through the in vitro ability of HUVECs to form tubules-like capillaries on a basement matrix (Figure 4). In vitro angiogenesis assay using HUVEC seeded on Matrigel is a well-established assay to screen the angiogenic effect of many substances. Among the tested compounds, 69 demonstrated a wide effect on tubules formation. The effects of 69 could be compared to tuftsin and were relatively close to those observed with bevacizumab, a humanized anti-VEGF monoclonal antibody used nowadays in clinic for cancer therapy and used here as positive control. Inhibition of tubules-like capillaries was also observed to a lesser extent with compound 61.

HUVECs ability to migrate through a basement membrane matrix was measured via an invasion assay and most of tested compounds showed an unexpected slight pro invasive effect (see SI, Figure S3).
Figure 4: Effect of the different compounds (c = 50 µM) on HUVECs ability to form capillaries-like structures on basement membrane matrix. A: Images were representative of three independent experiments of endothelial cells cultured on Matrigel™ in presence of the different compounds. The junctions number. B segments number C as well as the total segments length D were quantified by Angiogenesis Analyser for ImageJ. nt, non treated; tuf, tuftsin; beva, bevacizumab. Results are presented as mean ± standard error of the mean of three independent experiments and p<0.05 was considered to be statistically significant.

3. Discussion

The first series of analogs exhibited a VEGF-A165/NRP-1 inhibition which did not exceed 40%, except for compound 17. The effect of the linker length of separating both guanidium functions was difficult to evaluate, since compound 15 inhibited very weakly VEGF-A165/NRP-1 binding (entry 2, Table 1), while a modest activity was observed for 16 and 17 at 100 µM concentration (entries 3-4). Compound 27, based on sugar scaffold B and functionalized in the same manner as compound 1 was less active than this latter, suggesting a non-adapted spatial disposition of crucial residues (entry 5). Compounds 34, 33 and the corresponding diols 38 and 37 showed a weak affinity for NRP-1, suggesting that triazole moiety could be too rigid linker and deleterious for binding (entries 6-9).

The second series showed general binding inhibition properties higher than 40% at 100 µM, excepted for compound 63. Arginine anchored at C1 was beneficial for NRP-1 binding, as shown for compound 50 and 56 (entries 12-13). Compound 56 based on scaffold B showed a 67% binding inhibition at 100 µM and an IC50 of 39 µM, this result being in agreement with in silico predictions (see 2.2). However, the presence of amino-methyl and hydroxy-carboxymethyl groups recommended by molecular modeling was not beneficial for binding (entries 14-15), suggesting that the added chains were not long enough. Compound 69 obtained by conjugation with the 6-fluoroglycoside 68 displayed a 70% binding inhibition at 100 µM and an IC50 of 70 µM, pointing out that the presence of the sugar derivative was not deleterious for NRP-1 binding.

The biological properties of the compounds having the higher affinity were evaluated on HUVECs firstly through the downstream signaling pathways of NRP-1. All the compounds induced a similar change of ERK1/2 signaling pathways, which did not really impact the viability of endothelial cells even with a high concentration of 200 µM. Nevertheless, some compounds showed a significant effect on angiogenesis network formation. Unexpectedly, while compound 69 did not have the best affinity (IC50 = 69 µM), this latter demonstrated the most efficient properties toward inhibition of tubules formations. On the contrary, compound 56, the most affine compound (IC50 = 39 µM), showed a lack of anti-angiogenic effect, which could be explained by the significant increase of AKT phosphorylation with this compound (ratio 2.52). Indeed, AKT signaling pathway is well known to induce cell survival that limit the endothelial cell network remodeling during angiogenic process. The complexation of NRP-1 with its two well-known co-receptors, VEGFR and plexin (receptor of semaphorins), which have opposite effect on endothelial cell migration and tubulogenesis, could explain the results obtained with the different compounds. Indeed, the binding domains for semaphorin (a1/a2) are relatively close to the VEGF-A165 binding domains (b1/b2) and some compounds could also interact partially with a1/a2 semaphorin domains. Compounds 50, 56 and 61 which induced a slight invasive effect, could modify the signaling pathway involving the plexin/semaphorine/NRP-1 ternary complex. On the contrary, compound 69 seems to be more specific of the b1/b2 domains of VEGF due to its similar effect to tuftsin and to a lesser extent to bevacizumab. Consequently, despite a good affinity for NRP-1 attested by decrease of MAPK-induced activation, an evaluation of all...
signaling pathways regulated by NRP-1 could elicit elucidation of the different cellular effects observed with the tested compounds.

4. Conclusion

We have synthesized two series of carbohydrate-based peptidomimetics targeting NRP-1. The design of these ligands was based on rational modification of compound 1 to enhance binding interaction. Compound 56 inhibited VEGF-A165/NRP-1 binding with an IC50 = 39 µM, which is in the range of activity described by others working on NRP-1 inhibitors. Compound 56 demonstrated specificity for NRP-1 over VEGF-R2 binding more than 10-fold. No cytotoxic effects on HUVECs proliferation or viability were measured for all tested compounds. Interestingly, compound 69 (IC50 = 69µM) demonstrated a significant effect on tubules formation which could be compared to tuftsin and to a lesser extent to bevacizumab without promoting the endothelial cells migration. In addition to its use as a potential anti-angiogenic compound, this compound could be envisaged to target cells overexpressing NRP-1 as cancer cells.

5. Experimental section

5.1. Chemistry

Reagents, general methods, experimental procedures for synthesis and characterizations of all intermediates are given in SI. Compounds 2-6 have been prepared according to literature procedures.25

\[ \text{N}^\alpha-[2-C-(2,3-O-(1-methylthiylidine)-5-benzosulfonylamino-\alpha-D-galactofuranosyl(carbonylmethyl)}-L-arginine methyl ester 50 \]

To a solution of 49 (500 mg, 0.85 mmol) in THF (20 mL) and water (3 mL) was added LiOH (65 mg, 2.55 mmol, 3 eq.) and the mixture was stirred until completion of the reaction monitored by tlc. Amberlite® IR-120 was added until pH = 3-4 and the mixture was filtered off. The solvent were evaporated and the obtained carboxylic acid was solubilized in MeOH (20 mL) and Pd/C (10%) (40% w/w) was added. After stirring under H2 atmosphere (40 psi) until completion of the reaction, the mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo. The crude was purified by column chromatography (C-18 silica gel, H2O/MeOH) and lyophilized to provide 50 (367 mg, 82%).

```
61 \[ \text{N}^\alpha-[2-C-(2,3-O-(1-methylthiylidine)-5-benzosulfonylamino-\alpha-D-ribofuranosyl(carbonylmethyl)}-L-arginine methyl ester 56 \]
```

Prepared starting from 55 (150 mg, 0.26 mmol) following procedure described for 50 Yield: 70% (94 mg). White solid. Rf: 0.22 (H2O/Methanol: 1/1). HPLC (70/30): tR = 5.526 min. [α]D = +17.1c (c = 0.1, H2O). 1H NMR (250 MHz, D2O): δ = 1.37 (s, 3H, C(CH3)2), 1.52 (s, 3H, C(CH3)3), 1.59-1.95 (m, 4H, CH2 arg), 2.63 (dd, 1H, Jf = 14.5, Jh = 7.5 Hz), 2.72 (dd, 1H, Jf = 6.5 Hz, H-2'), 3.06 (dd, 1H, Jh = 14.0, Jh = 8.0 Hz, H-7'), 3.14 (dd, 1H, Jh = 6.0 Hz, H-7'), 3.23 (t, 2H, Jh = 7.0 Hz, CH2 arg), 4.12 (dd, 1H, Jh = 6.0 Hz, H-5), 4.84 (dd, 1H, Jh = 4.0 Hz, H-6), 7.66-7.80 (m, 3H, 2H-Arg), 7.91-7.95 (m, 2H, H-Arg). 13C NMR (62 MHz, D2O): δ = 23.6, 24.4, 25.0 (2C), 28.7 (CH2 arg), 35.2 (C-2), 40.7 (CH3 arg), 41.8 (C-1'), 54.7 (C-α), 77.3 (C-3), 79.1 (C-6), 80.3 (C-5), 81.1 (C-4), 112.8 (C(CH3)), 126.7 (C-2 Arg), 129.5 (C-2 Arg), 133.6 (C-Arg), 137.9 (C-Arg), 156.7 (C=N), 171.9 (C=O), 178.4 (C=O). IR (pellets): v = 3360, 3180, 2990, 2943, 2876, 1645, 1586. ESI-HRMS: m/z calcd for C23H21N5O13Na [M+Na]+ 550.1942; found 550.1956.

\[ \text{N}^\alpha-[2-C-(2,3-O-(1-methylthiylidine)-5-[(4-aminomethyl)benzo]sulfonylamino-\alpha-D-ribofuranosyl(carbonylmethyl)}-L-arginine methyl ester 61 \]

To a solution of 60 (80 mg, 0.14 mmol) in MeOH (5 mL), was added Pd/C (10%) (8 mg, 10% w/w). After stirring under H2 atmosphere (5 psi) until completion of the reaction, the mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo. The crude was purified by column chromatography (C-18 silica gel, H2O/Methanol) and lyophilized to provide 61 (65 mg, 87%). White solid. Rf: 0.38 (H2O/CH3CN/TFA: 7/3/0.1%). HPLC (70/30): tR = 3.681 min. [α]D = +6.9° (c = 0.1, H2O). 1H NMR (400 MHz, DMSO-d6): δ = 1.25 (s, 3H, C(CH3)2), 1.38 (s, 3H, C(CH3)3), 1.45-1.61 (m, 3H, CH2 arg), 1.73 (m, 1H, CH2 arg), 2.47-2.53 (m, 2H, 2 H-2'), 2.68 (m, 1H, H-7'), 3.07-3.11 (m, 2H, CH2 arg), 3.87 (t, 1H, Jh = 7.0 Hz, H-6'), 4.10-4.17 (m, 3H, 3H-3, 2H-CH2-NH2), 4.20 (td, 1H, Jh = 8.0, Jh = 5.0 Hz, H-α), 4.63 (dd, 1H, Jh = 6.0 Hz, H-5'), 4.66 (dd, 1H, Jh = 3.5 Hz, H-4'), 6.99 & 7.20 (brs, 4H, 4NH), 7.59 (t, 1H, J = 5.5 Hz, NH2), 7.67 (2H, J = 8.0 Hz, 2 H-Arg), 7.84-7.90 (m, 3H, 2H-Arg, NH2), 8.18 (d, 1H, Jf = 8.0 Hz, NH2), 8.28 (br s, 3H, CH2-CH2-NH3). 13C NMR (100 MHz, DMSO-d6): δ = 25.2 (CH2), 25.5 (CH3 arg), 26.6 (CH2 arg), 28.8 (CH3 arg), 35.3 (C-2), 40.7 (CH2 arg), 42.2 (C-7), 42.9 (CH2-NH3), 51.8 (C-α), 77.0 (C-3), 81.3 (C-4), 82.1 (C-6), 83.0 (C-5), 111.8
(C(CH3)2), 127.3 (2 C-Ar), 130.1 (2 C-Ar), 139.0 (C-Ar), 140.7 (C-Ar), 157.1 (C=N), 158.5 (TFA), 170.0 (C=O amide), 173.9 (C=O acid). IR (pellets) v: 3393, 3189, 2990, 2928, 1676, 1546. ESI-HRMS: m/z calcd for C32H47FN8O13SNa [M+Na]+ 825.2860; found 825.2845.

5.1.2. Compound 69

To a solution of alcyne 67 (33 mg, 0.04 mmol, 1 eq.) and azido sugar 68 (15 mg, 0.04 mmol, 1 eq.) in a water/iBuOH mixture (0.5 mL/0.5 mL) were added sodium ascorbate (2.4 mg, 0.008 mmol, 0.2 eq.) and Cu(OAc)2 (1.2 mg, 0.004 mmol, 0.1 eq.). The solution turned progressively pale green and the mixture was stirred at room temperature until completion of the reaction (the blue color reappeared). Chelex® resin (100 mg) was then added to the solution and the suspension was stirred until the solution became colorless. The resin was filtered off and the solvent were removed under reduced pressure. The crude was purified by column chromatography (C-18 silica gel, H2O/MEOH) and lyophilized to provide 69 (37 mg, 77%). White solid. Rf: 0.29 (H2O/MeOH: 1/1). HPLC (70/30): tR = 4.180 min. [α]D2 = -6.7° (c = 0.1, H2O). 1H NMR (400 MHz, D2O): δ = 1.31 (s, 3H, C(CH3)2), 1.46 (s, 3H, C(CH3)2), 1.53-1.61 (m, 2H, CH2 arg), 1.69 (m, 1H, CH2 arg), 1.84 (m, 1H, CH2 arg), 2.59 (dd, 1H, J6,7 = 15.0, J5,6 = 8.5 Hz, H-7), 3.08-3.17 (m, 3H, CH2 arg), 3.26 (m, 1H, H-11), 3.42-3.59 (m, 3H, H-12, H-13, H-14), 4.08 (dd, 1H, J7,8 = 8.5, J6,7 = 6.5 Hz, H-6), 4.14-4.25 (m, 3H, H-3, H-4, H-9), 4.33 (m, 1H, H-10), 4.61 (dd, 2H, J6,7 = 47.5, J6,7 = 2.0 Hz, H-15), 4.68 (d, 1H, J6,7 = 6.0 Hz, H-5), 4.73-4.77 (m, 3H, H-4, 2 H-8), 7.94 (d app., 2H, J = 8.0 Hz, H-Ar), 7.99 (d app., 2H, H-Ar), 8.49 (H-triazole). 13C NMR (62.9 MHz, D2O): δ = 23.9 (CH3), 24.6 (CH2 arg), 25.3 (CH3), 29.1 (CH2 arg), 35.6 (C-2), 40.9 (CH2 arg), 42.7 (C-2), 50.9 (C-8), 54.8 (C-α), 68.5 (d, JCF = 7.0 Hz, C-13), 68.7 (C-9), 73.1 (C-11), 74.5 (d, JCF = 18.0 Hz, C-14), 75.7 (C-12), 77.0 (C-3), 81.0, 82.4, 82.7 (C-4, C-5, C-6), 82.1 (d, JCF = 168.0 Hz, C-15), 102.9 (C-10), 113.3 (C(CH3)2), 124.4 (C-Ar), 126.7 (C-Ar), 127.8 (2 C-Ar), 134.7 (C-triazole), 138.5 (C-Ar), 146.0 (C-triazole), 156.9 (C=N), 172.0 (C=O), 178.7 (C=O) 19F NMR (235.2 MHz, D2O): δ = -235.16 (td, J1F = 47.0, J2F = 27.0 Hz). IR (ATR) v: 3346, 3236, 1655, 1505, 1150. ESI-HRMS: m/z calcd for C32H47FN8O13SNa [M+Na]+ 825.2860; found 825.2845.

5.2. Molecular modeling

The structures of the investigated compounds were prepared according to the CORINA48 (for the 3D conformers) and MOPAC49 softwares (for the atomic charges). The chemicals were firstly docked using the GOLD software (see SI for details). The protein target was the 3D structure of the b1 domain of NRP-1 as solved by X-ray when bound with the 3D structure of of compounds 27, 40, 50 and 56 (see SI for details). The crystal structure has been deposited at the Protein Data Bank51 with code 5C7G.

5.3. Biocrystallography

A crystallization screening of NRP-1-b1 fragment including a 6-His tag at the terminus was carried out according to the CORINA48 (for the 3D conformers) and MOPAC49 softwares (for the atomic charges). The crystals were firstly docked using the GOLD software (see SI for details). The protein target was the 3D structure of the b1 domain of NRP-1 as solved by X-ray when bound with the small molecule EG002290 (pdb code 3J97).

5.4. Biological assay

General procedure for receptor binding assays: The binding of new derivatives to recombinant NRP-1 protein was determined using a competition assay initially described by Tirand et al. (see details in SI).43

General procedure for HUVECs culture and treatments: HUVECs were collected from umbilical cords as previously described by Jaffe et al. (see details in SI).52

General procedure for NRP1 signaling pathways: For analysis of pAKT and pERK1/2 expression, western blotting was realized as previously described (see details in SI).53

General procedure for cell viability assays: Roche’s WST-1 cell proliferation reagent is a simple, colorimetric assay designed to measure the relative proliferation rates of cells in culture (see details in SI).

Angiogenesis assay: HUVECs were plated (90,000 cells/cm2) onto 24-well plate ibidi precoated with Matrigel™ Basement Membrane Matrix (BD Biosciences, France, see details in SI).

Acknowledgments

This work was supported by The Région Lorraine and Institut Jean Barriol. The Ministère de l’Enseignement Supérieur et de la Recherche is acknowledged for PhD funding to M. Richard. We thank S. Lamanndé-Langle for providing fluoro-β-D-glucopyranoside and C. Collet for help with HPLC analyses. We thank B. Fernette and F. Lachaud for technical assistance.
References and notes


Experimental procedures for synthesis and characterizations of all intermediates and copies of 1H and 13C NMR spectra, Supplementary Material experimental procedures for molecular modeling and biocrystallography. This material is available free of charge via the Internet at protein kinases 1 and 2.


Molecular Networks GmbH, Erlangen, Germany


Supplementary Material

Experimental procedures for synthesis and characterizations of all intermediates and copies of 1H and 13C NMR spectra, crystallographic summary of NRP-1/bicine structure, protein production and purification, biological assays and experimental procedures for molecular modeling and biocrystallography. This material is available free of charge via the Internet at

Abbreviations

MRI, magnetic resonance imaging; AKT, serine/threonine protein kinase; ERK1/2, extracellular signal-regulated protein kinases 1 and 2.
Supporting information

Carbohydrate-based peptidomimetics targeting neuropilin-1: synthesis, molecular docking study and biological activities

Mylène Richard, Alicia Chateau, Christian Jelsch, Claude Didierjean, Xavier Manival, Christophe Charron, Bernard Maigret, Muriel Barberi-Heyob, Yves Chapleur, Cédric Boura, Nadia Pellegrini-Moïse*

Experimental procedures for:
- Synthesis and characterizations of all compounds
- Molecular modeling
- Protein production, purification and biocrystallography
- Biological assays

Table and Figures:
- Table S1. Crystallographic summary of NRP-1/bicine structure
- Figure S1. Ribbon view of the NRP-1 b1 dimer in the tetragonal crystal
- Figure S2. a) Stereo view of the electron density showing the bicine molecule.
  b) Stereoview of the bicine molecule within the binding site surface
- Figure S3. Invasion assay

Copies of $^1$H and $^{13}$C NMR spectra of compounds 7-67, 69
Experimental procedures and characterizations of all compounds

Reagents and general methods
DMF was dried by distillation from calcium hydride. Other solvents and reagents were purchased from commercial sources and used without further purification. TLC analyses were performed using standard procedures on Kieselgel 60 F254 plates (Merck). Compounds were visualized using UV light (254 nm) and 30% methanolic H2SO4/heat as developing agent. Column chromatography was performed on silica gel SI 60 (63-200 µm) or Lichroprep RP-18 (40-63µm) (Merck). FTIR spectra were recorded on a Perkin-Elmer spectr um 1000 on NaCl windows (film) or KBr pellets (cm⁻¹). Melting points were determined with a Tottoli apparatus and are uncorrected. Optical rotations were measured on an Anton Paar MC300 polarimeter. 1H and 13C NMR spectra were recorded on a Bruker spectrometer DPX250 (250 MHz and 62.9 MHz, respectively) and DRX400 (400 MHz and 100.6 MHz, respectively). For complete assignment of 1H and 13C signals, two-dimensional 1H,1H COSY and 1H,13C correlation spectra were recorded. Mass spectra (MS) were recorded on a ESI/QqTOF Bruker spectrometer. Purity of the compounds for biological assays was confirmed to be greater than 95% by high-performance liquid chromatography. HPLC analyses were run on a Waters system (2695 ebpump, auto sampler injector, 2998 PDA detector and 2424 ELSD detector) controlled by the Empower software. Analyses were performed on a Platinium C18 (5 µm, 250x4.6 mm) from Grace with a AcCN/H2O/0.2%TFA mixture (proportions given in brackets) at 1 mL/min.

3,6-Anhydro-2-deoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-7-hydroxy-L-galacto-heptonamide 7
To a stirred solution of 6 (3.73 g, 8.40 mmol) in methanol (180 mL) at 0°C was added dropwise an aqueous solution of 1N HCl (60 mL). After stirring at room temperature until completion of the reaction, sat. aq. NaHCO3 was added until pH = 7. Half of the solvent was removed under vacuum and the product was extracted with CH2Cl2 (3 x 100 mL). The organic layer was dried over MgSO4, filtered and the solvent was evaporated. To a stirred solution of this diol in methanol (180 mL) was added NaIO4 (3.59 g, 16.8 mmol, 2 eq.) under argon. After stirring at room temperature until completion of the reaction, the solvent was removed by half in vacuo. The mixture was diluted with CH2Cl2 (200 mL). The organic layer was washed with water (3 x 75 mL), dried over MgSO4, filtered and the solvent was removed under reduced pressure. The product was used without further purification. To a stirred solution of the obtained aldehyde in MeOH (190 mL) was added NaBH4 (320 mg, 8.40 mmol, 1 eq.) at 0°C. After 1 h at room temperature, the solvent was removed under reduced pressure. The residue was diluted in CH2Cl2 (100 mL) and the organic layer was washed with a solution of 1N HCl (30 mL) and with water until pH = 7. The organic layer was dried over MgSO4, filtered and evaporated under reduced pressure. The crude product 7 (2.26 g, 72%) was used without further purification. Yellow powder. Rf: 0.38 (CH2Cl2/MeOH 9/1). [α]D= +3.0 (c = 1.50, CHCl3). Mp. = 94°C. 1H NMR (250 MHz, CDCl3): δ = 1.29 (s, 3H, C(CH3)3), 2.53-2.68 (m, 3H, 2H-2, OH), 3.00 (t, 2H, Jα,β = 6.5 Hz, 2H-β), 3.51-3.71 (m, 3H, H-6, 2H-α), 3.83-3.89 (m, 3H, H-3, 2H-7), 4.60 (dd, 1H, J4,5 = 6.0, J5,6 = 3.5 Hz, H-5), 4.70 (dd, 1H, J3,4 = 3.5 Hz, H-4), 6.22 (br s, 1H, NHamide), 7.09-7.26 (m, 3H, 3H-Ar), 7.41 (d, 1H, J = 8.0, H-Ar), 7.63 (d, 1H, J = 8.0, H-Ar), 8.55 (br s, 1H, NHindole). 13C NMR (62.9 MHz, CDCl3): δ = 23.4 (CH3), 23.9 (CH3), 24.7 (C-β), 35.1 (C-2), 38.6 (C-α), 59.8 (C-7), 76.9 (C-3), 80.1, 80.2, 80.5 (C-4, C-5, C-6), 110.2, 111.4, 111.6 (2C-Ar, C(CH3)3), 117.6 (C-Ar), 118.3 (C-Ar), 120.9 (C-Ar), 121.4 (C-Ar), 126.3 (C-Ar), 135.3 (C-Ar), 169.5 (C=O). IR (film) ν: 3327, 1645. ESI-HRMS: m/z calcd for C20H27N2O5 [M+H]+ 375.1914; found 375.1898.
3,6-Anhydro-2-deoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylthiethylidene)-7-(4-methylbenzenesulfonyl)-L-galacto-heptonamide 8

To a solution of 7 (1.94 g, 5.2 mmol) in CH₂Cl₂ (50 mL) were added Et₃N (1.8 mL, 13 mmol, 2.5 eq.), DMAP (61 mg, 0.5 mmol, 0.1 eq.) and tosyl chloride (2 g, 10.4 mmol, 2 eq.) and the mixture was stirred for 24 h at room temperature. The solution was washed with 1N HCl (20 mL), sat. aq. NaHCO₃ (20 mL) and brine (20 mL). The organic layer was dried over MgSO₄, filtered and the solvent was evaporated in vacuo. The compound was purified by flash chromatography (silica gel, cyH/EA) to provide 8 (2.47 g, 90%). White solid. Rf: 0.49 (CH₂Cl₂/MeOH 9/1). [α]D = +6.0 (c = 1.0, CHCl₃). Mp = 64°C. ¹H NMR (400 MHz, CDCl₃): δ = 1.21 (s, 3H, CH₃), 1.30 (s, 3H, CH₃), 2.44 (s, 3H, Ph-CH₃), 2.49-2.51 (m, 2H, H-2), 2.98 (t, 2H, J₆,β = 6.5 Hz, H-β), 3.49 (m, 1H, H-α), 3.62 (m, 1H, H-6), 3.68-3.77 (m, 2H, H-α, H-3), 4.12 (dd, 1H, J₆,γ = 7.5, J₇,γ = 11.0 Hz, H-7), 4.25 (dd, 1H, J₆,γ = 4.0 Hz, H-7'), 4.54 (dd, 1H, J₆,γ = 3.5, J₆,δ = 6.0 Hz, H-4), 4.59 (dd, 1H, Jₛ,δ = 4.0 Hz, H-5), 6.13 (t, 1H, J = 5.5 Hz, CONH), 7.08-7.12 (m, 2H, H-2, H-3), 7.19 (m, 1H, H-Ar), 7.33 (d, 2H, J = 8.0, H-Ar), 7.41 (d, 1H, J = 8.0, H-Ar), 7.59 (d, 1H, J = 8.0, H-Ar), 7.79 (d, 2H, J = 8.5 Hz, H-Ar), 8.58 (s, 1H, NH-Ar). ¹³C NMR (100 MHz, CDCl₃): δ = 21.7 (CH₃-Ph), 24.6 (CH₃), 24.9 (C-β), 25.7 (CH₃), 36.4 (C-2), 39.6 (C-α), 68.2 (C-7), 78.5 (C-3), 79.9 (C-6), 80.4 (C-5), 81.4 (C-4), 111.4 (C-Ar), 112.6 (C(CH₃)₂), 112.8 (C-Ar), 118.6 (C-Ar), 119.2 (C-Ar), 122.0 (C-Ar), 122.5 (C-Ar), 127.4 (C-Ar), 128.0 (2 C-Ar), 129.9 (2 C-Ar), 132.5 (C-Ar), 136.5 (C-Ar), 145.2 (C-Ar), 170.3 (C=O). IR (film) ν: 1654, 1535. ESI-HRMS: m/z calcd for C₂₇H₃₂N₂O₇SNa [M+Na]^+ 551.1822; found 551.1852.

3,6-Anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylthiethylidene)-7-[[[(phenylmethoxy)carbonyl]amino][propyl]amino]-L-galacto-heptonamide 9

A solution of 8 (422 mg, 0.8 mmol, 1 eq.) and amine (0.8 mmol, 4 eq.) in isopropanol (4 mL) was heated at 100°C for 48h in a sealed tube. The solvent was removed under reduced pressure and the residue was diluted with EtOAc (10 mL) and washed with sat. aq. NaHCO₃ (2x5 mL). The organic layer was dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. The compound was purified by flash chromatography (silica gel, cyH/EA) to provide 9 (324 mg, 72%). Colorless gum. Rf: 0.33 (CH₂Cl₂/MeOH 9/1). [α]D = +10.3 (c = 0.5, CHCl₃).

¹H NMR (250 MHz, CDCl₃): δ = 1.27 (s, 3H, CH₃), 1.42 (s, 3H, CH₃), 1.59-1.76 (m, 2H, H-9), 2.55-2.59 (m, 2H, H-2), 2.69-2.74 (m, 2H, H-10), 2.77-2.92 (m, 2H, H-7), 2.99 (t, 2H, J₆,β = 7.5 Hz, H-β), 3.13-3.23 (m, 2H, H-8), 3.44-3.79 (m, 4H, H-3, H-6, H-α), 4.55 (dd, 1H, J₄,δ = 6.0, Jₛ,δ = 3.5 Hz, H-5), 4.60 (dd, 1H, J₃,δ = 3.5 Hz, H-4), 5.12 (s, 2H, CH₂Ph), 5.50 (br s, 1H, NH), 6.29 (br s, 1H, NH), 7.05-7.23 (m, 3H, H-Ar), 7.32-7.42 (m, 6H, H-Ar), 7.61 (d, 1H, J = 8.0 Hz, H-Ar), 8.81 (br s, 1H, NH). ¹³C NMR (62.9 MHz, CDCl₃): δ = 24.7 (CH₃), 25.1 (C-β), 25.8 (CH₃), 29.2 (C-9), 36.4 (C-2), 39.5 (C-α), 39.7 (C-8), 47.6 (C-7), 47.9 (C-10), 66.7 (CH₂Ph), 78.1 (C-3), 80.4 (C-6), 81.0 (C-4), 81.5 (C-5), 111.4 (C-Ar), 112.3 (C(CH₃)₂), 112.9 (C-Ar), 118.7 (C-Ar), 119.3 (C-Ar), 122.0 (C-Ar), 122.2 (C-Ar), 127.5 (C-Ar), 128.1 (2 C-Ar), 128.5 (3 C-Ar), 136.4 (C-Ar), 136.6 (C-Ar), 156.8 (OCONH), 170.5 (CONH). IR (film) ν: 3312, 1714, 1645. ESI-HRMS: m/z calcd for C₃₁H₄₀N₆O₇SNa [M+Na]^+ 587.2840; found 587.2866.

3,6-Anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylthiethylidene)-7-[[[(phenylmethoxy)carbonyl]amino][butyl]amino]-L-galacto-heptonamide 10

Prepared starting from 8 (422 mg, 0.8 mmol) following procedure described for 9. Yield: 70% (323 mg). Colorless gum. Rf: 0.29 (CH₂Cl₂/MeOH 9/1). [α]D = +9.1 (c = 1.0, CHCl₃).

¹H NMR (400 MHz, CDCl₃): δ = 1.26 (s, 3H, CH₃), 1.41 (s, 3H, CH₃), 1.50-1.56 (m, 4H, H-9, H-10), 2.55 (dd, 2H, J₆,β = 6.5 Hz, J₂,δ = 4.0 Hz, J₂,δ = 2.0 Hz, H-2), 2.61-2.65 (m, 2H, H-11), 2.81 (dd, 1H, J₆,β = 12.5 Hz, J₆,δ = 7.5 Hz, H-7), 2.88 (dd, 1H, J₆,δ = 4.5 Hz, H-7'), 2.98 (t, 2H, J₆,β = 6.5 Hz, H-β), 3.17-3.21 (m, 2H, H-8), 3.52-3.61 (m, 2H, H-α, H-6), 3.65 (m, 1H, H-α), 3.74
(m, 1H, H-3), 4.49-4.55 (m, 2H, H-4, H-5), 5.10 (s, 2H, CH2-Ph), 5.47 (br s, 1H, NH), 6.26 (t, 1H, J = 5.5 Hz, NH), 7.05 (s, 1H, H-AR), 7.12 (m, 1H, H-AR), 7.20 (m, 1H, H-AR), 7.31-7.40 (m, 6H, H-AR), 7.61 (d, 1H, J = 8.0 Hz, H-AR), 8.72 (br s, 1H, NH). 13C NMR (100 MHz, CDCl3): δ = 24.7 (CH3), 25.1 (C-β), 25.8 (CH3), 26.6, 27.7 (C-9, C-10), 36.3 (C-2), 39.7 (C-α), 40.8 (C-8), 47.9 (C-7), 49.4 (C-11), 66.6 (CH2-Ph), 78.0 (C-3), 80.2 (C-6), 81.0, 81.5 (C-4, C-5), 111.4 (C-AR), 112.3 (C(CH3)2), 112.8 (C-AR), 118.7 (C-AR), 119.3 (C-AR), 122.0 (C-AR), 122.3 (C-AR), 127.4 (C-AR), 128.2 (2 C-AR), 128.5 (3 C-AR), 136.4 (C-AR), 136.7 (C-AR), 156.6 (OCNHN), 170.5 (CONH). IR (film) v: 3312, 1716, 1648. ESI-HRMS: m/z calcd for C32H42N6O6Na [M+Na]+ 601.2997; found 601.3002.

3.6-Anhydro-2,7-dideoxy-β-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-7-[[N-[[phenylmethoxy]carbonyl]amino][pentyl][amino]-1-galacto-heptonamide 11

Prepared starting from 8 (422 mg, 0.8 mmol) following procedure described for 9. Yield: 80% (378 mg). Colorless gum. Rf: 0.37 (CH2Cl2/MeOH 9/1). [α]D = +5.5 (c = 1.0, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.27 (s, 3H, CH3), 1.43 (s, 3H, CH3), 1.29-1.53 (m, 6H, H-9, H-10, H-11), 2.57 (d, 2H, Jα,β = 6.5 Hz, H-2), 2.62 (t, 2H, J1,1,12 = 7.0 Hz, H-12), 2.82-2.92 (m, 2H, H-7), 2.98 (t, 2H, Jα,β = 7.5 Hz, H-β), 3.16-3.21 (m, 2H, H-8), 3.54-3.65 (m, 3H, H-6, H-α), 3.81 (m, 1H, H-3), 4.56 (dd, 1H, Jq,α = 6.0, Jq,β = 3.5 Hz, H-5), 4.61 (dd, 1H, Jα,β = 3.5 Hz, H-4), 4.92 (br s, 1H, NH). 5.11 (s, 2H, CH2-Ph), 6.16 (t, 1H, J = 5.5 Hz, NH), 7.05 (s, 1H, H-AR), 7.12 (m, 1H, H-AR), 7.20 (m, 1H, H-AR), 7.31-7.39 (m, 6H, H-AR), 7.62 (d, 1H, J = 8.0 Hz, H-AR), 8.78 (br s, 1H, NH). 13C NMR (62.9 MHz, CDCl3): δ = 24.7 (C-10), 24.7 (CH3), 25.2 (C-β), 25.9 (CH3), 29.2, 29.7 (C-9, C-11), 36.3 (C-2), 39.7 (C-α), 40.9 (C-8), 48.0 (C-7), 49.7 (C-12), 66.6 (CH2-Ph), 78.0 (C-3), 80.5 (C-6), 81.1 (C-4), 81.5 (C-5), 111.3 (C-AR), 112.3 (C(CH3)2), 112.8 (C-AR), 118.7 (C-AR), 119.3 (C-AR), 122.0 (C-AR), 122.3 (C-AR), 127.4 (C-AR), 128.1 (2 C-AR), 128.5 (3 C-AR), 136.4 (C-AR), 136.7 (C-AR), 156.5 (OCNHN), 170.4 (CONH). IR (film) v: 3312, 1704, 1652. ESI-HRMS: m/z calcd for C33H44N4O6Na [M+Na]+ 615.3153; found 615.3137.

3.6-Anhydro-2,7-dideoxy-β-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-7-[[N-[[bis[phenylmethoxy]carbonyl]amino][methylomethyl]-N-[3-[bis[phenylmethoxy]carbonyl]guanidinopropyl][amino]-1-galacto-heptonamide 12

To a solution of 9 (282 mg, 0.5 mmol) in MeOH (10 mL) was added Pd/C (10%) (30 mg, 10% w/w). After stirring under H2 atmosphere (30 psi) until completion of the reaction, the mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo. The crude was solubilized in DMF (3 mL) and N,N'-bis(dibenzyloxycarbonyl)-S-methylisothiourea (1.0 mmol, 2eq.), HgCl2 (134 mg, 0.5 mmol, 1 eq.) and EtsN (153 mg, 1.5 mmol, 3 eq.) were added under argon atmosphere. After stirring 14 h at room temperature, the solvent was removed in vacuo and the residue was dissolved in EtOAc (40 mL). The organic layer was washed with water (5 mL), brine (5 mL), dried over MgSO4, filtered and the solvent was removed under reduced pressure. The compound was purified by column chromatography (silica gel, cyH/EA) to provide 12 (131 mg, 25%). White solid. Rf: 0.52 (EA). [α]D = +5.1 (c = 0.2, CHCl3). Mp = 65°C. 1H NMR (250 MHz, MeOH d4): δ = 1.21 (s, 3H, CH3), 1.31 (s, 3H, CH3), 1.80-1.92 (m, 2H, H-9), 2.46-2.49 (m, 2H, H-2), 2.91 (t, 2H, Jq,α = 7.0 Hz, H-β), 3.36-3.68 (m, 8H, H-7, H-8, H-10, H-α), 3.76-3.84 (m, 2H, H-3, H-6), 4.55 (dd, 1H, Jα,β = 6.0, Jα,α = 3.5 Hz, H-4), 4.62 (dd, 1H, Jα,α = 4.0 Hz, H-5), 4.96-5.03 (m, 4H, CH2-Ph), 5.07 (s, 2H, CH2-Ph), 5.12 (s, 2H, CH2-Ph), 6.94-7.09 (m, 3H, H-AR), 7.22-7.37 (m, 2H, H-AR), 7.55 (d, 1H, J = 8.0 Hz, H-AR). 13C NMR (62.9 MHz, MeOH d4): δ = 25.0 (CH3), 26.1 (C-β), 26.3 (CH3), 27.8 (C-9), 36.7 (C-2), 39.4 (C-10), 41.3 (C-α), 41.5 (C-8), 48.9 (C-7), 68.4 (CH2-Ph), 68.7 (2 CH2-Ph), 69.2 (CH2-Ph), 79.7 (C-3), 80.1 (C-6), 82.1 (C-4), 83.0 (C-5), 112.3 (C-AR), 113.3 (C-AR), 113.7 (C(CH3)2), 119.4 (C-AR), 119.7 (C-AR), 122.4 (C-AR), 123.5 (C-AR), 128.8 (C-AR), 128.9
(C-Ar), 129.0 (2 C-Ar), 129.1 (C-Ar), 129.2 (2 C-Ar), 129.3 (C-Ar), 129.5 (8 C-Ar), 129.6 (C-Ar), 129.7 (4 C-Ar), 136.5 (2 C-Ar), 138.1 (C-Ar), 138.2 (2 C-Ar), 154.6 (2 C=N), 157.4 (2 NHCOO), 164.8 (2 NHCOO), 172.7 (CONH). IR (film) v: 1754, 1730, 1640. ESI-HRMS: m/z calcd for C₇₇H₇₂N₈O₁₂Na [M+Na]+ 1073.4379; found 1073.4374.

3,6-Anhydro-2,7-dideoxy-N-[2-((1H-indol-3-yl)ethyl]-4,5-O-(1-methylethildiene)-7-[[N-[bis((phenylmethoxy)carbonyl)amino]iminomethyl]-N-[4-[bis((phenylmethoxy)carbonyl)guanidinobutyl]amino]-1-galacto-heptonamide 13

Prepared starting from 10 (289 mg, 0.5 mmol) following procedure described for 12. Yield: 25% (133 mg). White solid. Rp: 0.59 (EA). [α]₀ = +10.3 (c = 0.2, CHCl₃). Mp: 68°C. ¹H NMR (250 MHz, MeOH d₄): δ = 1.25 (s, 3H, CH₃), 1.35 (s, 3H, CH₃), 1.42-1.69 (m, 4H, H-9, H-10), 2.50-2.59 (m, 2H, H-2), 2.94 (t, 2H, J₉,β = 7.0 Hz, H-β), 3.37-3.54 (m, 6H, H-8, H-11, H-α), 3.61-3.71 (m, 2H, H-7), 3.76-3.84 (m, 2H, H-3, H-6), 4.59-4.68 (m, 2H, H-4, H-5), 5.04-5.06 (m, 4H, CH₂,Ph), 5.11 (s, 2H, CH₂-Ph), 5.20 (s, 2H, CH₂-Ph), 6.98-7.12 (m, 3H, H-3 Ar), 7.25-7.43 (m, 21H, H-Ar), 7.58 (d, 1H, J = 8.0 Hz, H-Ar). ¹³C NMR (62.9 MHz, MeOH d₄): δ = 25.1 (CH₃), 26.3 (CH₃), 26.4, 27.2, 28.2 (C-β, C-9, C-10), 36.9 (C-2), 41.5 (C-α), 41.7 (C-8), 50.0 (C-7, C-12), 68.5 (CH₂-Ph), 68.9 (2 CH₂-Ph), 69.4 (CH₂-Ph), 79.8 (C-3), 80.4 (C-6), 82.3 (C-4), 83.1 (C-5), 112.4 (C-Ar), 113.4 (C-Ar), 113.9 (C(CH₃)₂), 119.5 (C-Ar), 119.8 (C-Ar), 122.5 (C-Ar), 123.6 (C-Ar), 128.9 (2 C-Ar), 129.0 (2 C-Ar), 129.1 (C-Ar), 129.2 (2 C-Ar), 129.4 (C-Ar), 129.6 (8 C-Ar), 129.7 (C-Ar), 129.9 (4 C-Ar), 136.7 (2 C-Ar), 138.3 (2 C-Ar), 138.4 (C-Ar), 145.8 (C=N), 154.9 (C=N), 157.5 (2 NHCOO), 165.0 (2 NHCOO), 172.9 (CONH). IR (film) ν: 1728, 1638, 1622. ESI-HRMS: m/z calcd for C₅₉H₆₆N₈O₁₃Na [M+Na]+ 1087.4536; found 1087.4542.

3,6-Anhydro-2,7-dideoxy-N-[2-((1H-indol-3-yl)ethyl]-4,5-O-(1-methylethildiene)-7-[[N-[bis((phenylmethoxy)carbonyl)amino]iminomethyl]-N-[5-[bis((phenylmethoxy)carbonyl)guanidinopentyl]amino]-1-galacto-heptonamide 14

Prepared starting from 11 (296 mg, 0.5 mmol) following procedure described for 12. Yield: 35% (188 mg). White solid. Rp: 0.63 (EA). [α]₀ = +12.3° (c = 0.5, CHCl₃). Mp: 70°C. ¹H NMR (400 MHz, MeOH d₄): δ = 1.24 (s, 3H, CH₃), 1.26-1.33 (m, 2H, H-10), 1.35 (s, 3H, CH₃), 1.50-1.64 (m, 4H, H-9, H-11), 2.50-2.52 (m, 2H, H-2), 2.93 (t, 2H, J₉,β = 7.5 Hz, H-β), 3.30-3.51 (m, 6H, H-8, H-12, H-α), 3.58-3.68 (m, 2H, H-7), 3.74 (m, 1H, H-6), 3.84 (m, 1H, H-3), 4.59-4.64 (m, 2H, H-4, H-5), 5.03 (s, 4H, CH₂,Ph), 5.10 (s, 2H, CH₂-Ph), 5.19 (s, 2H, CH₂-Ph), 6.98-7.09 (m, 3H, H-Ar), 7.25-7.40 (m, 21H, H-Ar), 7.57 (d, 1H, J = 8.0 Hz, H-Ar). ¹³C NMR (100 MHz, MeOH d₄): δ = 24.7 (C-10), 24.9 (CH₃), 26.1 (C-β), 26.3 (CH₃), 27.6, 29.4, 30.7 (C-9, C-11), 36.7 (C-2), 41.4 (C-Ar), 41.8 (C-8), 49.1 (C-7), 50.1 (C-12), 68.4 (CH₂-Ph), 68.7 (2 CH₂-Ph), 69.3 (CH₂-Ph), 79.6 (C-3), 80.2 (C-6), 82.1 (C-4), 83.0 (C-5), 112.3 (C-Ar), 113.3 (C-Ar), 113.7 (C(CH₃)₂), 119.4 (C-Ar), 119.6 (C-Ar), 122.3 (C-Ar), 123.5 (C-Ar), 128.9 (C-Ar), 129.0 (4 C-Ar), 129.1 (4 C-Ar), 129.5 (8 C-Ar), 129.7 (4 C-Ar), 136.5 (2 C-Ar), 138.1 (C-Ar), 138.3 (2 C-Ar), 154.8 (2 C=N), 157.3 (2 NHCOO), 164.8 (2 NHCOO), 172.8 (CONH). IR (film) ν: 1759, 1728, 1643. ESI-IRMS: m/z calcd for C₅₈H₆₄N₈O₁₂Na [M+Na]+ 1101.4692; found 1101.4706.

3,6-Anhydro-2,7-dideoxy-N-[2-((1H-indol-3-yl)ethyl]-4,5-O-(1-methylethildiene)-7-[[N-(aminomethyl)-N-(3-guanidinopropyl)amino]-1-galacto-heptonamide 15

To a solution of 12 (110 mg, 0.1 mmol) in MeOH (10 mL) was added Pd/C (10%) (10% w/w). After stirring under H₂ atmosphere (30 ps) until completion of the reaction, the mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo. The crude was purified by column chromatography (C-18 silica gel, H₂O/MeCN/0.1%TFA) and lyophilized to provide 15 (46 mg, 90%). White solid. Rp: 0.27 (H₂O/MeCN/TFA: 6/4/0.1%). HPLC (60/40): tₚ = 4.920 min. [α]₀ = +11.2° (c = 0.1, MeOH). ¹H NMR (250 MHz, MeOH d₄): δ = 1.30 (s,
3H, CH3), 1.45 (s, 3H, CH3), 1.88-1.94 (m, 2H, H-9), 2.58 (d, 2H, Jgem = 7.0 Hz, H-2), 2.95 (t, 2H, Jαβ = 7.0 Hz, H-β), 3.18 (t, 2H, J9,10 = 7.0 Hz, H-10), 3.40-3.78 (m, 7H, H-6, H-7, H-8, H-α), 3.91 (m, 1H, H-3), 4.67 (dd, 1H, J4,5 = 6.0, J3,4 = 3.5 Hz, H-4), 4.77 (m, 1H, H-5), 6.96-7.11 (m, 3H, H-Ar), 7.33 (d, 1H, J = 8.0 Hz, H-Ar), 7.56 (d, 1H, J = 8.0 Hz, H-Ar). 13C NMR (62.9 MHz, MeOH d4): δ = 23.5 (CH3), 25.0, 25.1 (C-β, CH3), 25.8 (C-9), 35.3 (C-2), 38.6 (C-7), 40.4 (C-α), 45.0 (C-8), 48.7 (C-10), 78.8 (C-3), 79.0 (C-6), 81.1 (C-4), 81.6 (C-5), 111.1 (C-Ar), 112.1 (C-Ar), 112.6 (C(CHO2)), 118.1 (C-Ar), 118.4 (C-Ar), 121.2 (C-Ar), 122.3 (C-Ar), 127.7 (C-Ar), 137.0 (C-Ar), 157.6 (C=N), 157.7 (C=N), 171.7 (CONH). IR (film) υ: 3351, 1601, 1607. ESI-MS: m/z calcd for C25H38N8O4Na [M+Na]+ 537.2908; found 537.2930.

3,6-Anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-7-[N-(aminoiminomethyl)-N-(4-guanidinobutyl)amino]-l-galacto-heptonamide 16

Prepared starting from 13 (110 mg, 0.1 mmol) following procedure described for the synthesis of 15. Yield: 85% (45 mg). White solid. Rp: 0.23 (H2O/MeCN/TFA: 6/4/0.1%). HPLC (60/40): tR = 5.138 min. [α]D= +5.0° (c = 0.1, MeOH). 1H NMR (250 MHz, MeOH d4): δ = 1.30 (s, 3H, CH3), 1.46 (s, 3H, CH3), 1.59-1.71 (m, 4H, H-9, H-10), 2.57-2.61 (m, 2H, H-2), 2.95 (t, 2H, Jαβ = 7.0 Hz, H-β), 3.20 (t, 2H, H-11), 3.38-3.69 (m, 6H, H-7, H-8, H-α), 3.78 (m, 1H, H-6), 3.94 (m, 1H, H-3), 4.68 (dd, 1H, J1,4 = 6.0, J3,4 = 3.0 Hz, H-4), 4.83 (m, 1H, H-5), 6.97-7.11 (m, 3H, H-Ar), 7.34 (d, 1H, J = 8.0 Hz, H-Ar), 7.56 (d, 1H, J = 8.0 Hz, H-Ar). 13C NMR (62.9 MHz, MeOH d4): δ = 24.7 (CH3), 25.3 (CH3), 26.2, 26.3, 26.8 (C-β, C-9, C-10), 36.4 (C-2), 41.6 (C-α), 42.2 (C-7), 49.9 (C-8), 50.4 (C-11), 79.0 (C-3), 80.2 (C-6), 82.3 (C-4), 82.8 (C-5), 112.3 (C-Ar), 113.2 (C-Ar), 113.7 (C(CHO2)), 119.3 (C-Ar), 119.6 (C-Ar), 122.3 (C-Ar), 122.5 (C-Ar), 128.8 (C-Ar), 138.1 (C-Ar), 158.6 (C=N), 158.8 (C=N), 172.9 (CONH). IR (film) υ: 3379, 1681. ESI-MS: m/z calcd for C26H40N8O4Na [M+Na]+ 551.3065; found 551.3039.

3,6-Anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-7-[N-(aminoiminomethyl)-N-(5-guanidinopentyl)amino]-l-galacto-heptonamide 17

Prepared starting from 14 (160 mg, 0.15 mmol) following procedure described for the synthesis of 15. Yield: 94% (76 mg). White solid. Rp: 0.20 (H2O/MeCN/TFA: 6/4/0.1%). HPLC (60/40): tR = 5.404 min. [α]D= +6.9° (c = 0.2, MeOH). 1H NMR (250 MHz, MeOH d4): δ = 1.30 (s, 3H, CH3), 1.46 (s, 3H, CH3), 1.30-1.42 (m, 2H, H-9, H-10), 1.56-1.70 (m, 4H, H-9, H-11), 2.59 (d, 2H, J2,3 = 6.5 Hz, H-2), 2.94 (t, 2H, Jαβ = 7.5 Hz, H-β), 3.17 (t, 2H, J1,12 = 7.0 Hz, H-12), 3.36-3.67 (m, 6H, H-α, H-8, H-7), 3.76 (m, 1H, H-6), 3.94 (m, 1H, H-3), 4.68 (dd, 1H, J1,4 = 6.0, J5,6 = 3.5 Hz, H-5), 4.77 (dd, 1H, J3,4 = 3.5 Hz, H-4), 6.97-7.12 (m, 3H, H-Ar), 7.33 (d, 1H, J = 8.0 Hz, H-Ar), 7.57 (d, 1H, J = 8.0 Hz, H-Ar). 13C NMR (62.9 MHz, MeOH d4): δ = 24.6 (CH3), 24.7 (CH3), 26.2, 26.3 (C-β, C-10), 27.6, 29.6 (C-9, C-11), 36.5 (C-2), 41.6 (C-7), 42.3 (C-α), 49.7 (C-8), 50.7 (C-12), 79.9 (C-3), 80.2 (C-6), 82.3 (C-4), 82.8 (C-5), 112.3 (C-Ar), 113.3 (C(CHO2)), 113.7 (C-Ar), 119.3 (C-Ar), 119.6 (C-Ar), 122.3 (C-Ar), 122.4 (C-Ar), 128.8 (C-Ar), 138.2 (C-Ar), 158.7 (C=N), 158.8 (C=N), 172.8 (CONH). IR (film) υ: 3327, 1657, 1617. ESI-MS: m/z calcd for C27H42N8O4Na [M+Na]+ 565.3221; found 565.3200.

3,6-Anhydro-2-deoxy-4,5-O-(1-methylethylidene)-7-O-acetyl-d-altro-heptoanic acid methyl ester 18

To a stirred solution of 3 (2.5 g, 8.7 mmol) in EtOAc (20 mL) was added Pd/C (10%) (1 g, 40% w/w). The mixture was stirred under H2 atmosphere (30 psi) for 18h. The reaction mixture was then filtered through a pad of celite and the solvent was removed in vacuo. The compound was purified by column chromatography (silica gel, cyH/EA) to provide 18 (2.1 g, 85%). Colorless gum. Rp: 0.38 (cH/EA: 2/1). [α]D= -13.3° (c = 0.27, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.34 (s, 3H, CH3), 1.49 (s, 3H, CH3), 2.09 (s, 3H, OCH3), 2.72 (dd, 1H, Jgem = 16.0, J2,3 = 5.0 Hz, H-2), 2.81 (dd, 1H, J2,3 = 6.0 Hz, H-2'), 3.71 (s, 3H, COOCH3), 4.05 (dd, 1H,
the pH was adjusted to 2-3 with 1N HCl. The organic layer was washed with brine and dried by column chromatography (silica gel, cH/EA) to provide butyldimethylsilyl chloride (1.1g, 6.71 mmol, 1.1 eq.) and imidazole (1.04 g, 15.25 mmol, 2.5 eq.).

To a stirred solution of 18 (2.0 g, 6.9 mmol) in methanol (50 mL) was added a catalytic amount of sodium at 0°C under argon atmosphere. After completion of the reaction (approx. 1h), acidic resin Amberlite IR-120 was added until pH = 4. After filtration, the solvent was removed under reduced pressure to provide 19 (1.65 g, 97%). Colorless gum. Rf: 0.10 (cH/EA: 2/1). [α]D = +5.4° (c = 0.52, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.33 (s, 3H, C(CH3)2), 1.49 (s, 3H, C(CH3)2), 1.61 (bs, 1H, OH), 4.27 (dd, 1H, Jgem = 16.5, J2,3 = 7.0 Hz, H-2), 4.41 (dd, 1H, J6,7 = 7.0, J5,6 = 1.0 Hz, H-6), 4.39 (td, 1H, J3,4 = 4.0 Hz, H-3), 4.66 (dd, 1H, J4,5 = 6.0 Hz, H-5), 4.78 (dd, 1H, H-4). 13C NMR (62.9 MHz, CDCl3): δ = 25.2 (CH3), 26.4 (CH3), 34.5 (C-2), 51.9 (OCH3), 62.1 (C-7), 77.2 (C-3), 81.5, 82.6, 84.3 (C-4, C-5, C-6), 113.0 (C(CH3)2), 171.8 (C=O). IR (film): ν: 3464, 2990, 2943, 1737. ESI-HRMS: m/z calcd for C13H20O7Na [M+Na]+ 311.1101; found 311.1119.

3.6-Anhydro-2-deoxy-4,5-O-(1-methylethylidene)-d-altro-heptonic acid methyl ester 19

To a stirred solution of 18 (2.0 g, 6.9 mmol) in methanol (50 mL) was added a catalytic amount of sodium at 0°C under argon atmosphere. After completion of the reaction (approx. 1h), acidic resin Amberlite IR-120 was added until pH = 4. After filtration, the solvent was removed under reduced pressure to provide 19 (1.65 g, 97%). Colorless gum. Rf: 0.10 (cH/EA: 2/1). [α]D = +5.4° (c = 0.52, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.33 (s, 3H, C(CH3)2), 1.49 (s, 3H, C(CH3)2), 1.61 (bs, 1H, OH), 4.27 (dd, 1H, Jgem = 16.5, J2,3 = 7.0 Hz, H-2), 4.41 (dd, 1H, J6,7 = 7.0, J5,6 = 1.0 Hz, H-6), 4.39 (td, 1H, J3,4 = 4.0 Hz, H-3), 4.66 (dd, 1H, J4,5 = 6.0 Hz, H-5), 4.78 (dd, 1H, H-4). 13C NMR (62.9 MHz, CDCl3): δ = 25.2 (CH3), 26.4 (CH3), 34.5 (C-2), 51.9 (OCH3), 62.1 (C-7), 77.2 (C-3), 81.5, 82.6, 84.3 (C-4, C-5, C-6), 113.0 (C(CH3)2), 171.8 (C=O). IR (film): ν: 3464, 2990, 2943, 1737. ESI-HRMS: m/z calcd for C13H20O7Na [M+Na]+ 311.1101; found 311.1119.

3,6-Anhydro-2-deoxy-4,5-O-(1-methylethylidene)-d-altro-heptonic acid methyl ester 20

To a stirred solution of 19 (1.5 g, 6.1 mmol, 1 eq.) in DMF (12 mL), were added tert-butyldimethylsilyl chloride (1.1g, 6.71 mmol, 1.1 eq.) and imidazole (1.04 g, 15.25 mmol, 2.5 eq.) and the mixture was stirred at room temperature until completion of the reaction. The reaction was quenched by addition of EtOH and the solvent were removed in vacuo. The residue was diluted in EtOAc (40 mL) and washed with water (20 mL) and brine (20 mL). The organic layer was dried over MgSO4, filtered and concentrated in vacuo. The compound was purified by column chromatography (silica gel, cyH/EA) to provide 20 in quantitative yield (2.2 g). Colorless gum. Rf: 0.76 (cH/EA: 2/1). [α]D = -12.7° (c = 0.45, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 0.06 (s, 6H, CH3), 0.89 (s, 9H, C(CH3)3), 1.34 (s, 3H, CH3), 1.48 (s, 3H, CH3), 2.67 (dd, 1H, Jgem = 16.0, J3,4 = 4.5 Hz, H-2), 2.75 (dd, 1H, J2,3 = 4.0, H-2'), 3.69 (s, 3H, COOCH3), 3.64-3.75 (m, 2H, H-7, H-7'), 4.07 (t, 1H, J6,7 = 3.5 Hz, H-6), 4.50 (m, 1H, H-3), 4.75 (m, 1H, H-5), 4.82 (d, 1H, J4,5 = 6.0 Hz, H-4). 13C NMR (62.9 MHz, CDCl3): δ = -5.5 (Si(CH3)2), 18.2 (C(CH3)3), 25.1 (C(CH3)2), 25.9 (C(CH3)2), 26.3 (C(CH3)2), 34.9 (C-2), 51.7 (CH3OCCO), 64.9 (C-7), 78.6, 81.9, 83.3, 84.3 (C-3, C-4, C-5, C-6), 113.0 (C(CH3)2), 171.8 (C=O). IR (film): ν: 2976, 2957, 2933, 2853, 1742. ESI-HRMS: m/z calcd for C17H32O6SiNa [M+Na]+ 383.1860; found 383.1864.

3,6-Anhydro-7-O-[(tert-butyldimethyl)silyl]-2-deoxy-4,5-O-(1-methylethylidene)-d-altro-heptonic acid 21

To a solution of 20 (500 mg, 1.39 mmol) in THF (15 mL) and water (5 mL) was added LiOH (100 mg, 4.17 mmol, 3 eq.) and the mixture was stirred until completion of the reaction monitored by tlc. THF was removed under reduced pressure, CH2Cl2 (20 mL) was added and the pH was adjusted to 2-3 with 1N HCl. The organic layer was washed with brine and dried over MgSO4, filtered and concentrated to provide 21 (312 mg, 65%). Colorless gum. Rf: 0.56 (cH/EA: 2/1). [α]D = -21.1° (c = 0.1, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 0.06 (s, 6H, CH3), 0.89 (s, 9H, C(CH3)3), 1.35 (s, 3H, CH3), 1.50 (s, 3H, CH3), 2.71 (dd, 1H, Jgem = 16.0, J2,3 = 4.5 Hz, H-2), 2.80 (dd, 1H, J2,3 = 4.0, H-2'), 3.69 (dd, 1H, Jgem = 11.0, J6,7 = 3.5 Hz, H-
3.6-anhydro-7-O-[(tert-butyldimethyl)silyl]-2-deoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-d-altro-heptonamide 22

To a stirred solution of 21 (260 mg, 0.75 mmol, 1 eq.) in CH2Cl2 (10 mL) were added tryptamine (132 mg, 0.83 mmol, 1.1 eq.) and EDC (159 mg, 0.83 mmol, 1.1 eq.) at 0°C under reduced pressure. The residue was diluted with CH2Cl2 (20 mL) and the organic layer was washed with a saturated aqueous solution of NaHCO3 (10 mL), dried over MgSO4, filtered and the solvent was removed **in vacuo**. The product was purified by flash chromatography (silica gel, cyH/EA) to provide 22 (292 mg, 80%). Colorless gum. *Rf*; 0.75 (CH2Cl2/MeOH 9/1). [α]D = +10.7 (c = 1.0, CHCl3). Mp = 64°C. 1H NMR (250 MHz, CDCl3): δ = -5.5 (Si(CH3)), -5.4 (Si(CH3)), 18.3 (C(CH3)3), 24.9 (C(CH3)2), 25.4 (C-β), 26.0 (C(CH3)3), 26.3 (C(CH3)2), 37.6 (C-2), 39.9 (C-α), 64.4 (C-7), 79.0, 82.1, 83.1, 84.5 (C-3, C-4, C-5, C-6), 111.3 (C(CH3)2), 112.3, 113.2, 118.9, 119.5, 122.1, 122.2, 127.5, 136.5 (8 C-Ar), 170.9 (C=O). IR (film) v: 3336, 2985, 2943, 2876, 1650. ESI-HRMS: m/z calcd for C26H40N2O5SiNa [M+Na]+ 551.2599; found 551.2549.

3.6-Anhydro-2-deoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-d-altro-heptonamide 23

To a solution of 22 (248 mg, 0.51 mmol, 1 eq.) in dry THF (3 mL) was added dropwise TBAF (1M in THF) (0.77 mL, 0.77 mmol, 1.5 eq.) at 0°C under argon atmosphere. Water (5 mL) was added and the aqueous layer was extracted with EtOAc (2x15 mL). The combined organic layers were washed with brine (10 mL), dried over MgSO4, filtered and the solvent was removed **in vacuo**. The product was purified by flash chromatography (silica gel, cyH/EA) to provide 23 (181 mg, 95%). Colorless gum. *Rf*; 0.61 (CH2Cl2/MeOH 9/1). [α]D = -6.3 (c = 0.09, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.27 (s, 3H, CH3), 1.44 (s, 3H, CH3), 2.40 (br s, 1H, OH), 2.53 (d, 2H, J3,3 = 6.5 Hz, H-2, H-2’), 2.97 (t, 2H, J = 6.5 Hz, H-β), 3.51 (d, 2H, J6,7 = 5.5 Hz, H-7, H-7’), 3.56-3.66 (m, 2H, H-α), 4.06 (t, 1H, H-6), 4.30 (m, 1H, H-3), 4.55-4.63 (m, 2H, H-5, H-4), 6.19 (t, 1H, J = 5.0 Hz, CONH), 7.04-7.23 (m, 3H, H-Ar), 7.37 (d, 1H, H-Ar), 7.62 (d, 1H, H-Ar), 8.03 (br s, 1H, NH). 13C NMR (62.9 MHz, CDCl3): δ = 24.9 (C(CH3)2), 25.2 (C-β), 26.3 (C(CH3)3), 37.1 (C-2), 39.9 (C-α), 62.3 (C-7), 77.7, 81.8, 82.6, 84.7 (C-3, C-4, C-5, C-6), 111.4 (C(CH3)3), 112.6, 112.9, 118.8, 119.5, 122.2, 122.4, 127.5, 136.5 (8 C-Ar), 171.1 (C=O). IR (film) v: 3336, 2985, 2943, 2876, 1650. ESI-HRMS: m/z calcd for C26H30N2O5SiNa [M+Na]+ 397.1734; found 397.1731.

3.6-Anhydro-2-deoxy-N-[2-(1H-indol-3-yl)ethyl]-7-(4-methylbenzenesulfonylate)-4,5-O-(1-methylethylidene)-d-altro-heptonamide 24

Prepared starting from 23 (160 mg, 0.43 mmol) following procedure described for 8. Yield: 87% (197 mg). White solid. *Rf*; 0.67 (EA). [α]D = +10.7 (c = 1.0, CHCl3). Mp = 64°C. 1H NMR (250 MHz, CDCl3): δ = 1.22 (s, 3H, CH3), 1.42 (s, 3H, CH3), 2.48 (s, 3H, Ph-CH3), 2.46-2.52
(m, 2H, H-2), 3.02 (t, 2H, J_{α,β} = 6.5 Hz, H-β), 3.60-3.68 (m, 2H, H-α), 3.92 (dd, 1H, J_{6,7} = 4.5, J_{gem} = 11.0 Hz, H-7), 4.03 (dd, 1H, J_{6,7} = 5.0 Hz, H-7'), 4.13-4.23 (m, 2H, H-6, H-3), 4.45 (dd, 1H, J_{α,β} = 6.0, J_{6,7} = 4.0 Hz, H-5), 4.60 (d, 1H, H-4), 6.11 (t, 1H, J = 5.5 Hz, CONH), 7.09-7.44 (m, 6H, H-Ar), 7.63 (d, 1H, J = 8.0 Hz, H-Ar), 7.81 (d, 2H, J = 8.5 Hz, H-Ar), 8.33 (s, 1H, NHe-Ar). 13C NMR (62.9 MHz, CDC13): δ = 21.7 (CH3Ph), 24.7 (CH3), 25.0 (CH3), 26.1 (C-β), 37.2 (C-2), 39.5 (C-α), 69.5 (C-7), 78.8 (C-3), 81.4 (C-6, C-4), 82.3 (C-5), 111.3 (C(CH3)2), 112.8 (C-Ar), 112.9 (C-Ar), 118.7 (C-Ar), 119.4 (C-Ar), 122.1 (C-Ar), 122.3 (C-Ar), 127.4 (C-Ar), 127.9 (2C-Ar), 130.1 (2C-Ar), 132.2 (C-Ar), 136.4 (C-Ar), 145.4 (C-Ar), 170.1 (C=O). IR (film) v: 3407, 3308, 1652. ESI-MS: m/z = calcd for C27H32N2O7SNa [M+Na]+ 551.1822; found 551.1853.

3.6-Anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methyllethyldiene)-7-[[phenylmethoxy]carbonyl]amino[ethyl]amino-[D-altro]-heptonamide 25

Prepared starting from 24 (170 mg, 0.32 mmol) following procedure described for 9. Yield: 82% (144 mg). Colorless gum. RF: 0.35 (CH2Cl2/Methanol 9/1). [α]D = +3.5 (c = 0.5, CHCl3). 1H NMR (400 MHz, CDCl3): δ = 1.28 (s, 3H, C(CH3)2), 1.45 (s, 3H, C(CH3)2), 2.07 (br s, 1H, NH), 2.47 (dd, 1H, J_{gem} = 12.5, J_{6,7} = 5.0 Hz, H-7), 2.53-2.59 (m, 3H, 2H-2, H-7), 2.71 (t, 2H, J_{6,7} = 6.0 Hz, H-8), 2.97 (t, 2H, J_{α,β} = 6.5 Hz, H-β), 3.23-3.27 (m, 2H, H-9), 3.58-3.64 (m, 2H, H-4), 4.09 (m, 1H, H-6), 4.14 (m, 1H, H-3), 4.47 (d, 1H, J_{α,β} = 6.0 Hz, H-5), 4.56 (dd, 1H, J_{4,5} = 4.0 Hz, H-4), 5.12 (2s, 2H, CH2-Ph), 5.39 (t, 1H, J = 5.0 Hz, CONH), 6.12 (t, 1H, J = 5.0 Hz, CONH), 7.05 (d, 1H, J = 2.0 Hz, H-7), 7.13 (m, 1H, H-4), 7.21 (m, 1H, H-Ar), 7.32-7.39 (m, 6H, H-Ar), 7.64 (d, 1H, J = 8.0 Hz, H-Ar), 8.52 (br s, 1H, NH). 13C NMR (100 MHz, CDCl3): δ = 24.3 (C(CH3)2), 25.1 (C-β), 26.2 (C(CH3)2), 36.5 (C-2), 39.7 (C-α), 40.3 (C-9), 48.3 (C-7), 48.6 (C-8), 66.7 (CH2-Ph), 76.5 (C-3), 81.3 (C-4), 82.9 (C-6), 83.7 (C-5), 111.3 (C-Ar), 112.6 (C(CH3)2), 112.9 (C-Ar), 118.7 (C-Ar), 119.4 (C-Ar), 122.1 (C-Ar), 122.2 (C-Ar), 127.4 (C-Ar), 128.1 (C-Ar), 128.2 (2C-Ar), 128.5 (2C-Ar), 136.4 (C-Ar), 136.6 (C-Ar), 156.7 (OCONH), 170.5 (CONH). IR (film) v: 3327, 1709, 1650. ESI-MS: m/z = calcd for C30H38N4O6Na [M+Na]+ 573.2684; found 573.2707.

3.6-Anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methyllethyldiene)-7-[[N-[bis[(phenylmethoxy)carbonyl]aminoiminomethyl]-N-[2-[bis[(phenylmethoxy)carbonyl]guanidinoethyl]amino]-D-altro]-heptonamide 26

Prepared starting from 25 (120 mg, 0.22 mmol) following procedure described for 12. Yield: 30% (68 mg). White solid. RF: 0.50 (EA). [α]D = +11.4 (c = 0.5, CHCl3). Mp = 70°C. 1H NMR (400 MHz, MeOH-d4): δ = 1.23 (s, 3H, C(CH3)2), 1.39 (s, 3H, C(CH3)2), 2.47 (dd, 1H, J_{gem} = 15.5, J_{2,3} = 5.5 Hz, H-2), 2.56 (dd, 1H, J_{2,3} = 8.0 Hz, H-2'), 2.90 (t, 2H, J_{α,β} = 6.5 Hz, H-β), 3.44-3.72 (m, 8H, 2H-α, 2H-7, 2H-8, 2H-9), 4.25-4.29 (m, 2H, H-6, H-3), 4.43 (d, 1H, J_{4,5} = 6.0 Hz, H-5), 4.51 (dd, 1H, J_{3,4} = 3.5 Hz, H-4), 5.00-5.01 (m, 6H, CH2-Ph), 5.08 (s, 2H, CH2-Ph), 6.96-7.08 (m, 3H, H-Ar), 7.19-7.36 (m, 2H, H-Ar), 7.54 (d, 1H, J = 8.0 Hz, H-Ar). 13C NMR (100 MHz, MeOH-d4): δ = 25.1 (C(CH3)2), 26.1 (C-β), 26.6 (C(CH3)2), 36.9 (C-2), 39.7 (C-7), 41.3 (C-α), 48.8 (C-8), 68.3 (CH2-Ph), 68.8 (CH2-Ph), 69.2 (2CH2-Ph), 78.0 (C-3, C-6), 82.6 (C-4), 84.5 (C-5), 112.3 (C-Ar), 113.3 (C-Ar), 113.6 (C(CH3)2), 119.4 (C-Ar), 119.6 (C-Ar), 122.3 (C-Ar), 123.5 (C-Ar), 128.8 (C-Ar), 129.0 (2C-Ar), 129.2 (8C-Ar), 129.4 (4C-Ar), 129.5 (4C-Ar), 129.6 (4C-Ar), 136.4 (C-Ar), 138.1 (2C-Ar), 154.4 (2C-gua), 157.7 (2OCONH), 164.7 (2OCONH), 172.8 (CONH). IR (film) v: 3327, 1742, 1636, 1619. ESI-MS: m/z = calcd for C56HooN8O12Na [M+Na]+ 1059.4223; found 1059.4244.

3.6-anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methyllethyldiene)-7-[[N-(aminoiminomethyl)-N-(2-guanidinoethyl)amino]-D-altro]-heptonamide 27
Prepared starting from 26 (50 mg, 0.05 mmol) following procedure described for the synthesis of 15. Yield: 90% (20 mg). White solid. Rf: 0.29 (H₂O/MeCN/TFA: 6/4/0.1%). HPLC (60/40): Rᵣ = 4.740 min. [α]D = +23.0 (c = 0.2, CH₂Cl₂). 

1H NMR (250 MHz, CDCl₃): δ = 1.13 (s, 3H, C(CH₃)₂), 1.48 (s, 3H, C(CH₃)₂), 2.52-2.66 (m, 2H, H-2), 2.97 (t, 2H, Jα,β = 7.0 Hz, H-β), 3.38-3.62 (m, 8H, H-α, H-7, H-8, H-9), 4.24 (m, 1H, H-6), 4.39 (m, 1H, H-3), 4.60 (d, 1H, J4,5 = 6.0 Hz, H-5), 4.72 (dd, 1H, J3,4 = 4.0 Hz, H-4), 6.99-7.14 (m, 3H, H-Ar), 7.36 (d, 1H, J = 8.0 Hz, H-Ar), 7.59 (d, 1H, J = 8.0 Hz, H-Ar). 13C NMR (62.9 MHz, CDCl₃): δ = 17.5 (CH₃), 26.2 (C(CH₃)₂), 36.7 (C(CH₃)₂), 40.2 (C-α), 41.6 (C-7), 49.7, 49.8 (C-8, C-9), 78.0 (C-3), 82.7, 84.0, 84.5 (C-4, C-5, C-6), 112.3 (C-Ar), 113.3 (C(CH₃)₂), 113.9 (C-Ar), 119.3 (C-Ar), 119.6 (C-Ar), 122.3 (C-Ar), 123.5 (C-Ar), 128.8 (C-Ar), 138.1 (C-Ar), 159.2, 159.7 (2 C=N), 172.9 (CONH). IR (film) ν: 3374, 3189, 1674, 1607. ESI-HRMS: m/z calcd for C₂₁H₂₂N₃O₄Na [M+Na]⁺ 380.1594. Found 380.1592.

**N,N’-[Bis(phenylmethoxy)carbonyl]-N’’-(but-3-ynyl)-guanidine 28**

To a cooled solution of butyn-4-ol (151 µL, 2.0 mmol, 1 eq.) in dry toluene (10 mL), was added dropwise over 5 minutes a solution of bis(benzyloxycarbonyl)guanidine (650 mg, 2.0 mmol, 1 eq.) and PPh₃ (630 mg, 2.4 mmol, 1.2 eq.) in dry toluene (10 mL), was added dropwise over 5 minutes a solution of diethylazodicarboxylate (DEAD, 378 µL, 2.4 mmol, 1.2 eq.) in toluene (5 mL) under argon atmosphere. The mixture was stirred at room temperature for 4h and the solvent was removed in vacuo. The crude was purified by flash chromatography (silica gel, cyc/H/EA) to provide 

**N,N’-[Bis(phenylmethoxy)carbonyl]-N’’-(pent-4-ynyl)-guanidine 29**

Prepared starting from pentyln-5-ol (185 µL, 2.0 mmol) following procedure described for 

3.6-Anhydro-7-azido-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-1-galacto-heptonamide 30

To a solution of 8 (1.0 g, 1.9 mmol) in DMF (10 mL) was added sodium azide (1.0 g, 15.2 mmol, 8 eq.) and the mixture was stirred for 2 days at 80°C. The solvent was removed in vacuo and the residue was diluted with EtOAc (50 mL) and washed with H₂O (20 mL) and brine (20 mL). The organic layer was dried over MgSO₄ filtered and the solvent was evaporated under reduced pressure. The compound was purified by flash chromatography (silica gel, cyc/H/EA) to provide 30 (667 mg, 88%). Colorless gum. Rf: 0.49 (CH₂Cl₂/MeOH/9:1), [α]D = +9.7° (c = 0.4, CHCl₃). 1H NMR (250 MHz, CDCl₃): δ = 1.27 (s, 3H, C(CH₃)), 1.42 (s, 3H, C(CH₃)), 2.57 (d, 2H, J2,3 = 6.5 Hz, H-2), 2.99 (t, 2H, Jα,β = 6.5 Hz, H-β), 3.44 (dd, 2H, Jγ,δ = 7.5, J₆,₇ = 3.0 Hz,
3.6-Anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-7-[4-[3-[N-bis(phenylmethoxy)carbonyl]guanidinopropyl]-1H-1,2,3-triazol-1-yl]-1-galactoheptonamide 31

To a solution of 30 (300 mg, 0.75 mmol, 1 eq.) and 28 (284 mg, 0.75 mmol, 1 eq.) in CH2Cl2 (2x20 mL) and the combined organic layers were dried over MgSO4, filtered and the solvent was removed under reduced pressure. The compound was purified by column chromatography (silica gel, eluent/EtOAc) to provide 31 (466 mg, 80%). White solid. Rf: 0.46 (EtOA). [α]D = -3.5° (c = 0.2, CHCl3). Mp = 72°C. 1H NMR (250 MHz, MeOH d4): δ = 1.29 (s, 3H, C(CH3)), 1.47 (s, 3H, C(CH3)), 2.53-2.58 (m, 2H, H-2), 6.39 (dd, 1H, Jα,β = 7.5 Hz, H-β), 3.38-3.50 (m, 2H, H-α), 3.78 (m, 1H, H-6), 3.85 (m, 1H, H-3), 4.11-4.19 (m, 2H, H-10), 4.57 (dd, 1H, Jα,β = 7.7, 6.7 Hz, H-7), 4.66-4.69 (m, 2H, H-4, H-5), 5.10 (s, 2H, CH2-Ph), 5.11 (s, 2H, CH2-Ph), 6.94-7.08 (m, 3H, H-3, 4, 5), 7.23-7.39 (m, 11H, H-3, 4, 5), 7.53 (d, 1H, Jα,β = 8.0 Hz, H-6), 7.59 (s, 1H, H-triazole). 13C NMR (62.9 MHz, CDCl3): δ = 24.7 (CH3), 30.3, 30.4 (CH3), 51.2, 51.3 (C-5), 75.4 (C-3), 108.2 (C-2), 122.3 (C-3), 123.5 (C-4), 127.6 (C-2), 128.9 (4C), 148.2 (C-7), 164.9 (NCOO), 172.9 (C=O). IR (film) ν: 1215, 1583, 1615. ESI-HRMS: m/z calcd for C20H25N5O4Na [M+Na]+ 422.1799; found 422.1821.
3.6-Anhydro-2,7-dideoxy-β-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-7-[4-(2-guanidinoethyl)-1H-1,2,3-triazol-1-yl]-l-galacto-heptonamide 33

Prepared starting from 31 (150 mg, 0.19 mmol) following procedure described for 15. Yield: 90% (87 mg). White solid. 

[α]D = -5.8° (c = 0.1, MeOH).

The title compound was obtained as a white solid. Rf: 0.0 (EA).

1H NMR (400 MHz, MeOH-d4): δ = 1.32 (s, 3H, C(CH3)), 1.49 (s, 3H, C(CH3)), 1.83-1.89 (m, 2H, H-9), 2.56-2.61 (m, 2H, H-2), 2.68 (t, 2H, J8,9 = 8.0 Hz, H-8), 2.91 (t, 2H, J9,β = 7.5 Hz, H-β), 3.11 (t, 2H, J9,10 = 7.0 Hz, H-10), 3.43-3.49 (m, 2H, H-α), 3.86-3.95 (m, 2H, H-3, H-6), 4.51 (dd, 1H, Jgem = 14.5, J6,7 = 8.5 Hz, H-7), 4.68 (m, 1H, H-7'), 4.73-4.80 (m, 2H, H-4, H-5), 6.96-7.11 (m, 3H, H-Ar), 7.33 (d, 1H, J = 8.0 Hz, H-Ar), 7.55 (d, 1H, J = 8.0 Hz, H-Ar), 7.73 (s, 1H, H-triazole).

13C NMR (62.9 MHz, MeOH-d4): δ = 23.2 (C-8), 24.9 (CH3), 26.2 (CH3, C-β), 36.5 (C-2), 41.5 (C-α), 41.9 (C-9), 50.6 (C-7), 79.7 (C-3), 80.7 (C-6), 82.2, 89.2 (C-4, C-5), 112.3 (C-Ar), 113.2 (C-Ar), 113.8 (C(CH3)2), 119.3 (C-Ar), 119.6 (C-Ar), 122.3 (C-Ar), 123.5 (C-Ar), 124.8 (C-Ar), 128.8 (C-Ar), 138.1 (C-Ar), 145.3 (C-Ar), 158.9 (C=N), 172.9 (C=O). IR (pellets): υ = 3327, 1652. ESI-HRMS: m/z calcd for C25H34N8O4Na+ [M+Na]+ 533.2595; found 533.2597.

3.6-Anhydro-2,7-dideoxy-β-N-[2-(1H-indol-3-yl)ethyl]-4,5-O-(1-methylethylidene)-7-[4-(3-guanidinopropyl)-1H-1,2,3-triazol-1-yl]-l-galacto-heptonamide 34

Prepared from 32 (150 mg, 0.19 mmol) following procedure described for 15. Yield: 90% (90 mg). White solid. Rf: 0.0 (EA).

1H NMR (500 MHz, MeOH-d4): δ = 1.19 (s, 3H, CH2-Ph), 5.11 (s, 3H, C(CH3)2). The title compound was obtained as a white solid. Rf: 0.23 (EA/MeOH: 9/1).

Colorless gum. 

[α]D = -11.6° (c = 0.2, MeOH).

1H NMR (250 MHz, MeOH-d4): δ = 2.46-2.58 (m, 2H, H-2), 2.89 (t, 4H, H-8, H-β), 3.46 (t, 2H, Jα,β = 7.0 Hz, H-α), 4.01-4.18 (m, 5H, H-4, H-5, H-6, H-9), 4.30 (t, 1H, J3,3 = 5.5 Hz, H-3), 4.41 (dd, 1H, Jgem = 14.5, J6,7 = 9.0 Hz, H-7), 4.56 (dd, 1H, J6,7 = 3.5 Hz, H-7'), 5.08 (s, 3H, C(CH3)2), 5.11 (s, 3H, C(CH3)2), 6.92-7.08 (m, 3H, H-Ar), 7.25-7.37 (m, 11H, H-Ar), 7.52 (d, 1H, J = 8.0 Hz, H-Ar), 7.58 (s, 1H, H-triazole). IR (pellets): υ = 3355, 1650. ESI-HRMS: m/z calcd for C26H36N8O4Na+ [M+Na]+ 547.2752; found 547.2752.

3.6-Anhydro-2,7-dideoxy-β-N-[2-(1H-indol-3-yl)ethyl]-7-[4-[2-[N-bis(phenylmethoxy)carbonyl]guanidinoethyl]-1H-1,2,3-triazol-1-yl]-l-galacto-heptonamide 35

To a stirred solution of 31 (250 mg, 0.32 mmol) in H2O (2 ml) at 0°C was added trifluoroacetic acid (2 ml). After completion of the reaction (tlc monitoring), the solution mixture was diluted with EtOAc (50 mL) and NaHCO3 sat. aq. was added until pH 5-6. The product was extracted with EtOAc (2x15 ml) and the combined organic layers were dried over MgSO4, filtered and the solvent was removed under reduced pressure. The compound was purified by column chromatography (silica gel, EA/MeOH) to provide 35 (200 mg, 85%). Colorless gum. Rf: 0.23 (EA/MeOH: 9/1). [α]D = -5.8° (c = 0.1, MeOH).

1H NMR (250 MHz, MeOH-d4): δ = 2.46-2.58 (m, 2H, H-2), 2.89 (t, 4H, H-8, H-β), 3.46 (t, 2H, Jα,β = 7.0 Hz, H-α), 4.01-4.18 (m, 5H, H-4, H-5, H-6, H-9), 4.30 (t, 1H, J3,3 = 5.5 Hz, H-3), 4.41 (dd, 1H, Jgem = 14.5, J6,7 = 9.0 Hz, H-7), 4.56 (dd, 1H, J6,7 = 3.5 Hz, H-7'), 5.08 (s, 3H, C(CH3)2), 5.11 (s, 3H, C(CH3)2), 6.92-7.08 (m, 3H, H-Ar), 7.25-7.37 (m, 11H, H-Ar), 7.52 (d, 1H, J = 8.0 Hz, H-Ar), 7.58 (s, 1H, H-triazole). IR (pellets): υ = 3355, 1650. ESI-HRMS: m/z calcd for C26H36N8O4Na+ [M+Na]+ 547.2752; found 547.2752.
(C-Ar), 119.3 (C-Ar), 119.6 (C-Ar), 122.3 (C-Ar), 123.4 (C-Ar), 124.6 (C-Ar), 128.9 (4 C-Ar), 129.4 (4 C-Ar), 129.7 (4 C-Ar), 136.4 (C-Ar), 138.1 (C-Ar), 138.5 (C-Ar), 156.7 (C=N), 161.7 (2 NHCOC), 162.9, 163.2 (TFA), 173.9 (C=O). IR (peppers): 3325, 1665, 1632. ESI-HRMS: m/z calcd for C_{38}H_{42}N_{8}O_{8}Na [M+Na]^+ 761.3018; found 761.3011.

3,6-Anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-7-[4-[3-[N-[[bis(phenylmethoxy)carbonyl][guanidinopropyl]-1H-1,2,3-triazol-1-yl]-l-galacto-heptanamide 36

Prepared starting from 32 (250 mg, 0.32 mmol) following procedure described for 35. Yield: 53% (127 mg). Colorless gum. Rf: 0.34 (EA/MeOH: 9:1). [α]_D = -2.0° (c = 0.2, MeOH). ¹H NMR (250 MHz, MeOH-d₄): δ = 1.82-1.93 (m, 2H, H-9), 2.47-2.61 (m, 4H, H-2, H-8), 2.85-2.90 (m, 2H, H-β), 3.44 (t, 2H, J_{6,8} = 7.0 Hz, H-α), 3.89 (t, 2H, J_{9,10} = 7.5 Hz, H-10), 4.04-4.15 (m, 3H, H-4, H-5, H-6), 4.29 (t, 1H, J_{2,3} = 5.5 Hz, H-3), 4.42 (dd, 1H, J_{gem} = 14.5, J_{6,7} = 9.0 Hz, H-7), 4.54 (dd, 1H, J_{6,7} = 3.5 Hz, H-7'), 5.11 (s, 2H, CH₂-Phe), 5.18 (s, 2H, CH₂-Phe), 6.92-7.07 (m, 3H, H-Ar), 7.25-7.38 (m, 11H, H-Ar), 7.52 (d, 1H, J = 8.0 Hz, H-Ar), 7.69 (s, 1H, H-triazole). ¹³C NMR (62.9 MHz, MeOH-d₄): δ = 23.3 (C-9), 26.3, 28.7 (C-8, C-β), 38.5 (C-2), 41.5 (C-α), 45.8 (C-10), 52.7 (C-7), 68.5, 70.1 (CH₂-Phe), 73.3 (C-4, C-5), 79.0 (C-3), 79.9 (C-6), 112.2 (C-Ar), 113.2 (C-Ar), 119.3 (C-Ar), 119.6 (C-Ar), 122.3 (C-Ar), 123.5 (C-Ar), 124.2 (C-Ar), 128.7 (C-Ar), 129.0 (3 C-Ar), 129.4 (2 C-Ar), 129.5 (2 C-Ar), 129.7 (4 C-Ar), 136.4 (C-Ar), 138.1 (C-Ar), 138.2 (C-Ar), 156.5 (C=N), 161.4 (2 NHCOC), 163.0 (TFA), 173.9 (C=O). IR (peppers): 3368, 1668, 1645. ESI-HRMS: m/z calcd for C_{39}H_{44}N_{8}O_{8}Na [M+Na]^+ 775.3174; found 775.3172.

3,6-anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-7-[4-(3-guanidinoethyl)-1H-1,2,3-triazol-1-yl]-l-galacto-heptanamide 37

Prepared starting from 35 (150 mg, 0.2 mmol) following procedure described for 15. Yield: 80% (75 mg). Colorless gum. Rf: 0.58 (H₂O/MeCN/TFA: 4:6/0.1%). HPLC (60/40): tR = 3.678 min. [α]_D = -9.0° (c = 0.1, MeOH). ¹H NMR (250 MHz, MeOH-d₄): δ = 2.56-2.61 (m, 2H, H-2), 2.85-2.96 (m, 4H, H-8, H-β), 3.40-3.51 (m, 4H, H-α, H-9), 4.11-4.19 (m, 3H, H-4, H-5, H-6), 4.37 (t, 1H, J_{2,3} = 5.5 Hz, H-3), 4.52 (dd, 1H, J_{gem} = 14.0, J_{6,7} = 9.0 Hz, H-7), 4.65 (dd, 1H, J_{6,7} = 3.0 Hz, H-7'), 6.96-7.11 (m, 3H, H-Ar), 7.32 (d, 1H, J = 8.0 Hz, H-Ar), 7.55 (d, 1H, J = 8.0 Hz, H-Ar), 7.77 (s, 1H, H-triazole). ¹³C NMR (62.9 MHz, MeOH-d₄): δ = 24.9, 25.1 (C-8, C-β), 37.3 (C-2), 40.4 (C-α), 40.7 (C-9), 51.7 (C-7), 72.2 (C-4, C-5), 78.0 (C-3), 78.8 (C-6), 111.1 (C-Ar), 112.1 (C-Ar), 118.1 (C-Ar), 118.4 (C-Ar), 121.2 (C-Ar), 122.3 (2 C-Ar), 123.9 (C-Ar), 127.6 (C-Ar), 137.0 (C-Ar), 157.5 (C=N), 172.7 (C=O). IR (peppers): 3339, 3210, 2657, 2922, 1665, 1649. ESI-HRMS: m/z calcd for C_{22}H_{30}N_{8}O_{4}Na [M+Na]^+ 493.2282; found 493.2274.

3,6-anhydro-2,7-dideoxy-N-[2-(1H-indol-3-yl)ethyl]-7-[4-(3-guanidinopropyl)-1H-1,2,3-triazol-1-yl]-l-galacto-heptanamide 38

Prepared starting from 36 (100 mg, 0.13 mmol) following procedure described for 15. Yield: 95% (60 mg). Colorless gum. Rf: 0.34 (H₂O/MeCN/TFA: 6:4/0.1%). HPLC (60/40): tR = 3.692 min. [α]_D = -6.5° (c = 0.2, MeOH). ¹H NMR (250 MHz, D₂O): δ = 1.63-1.75 (m, 2H, H-9), 2.38-2.59 (m, 4H, H-2, H-8), 2.81-2.94 (m, 4H, H-10, H-β), 3.33-3.51 (m, 2H, H-α), 4.01-4.21 (m, 3H, H-4, H-5, H-6), 4.33-4.45 (m, 2H, H-3, H-7), 4.54 (dd, 1H, J_{gem} = 14.5, J_{6,7} = 3.0 Hz, H-7), 6.98-7.18 (m, 3H, H-Ar), 7.39 (d, 1H, J = 8.0 Hz, H-Ar), 7.54-7.57 (m, 2H, H-Ar, H-triazole). ¹³C NMR (62.9 MHz, D₂O): δ = 21.7 (C-9), 24.2, 27.4 (C-8, C-β), 37.0 (C-2), 40.3 (C-α, C-10), 51.3 (C-7), 71.6, 71.8 (C-4, C-5), 77.2 (C-3), 77.9 (C-6), 111.7 (C-Ar), 111.8 (C-Ar), 118.4 (C-Ar), 119.1 (C-Ar), 121.8 (C-Ar), 123.3 (2 C-Ar), 123.7 (C-Ar), 126.9 (C-Ar),
136.1 (C-Ar), 156.5 (C=N), 173.5 (C=O). IR (pellets) ν: 3279, 1622, 1549. ESI-HRMS: m/z calcd for C23H32N8O4Na [M+Na]+ 507.2439; found 507.2445.

**N⁵-[2-C-[2,3:5,6-Bis-O-(1-methyllethylidene)-α-D-gulofuranosyl(carboxymethyl)]-L-nitro-arginine methyl ester 39**

To a solution of N⁵-NO₂-L-arg-OMe (296 mg, 1.1 mmol, 1 eq.) and 5 (300 mg, 1 mmol, 1 eq.) in DMF (10 mL) were added HATU (570 mg, 1.5 mmol, 1.5 eq.) and DIEA (363 µL, 2.1 mmol, 2.1 eq.) at 0°C under argon atmosphere. After stirring at room temperature for 24h, the solvent was evaporated. The residue was dissolved in ethyl acetate (10 mL), washed with 1N HCl (5 mL), NaHCO₃ sat. aq. (5 mL) and brine (5 mL). The organic layer was dried over MgSO₄, filtered and concentrated. The product was purified by column chromatography (silica gel, cyclohexane/AcOEt) to provide 39 (258 mg, 50%). Colorless oil. *Rf*: 0.36 (CH₂Cl₂/MeOH: 9/1). [α]D = -22.2° (c = 0.2, CHCl₃). ¹H NMR (250 MHz, DMSO-d₆): δ = 1.20 (s, 3H, C(CH₃)₂), 1.25 (s, 3H, C(CH₃)₂), 1.30 (s, 3H, C(CH₃)₂), 1.36 (s, 3H, C(CH₃)₂), 1.45-1.73 (m, 4H, CH₂ arg), 2.49-2.54 (m, 2H, H-2), 3.10-3.17 (m, 2H, CH₂ arg), 3.41 (dd, 1H, J₆,₇ = 8.5, J₅,₆ = 3.5 Hz, H-6), 3.62 (s, 3H, OCH₃), 3.66 (t, 1H, J₁,₂ = 7.5 Hz, H-8), 3.85 (td, 1H, J₂,₃ = 6.5, J₃,₄ = 3.0 Hz, H-3), 4.01 & 4.01 (2m, 2H, H-7, H-8’), 4.26 (m, 1H, H-α), 4.60 (dd, 1H, J₉,₁₀ = 6.0 Hz, H-9), 4.66 (dd, 1H, H-5), 7.91 (br s, 2H, NH₂), 8.32 (dd, 1H, J = 7.5 Hz, NH), 8.46 (br s, 1H, NH). ¹³C NMR (62.9 MHz, DMSO-d₆): δ = 24.7 (CH₃, CH₂ arg), 25.3 (CH₃), 25.8 (CH₃), 26.5 (CH₃), 28.2 (CH₂ arg), 34.3 (C-2), 40.1 (CH₂ arg), 51.5 (C-α), 51.8 (OCH₃), 65.2 (C-8), 75.2 (C-7), 77.4 (C-3), 80.6 (C-5), 81.1 (C-4), 82.4 (C-6), 108.6 (C(CH₃)₂), 111.3 (C(CH₃)₂), 159.3 (C=N), 169.7 (C=O), 172.5 (C=O). IR (film): ν: 3317, 2985, 2928, 2862, 1742, 1650, 1629, 1598, 1539. ESI-HRMS: m/z calcd for C₂₁H₂₅N₁₀O₁₀Na [M+Na]+ 540.2276; found 540.2294.

**N⁵-[2-C-[2,3:5,6-Bis-O-(1-methyllethylidene)-α-D-gulofuranosyl(carboxymethyl)]-L-arginine methyl ester 40**

To a solution of 39 (200 mg, 0.39 mmol) in THF (9 mL) and water (3 mL) was added LiOH (30 mg, 1.17 mmol, 3 eq.) and the mixture was stirred until completion of the reaction monitored by tlc. Amberlite® IR-120 was added until pH = 3-4 and the mixture was filtered off. The solvent were evaporated and the obtained carboxylic acid was solubilized in MeOH (10 mL) and Pd/C (10%) (80 mg, 40% w/w) was added. After stirring under H₂ atmosphere (40 psi) until completion of the reaction, the mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo. The crude was purified by column chromatography (C-18 silica gel, H₂O/MeOH) and lyophilized to provide 40 (125 mg, 70%). White foam. *Rf*: 0.32 (H₂O/MeOH: 1/1). HPLC (70/30): tᵣ = 4.436 min. [α]D = -1.0° (c = 0.2, H₂O). ¹H NMR (400 MHz, D₂O): δ = 1.26 (s, 3H, C(CH₃)₂), 1.32 (s, 3H, C(CH₃)₂), 1.38 (s, 3H, C(CH₃)₂), 1.42 (s, 3H, C(CH₃)₂), 1.51-1.60 (m, 2H, CH₂ arg), 1.66 (m, 1H, CH₂ arg), 1.77 (m, 1H, CH₂ arg), 2.70 (dd, 2H, J₂,₃ = 7.0 Hz, H-2), 3.12 (t, 2H, J = 7.0 Hz, CH₂ arg), 3.64 (dd, 1H, J₆,₇ = 8.5, J₅,₆ = 3.5 Hz, H-6), 3.80 (dd, 1H, J₆,₇ = 9.0, J₅,₆ = 6.5 Hz, H-8), 3.99 (td, 1H, J₃,₄ = 3.5 Hz, H-3), 4.12 (dd, 1H, J = 8.0, J = 5.0 Hz, H-α), 4.17 (dd, 1H, J₇,₈ = 7.0 Hz, H-8’), 4.33 (m, 1H, H-7), 4.77 (dd, 1H, J₉,₁₀ = 6.0 Hz, H-9), 4.81 (dd, 1H, H-5), 13C NMR (100 MHz, D₂O): δ = 23.6 (CH₃), 24.1 (CH₃), 24.4 (CH₂ arg), 24.7 (CH₃), 25.5 (CH₃), 28.7(CH₂ arg), 34.6 (C-2), 40.6 (CH₂ arg), 54.6 (C-α), 65.2 (C-8), 74.6 (C-7), 78.0 (C-3), 80.4 (C-5), 81.1 (C-4), 82.6 (C-6), 110.4 (C(CH₃)₂), 113.1 (C(CH₃)₂), 156.7 (C=N), 171.8 (C=O), 178.6 (C=O). IR (pellets): ν: 3364, 2985, 2938, 2867, 1652, 1579. ESI-HRMS: m/z calcd for C₂₀H₂₄N₇O₁₁Na [M+Na]+ 481.2269; found 481.2275.

**(E)-3,6-Anhydro-2-deoxy-4,5:7,8-bis-O-(1-methyllethylidene)-d-gulo-Oct-2-enonic acid methyl 41**

To a solution of 2 (630 mg, 2 mmol, 1 eq.) in pyridine (15 mL) was added LiI (2.6 g, 20 mmol, 10 eq.) and the mixture was stirred at 120 °C until completion of the reaction monitored
by tlc. The solvent was removed under reduced pressure, CH₂Cl₂ (20 mL) was added and the pH was adjusted to 2-3 with 1N HCl. The layers were separated and the organic layer was washed with brine and dried over MgSO₄, filtered and concentrated. The crude residue was purified by column chromatography (silica gel, cycH/EA) to provide 41 (300 mg, 50%). White solid. Rf: 0.21 (cH/EA: 1/1). [α]D = -36.5° (c = 1.0, CHCl₃). Mp = 201°C. ¹H NMR (250 MHz, CDCl₃): δ = 1.37 (s, 3H, CH₃), 1.53 (s, 3H, CH₃). 1.43 (s, 3H, CH₃), 1.46 (s, 3H, CH₃), 3.77 (dd, 1H, J₆₋₇ = 9.0, J₇₋₈ = 7.0 Hz, H-8), 4.18-4.26 (m, 1H, H-4), 4.42 (dd, 1H, H-7, 4.75 (dd, 1H, J₄₋₅ = 6.0, J₅₋₆ = 4.0 Hz, H-5), 5.51 (dd, 1H, H-4), 10.66 (br s, 1H, COOH). ¹³C NMR (62.9 MHz, CDCl₃): δ = 25.2 (CH₃), 26.4 (CH₃), 26.7 (CH₃), 65.7 (C-8), 75.5, 77.5, 79.9, 86.5 (C-4, C-5, C-6, C-7), 94.9 (C-2), 110.3, 113.7 (2 C(CH₃)₂), 172.0 (C-3), 173.1 (C=O). IR (film) ν: 3061, 2985, 2933, 2563, 1685, 1655. ESI-HRMS: m/z calcd for C₁₄H₂₀O₇Na [M+Na]+ 323.1101; found 323.1102.

**Compound 42**

To a solution of HCl.H-L-arg-OMe (191 mg, 0.73 mmol, 1.1 eq.) and 41 (200 mg, 0.67 mmol, 1 eq.) in DMF (10 mL) were added HATU (380 mg, 1.0 mmol, 1.5 eq.) and DIEA (243 µL, 1.41 mmol, 2.1 eq.) at 0°C under argon atmosphere. After stirring at room temperature for 24h, the product was purified by column chromatography (silica gel, cycH/EA) to provide 42 (96 mg, 33%). White solid. Rf: 0.41 (H₂O/MeOH: 1/1). HPLC (70/30): tR = 4.782 min. [α]D = -44.6° (c = 0.08, H₂O). ¹H NMR (250 MHz, D₂O): δ = 1.47 (s, 3H, CH₃), 1.48 (s, 3H, CH₃), 1.53 (s, 3H, CH₃), 1.54 (s, 3H, CH₃), 1.63-1.75 (m, 2H, CH₂ arg), 1.79-1.99 (m, 2H, CH₂ arg), 3.26 (t, 2H, J = 7.0 Hz, CH₂ arg), 4.08 (dd, 1H, J₆₋₇ = 9.0, J₇₋₈ = 6.0 Hz, H-8), 4.32-4.39 (m, 2H, H-6, H-8'), 4.62 (m, 1H, H-7), 4.73 (m, 1H, H-α), 5.07 (dd, 1H, J₄₋₅ = 6.0, J₅₋₆ = 4.0 Hz, H-5), 5.22 (d, 1H, J₆₋₇ = 1.0 Hz, H-2), 5.49 (dd, 1H, H-4), 13C NMR (62.9 MHz, D₂O): δ = 24.6 (CH₃, CH₂ arg), 25.0 (CH₃), 25.9 (CH₃), 26.0 (CH₃), 29.4 (CH₂ arg), 41.0 (CH₂ arg), 54.6 (C-α), 65.6 (C-8), 74.9 (C-7), 77.2 (C-5), 81.6 (C-4), 87.0 (C-6), 96.0 (C-2), 111.0 (C(CH₃)₂), 115.2 (C(CH₃)₂), 157.0 (C=O), 165.5 (C-3), 167.3 (C=O), 178.9 (C=O). IR (pellets) ν: 3132, 3165, 2981, 2937, 2725, 2860, 1672, 1583. ESI-HRMS: m/z calcd for C₂₀H₃₉NaO₈ [M+Na]+ 457.2293; found 457.2278.

**3,6-Anhydro-2-deoxy-4,5-O-(1-methyleneylidine)-D-glycero-1-galacto-octonic acid methyl ester 43**

To a stirred solution of 4 (3.16 g, 10.0 mmol) in methanol (200 mL) at 0°C was added an aqueous solution of 1N HCl (70 mL). After stirring at room temperature until completion of the reaction, sat. aq. NaHCO₃ was added until pH = 7. Half of the solvent was removed under vacuo and the product was extracted with CH₂Cl₂ (3 x 100 mL). The organic layer was dried over MgSO₄, filtered and concentrated. The crude residue was purified by column chromatography (silica gel, cyH/EA) to provide 43 (2.54 g, 92%). White solid. Rf: 0.29 (EA). [α]D = -5.6° (c = 1.0, CHCl₃). Mp = 50°C. ¹H NMR (250 MHz, CDCl₃): δ = -1.31 (s, 3H, C(CH₃)₂), 1.47 (s, 3H, C(CH₃)₂), 2.78 (dd, 1H, J₆₋₇ = 17.0, J₇₋₈ = 7.0 Hz, H-2), 2.86 (dd, 1H, J₂₋₃ = 7.0 Hz, H-2'), 3.57 (dd, 1H, J₆₋₇ = 6.5, J₅₋₆ = 3.0 Hz, H-6), 3.72 (s, 3H, O-CH₃), 3.74 (dd, 1H, J₆₋₇ = 11.5, J₇₋₈ = 5.0 Hz, H-8), 3.81 (dd, 1H, J₇₋₈ = 4.0 Hz, H-8'), 3.95 (td, 1H, J₃₋₄ = 3.5 Hz, H-3), 4.09 (m, 1H, H-7), 4.75 (dd, 1H, J₄₋₅ = 6.0 Hz, H-4), 4.79 (dd, 1H, H-5). ¹³C NMR (62.9 MHz, CDCl₃): δ = 24.6 (CH₃), 25.7 (CH₃), 33.3 (C-2), 35.1 (OCH₃), 63.4 (C-7), 70.7 (C-8), 77.3 (C-3), 81.0, 81.3 (C-4, C-5, C-6), 112.6 (C(CH₃)₂), 171.5 (C=O). IR (film) ν: 3440, 2990, 2938, 2876, 1735. ESI-HRMS: m/z calcd for C₁₂H₂₀O₇K [M+K]+ 315.0841; found 315.0858.
3.6-Anhydro-2-deoxy-4,5-O-(1-methylethylidene)-L-galacto-heptonic acid methyl ester 44

To a stirred solution of 43 (2.2 g, 7.97 mmol) in methanol (180 mL) was added NaIO₄ (3.42 g, 16.0 mmol, 2 eq.) under argon. After stirring at room temperature until completion of the reaction, the solvent was removed by half in vacuo. The mixture was diluted with CH₂Cl₂ (200 mL). The organic layer was washed with water (3 x 75 mL), dried over MgSO₄, filtered and the solvent was removed under reduced pressure. The product was used without further purification.

To a stirred solution of the obtained aldehyde in MeOH (190 mL) was added NaBH₄ (305 mg, 7.97 mmol, 1 eq.) at 0°C. After 1 h at room temperature, the solvent was removed under reduced pressure. The residue was diluted in CH₂Cl₂ (100 mL) and the organic layer was washed with a solution of 1N HCl (30 mL) and with water until pH = 7. The organic layer was dried over MgSO₄, filtered and evaporated under reduced pressure. The crude product 44 was used without further purification. Yield: 93% (2.24 g). Colorless oil.

3,6-Anhydro-2-deoxy-4,5-O-(1-methylethylidene)-7-(4-methylbenzenesulfonyl)amide 45

Prepared starting from 44 (1.50 g, 6.1 mmol) following procedure described for 8. Yield: 92% (2.24 g). Colorless oil. Rf: 0.47 (EA). [α]D = -1.4° (c = 0.2, CHCl₃). ¹H NMR (250 MHz, CDCl₃): δ = 1.32 (s, 3H, C(CH₃)₂), 1.47 (s, 3H, C(CH₃)₂), 1.94 (br s, 1H, OH), 2.77 (dd, 1H, J₆,₇ = 7.0 Hz, H-2'), 2.85 (dd, 1H, J₅,₆ = 3.5 Hz, H-3'), 3.66 (m, 1H, H-6), 3.71 (s, 3H, OCH₃), 3.89 (dd, 1H, J₆,₇ = 11.0, J₅,₆ = 5.0 Hz, H-7'), 3.96 (dd, 1H, J₆,₇ = 4.0 Hz, H-7'), 3.97 (m, 1H, H-3), 4.75 (dd, 1H, J₄,₅ = 6.0 Hz, H-4), 4.79 (dd, 1H, H-5). ¹³C NMR (62.9 MHz, CDCl₃): δ = 24.7 (CH₃), 25.8 (CH₃), 33.3 (C-2), 51.8 (OCH₃), 61.0 (C-7), 77.4 (C-3), 80.9, 81.3, 81.4 (C-4, C-5, C-6), 112.7 (C(CH₃)₂), 171.4 (C=O). IR (film) ν: 3459, 2985, 2928, 2862, 1737. ESI-MS: m/z calcd for C₁₁H₁₈O₆Na [M+Na]+ 269.0996; found 269.1030.

3.6-Anhydro-2,7-dideoxy-4,5-O-(1-methylethylidene)-7-(4-methylbenzenesulfonyl)amide 46

Prepared starting from 45 (2.0 g, 5.0 mmol) following procedure described for 30. Yield: 84% (1.14 g). Colorless oil. Rf: 0.51 (CH/EA: 1/1). [α]D = + 6.0° (c = 0.1, CHCl₃). ¹H NMR (250 MHz, CDCl₃): δ = 1.32 (s, 3H, C(CH₃)₂), 1.46 (s, 3H, C(CH₃)₂), 2.76 (dd, 1H, J₁,₂ = 17.0, J₃,₄ = 7.0 Hz, H-2'), 2.84 (dd, 1H, J₂,₃ = 7.0 Hz, H-2'), 3.51 (dd, 1H, J₆,₇ = 6.5 Hz, H-7'), 3.57 (dd, 1H, J₆,₇ = 6.5 Hz, H-7'), 3.67 (m, 1H, H-6), 3.71 (s, 3H, OCH₃), 3.96 (td, 1H, J₄,₅ = 3.5 Hz, H-3), 4.70 (dd, 1H, J₄,₅ = 6.0 Hz, H-4), 4.78 (dd, 1H, J₅,₆ = 3.5 Hz, H-5). ¹³C NMR (62.9 MHz, CDCl₃): δ = 25.1 (CH₃), 26.0 (CH₃), 33.5 (C-2), 49.8 (C-7), 52.0 (OCH₃), 77.9 (C-3), 79.9 (C-6), 81.0, 81.3 (C-4, C-5), 113.0 (C(CH₃)₂), 171.5 (C=O). IR (film) ν: 2985, 2943, 2867, 2099, 1742. ESI-MS: m/z calcd for C₁₁H₁₇N₃O₃Na [M+Na]+ 294.1060; found 294.1065.

3.6-anhydro-2,7-dideoxy-4,5-O-(1-methylethylidene)-7-benzosulfonylamino-L-galacto-heptonic acid methyl ester 47
To a solution of 46 (500 mg, 1.84 mmol, 1 eq.) in MeOH (10 mL) was added Pd/C (10%) (50 mg, 10% w/w). After stirring under H₂ atmosphere (15 psi) until completion of the reaction, the mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo. The crude amine was solubilized in pyridine (3 mL) and this solution was added dropwise under argon atmosphere to a stirred solution of benzylsulfonyl chloride (28 µL, 2.21 mmol, 1.2 eq.) in pyridine (6 mL). After stirring at room temperature until completion of the reaction, the solvent was removed under reduced pressure. The residue was diluted with EtOAc (15 mL) and washed with H₂O (2x5 mL). The organic layer was dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. The compound was purified by flash chromatography (silica gel, cyh/EA) to provide 47 (566 mg, 80%). Colorless oil. Rf: 0.22 (cH/EA: 7/3), [α]₀= +12.2° (c = 0.5, CHCl₃). ¹H NMR (250 MHz, CDCl₃): δ = 1.27 (s, 3H, C(CH₃)₂), 1.36 (s, 3H, C(CH₃)₂), 2.64 (dd, 1H, J₆₋₇ = 6.5 Hz, H-2), 2.73 (dd, 1H, J₅₋₆ = 6.5 Hz, H-2'), 3.27 (t, 2H, J₃₋₄ = 6.5, J₆₋₇ = 6.5 Hz, H-7), 3.63 (td, 1H, J₅₋₆ = 3.5 Hz, H-6), 3.70 (s, 3H, OCH₃), 3.88 (td, 1H, J₅₋₆ = 3.5 Hz, H-3), 4.63 (dd, 1H, J₆₋₇ = 6.0 Hz, H-4), 4.71 (dd, 1H, H-5), 4.88 (t, 1H, NH), 7.48-7.61 (m, 3H, H-Ar), 7.85-7.89 (m, 2H, H-Ar). ¹³C NMR (62.9 MHz, CDCl₃): δ = 24.7 (CH₃), 25.7 (CH₃), 33.3 (C-2), 42.1 (C-7), 51.8 (OCH₃), 77.5 (C-3), 79.1 (C-6), 80.8, 81.2 (C-4, C-5), 112.7 (C(CH₃)₂), 127.1 (2-C-Ar), 129.1 (2-C-Ar), 132.6 (C-Ar), 140.0 (C-Ar), 171.2 (C=O). IR (film) ν: 3284, 2985, 2938, 2872, 1737. ESI-HRMS: m/z calcd for C₁₁H₁₃NO₃Na [M+Na]⁺ 408.1087; found 408.1122.

3,6-anhydro-2,7-dideoxy-4,5-O-(1-methylethylidene)-7-benzosulfonylamino-L-galacto-heptonic acid 48

Prepared starting from 47 (500 mg, 1.30 mmol) following procedure described for 21. Yield: 90% (434 mg). Colorless gum. Rf: 0.10 (cH/EA: 1/2), [α]₀= +11.3° (c = 0.08, CHCl₃). ¹H NMR (250 MHz, CDCl₃): δ = 1.28 (s, 3H, C(CH₃)₂), 1.37 (s, 3H, C(CH₃)₂), 2.69 (dd, 1H, J₆₋₇ = 17.0, J₅₋₆ = 6.5 Hz, H-2), 2.79 (dd, 1H, J₅₋₆ = 6.5 Hz, H-2'), 3.28 (t, 2H, J₃₋₄ = 6.5, J₆₋₇ = 6.5 Hz, H-7), 3.66 (td, 1H, J₃₋₄ = 3.5 Hz, H-6), 3.88 (td, 1H, J₃₋₄ = 3.5 Hz, H-3), 4.65 (dd, 1H, J₆₋₇ = 6.0 Hz, H-4), 4.71 (dd, 1H, H-5), 5.02 (t, 1H, NH), 7.48-7.61 (m, 3H, H-Ar), 7.85-7.89 (m, 2H, H-Ar). ¹³C NMR (62.9 MHz, CDCl₃): δ = 24.7 (CH₃), 25.6 (CH₃), 33.3 (C-2), 42.1 (C-7), 77.2 (C-3), 79.3 (C-6), 80.8, 81.1 (C-4, C-5), 112.9 (C(CH₃)₂), 127.0 (2-C-Ar), 129.1 (2-C-Ar), 132.7 (C-Ar), 139.9 (C-Ar), 175.7 (C=O). IR (film) ν: 3270, 2985, 2928, 2857, 1716. ESI-HRMS: m/z calcd for C₁₆H₂₁NO₃Na [M+Na]⁺ 394.0931; found 394.0953.

Ν°-[2-C-[2,3-O-(1-methylethylidene)-5-benzosulfonylamino-α-D-galactofuranosy]carboxymethyl]-l-nitro-arginine methyl ester 49

Prepared starting from 48 (400 mg, 1.08 mmol) following procedure described for 39. Yield: 84% (531 mg). White foam. Rf: 0.4 (EA/MeOH: 9/1), [α]₀= +5.1° (c = 0.4, CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ = 1.25 (s, 3H, C(CH₃)₂), 1.38 (s, 3H, C(CH₃)₂), 1.65-1.76 (m, 3H, CH₂ arg), 1.93 (m, 1H, CH₂ arg), 2.54 (dd, 1H, J₆₋₇ = 15.0, J₅₋₆ = 3.5 Hz, H-2), 2.71 (dd, 1H, J₅₋₆ = 9.0 Hz, H-2'), 3.22 (dd, 1H, J₆₋₇ = 13.0, J₆₋₇ = 8.0 Hz, H-7), 3.30-3.37 (m, 2H, H-7'), CH₂ arg), 3.56 (m, 1H, CH₂ arg), 3.68 (m, 1H, H-6), 3.79-3.92 (m, 4H, H-3, OCH₃), 4.56-4.65 (m, 3H, H-α, H-4, H-5), 5.77 (br s, 1H, NH), 7.13 (d, 1H, J = 7.0 Hz, NH), 7.52-7.62 (m, 5H, 3 H-Ar, 2 NH), 7.90 (d, 2H, J = 7.5 Hz, H-Ar), 8.66 (br s, 1H, NH). ¹³C NMR (100 MHz, CDCl₃): δ = 24.5 (CH₂ arg, CH₃), 25.7 (CH₃), 30.5 (CH₂ arg), 36.1 (C-2), 40.5 (CH₂ arg), 42.3 (C-7), 51.1 (C-α), 53.1 (OCH₃), 77.7 (C-3), 79.9 (C-6), 80.8, 81.6 (C-4, C-5), 112.9 (C(CH₃)₂), 127.0 (2-C-Ar), 129.2 (2-C-Ar), 132.7 (C-Ar), 139.9 (C-Ar), 159.3 (C= N), 172.1 (C=O), 173.0 (C=O). IR (film) ν: 3360, 2933, 2867, 1735, 1633, 1539. ESI-HRMS: m/z calcd for C₂₃H₂₄N₆O₁₀Na [M+Na]⁺ 609.1949; found 609.1969.
3,6-Anhydro-2-deoxy-4,5-O-(1-methylideneidene)-7-(4-methylbenzenesulfonate)-D-altro-heptonic acid methyl ester 51

Prepared starting from 19 (1.0 g, 4.07 mmol) following procedure described for 8. Yield: 98% (1.59 g). Colorless oil. Rf: 0.71 (cH/EA: 1/1); 0.50 (cH/EA: 2/1). [α]D = +11.0° (c = 0.1, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 2.18 (Ph-CH3), 2.50 (2OCH3), 4.00 (dd, 1H, Jgem = 17.0, JH = 5.0 Hz). 13C NMR (62.9 MHz, CDCl3): δ = 21.8 (Ph-CH3), 25.0 (CH3), 26.3 (CH3), 34.5 (C-2), 51.9 (OCH3), 69.6 (C-7), 78.4, 81.4, 82.7 (C-3, C-4, C-5, C-6), 113.1 (C(CH3)2), 128.2 (2 C-Ar), 130.2 (2 C-Ar), 132.6 (C-Ar), 145.3 (C-Ar), 171.4 (C=O). IR (film) ν: 2985, 2943, 1737, 1596. ESI-MS: m/z calcd for C18H24O8SNa [M+Na]+ 423.1084; found 423.1087.

3,6-Anhydro-2,7-dideoxy-4,5-O-(1-methylidenediene)-7-(1-methylethylidene)-D-altro-heptonic acid methyl ester 52

Prepared starting from 51 (1.4 g, 3.5 mmol) following procedure described for 30. Yield: 97% (920 mg). Colorless oil. Rf: 0.50 (cH/EA: 2/1). [α]D = +18.5° (c = 0.2, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.33 (s, 3H, C(CH3)2), 1.49 (s, 3H, C(CH3)2), 2.73 (dd, 1H, Jgem = 17.0, JH = 7.0 Hz). 13C NMR (62.9 MHz, CDCl3): δ = 25.2 (CH3), 26.4 (CH3), 34.5 (C-2), 51.8, 52.0 (C-7, OCH3), 77.6 (C-3), 81.5, 82.9, 83.4 (C-4, C-5, C-6), 113.2 (C(CH3)2), 171.5 (C=O). IR (film) ν: 2990, 2938, 2909, 1737. ESI-MS: m/z calcd for C18H17N3O8Na [M+Na]+ 294.1060; found 294.1073.

3,6-Anhydro-2,7-dIDEOxy-4,5-O-(1-methylidene)-7-benzosulfonamido-D-altro-heptonic acid methyl ester 53

Prepared starting from 52 (200 mg, 0.74 mmol) following procedure described for 47. Yield: 83% (235 mg). Colorless oil. Rf: 0.29 (cH/EA: 7/3). [α]D = +26.2° (c = 0.14, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.29 (s, 3H, C(CH3)2), 1.44 (s, 3H, C(CH3)2), 2.64 (dd, 1H, Jgem = 16.0, JH = 7.0 Hz). 13C NMR (62.9 MHz, CDCl3): δ = 25.2 (CH3), 26.4 (CH3), 34.5 (C-2), 51.8, 52.0 (C-7, OCH3), 77.6 (C-3), 81.5, 82.9, 83.4 (C-4, C-5, C-6), 113.2 (C(CH3)2), 171.5 (C=O). IR (film) ν: 2990, 2938, 2909, 1737. ESI-MS: m/z calcd for C18H17N3O8Na [M+Na]+ 294.1060; found 294.1073.
13C NMR (62.9 MHz, CDCl3): δ = 25.1 (CH3), 26.3 (CH3), 34.1 (C-2), 42.8 (C-7), 52.0 (OCH3), 76.5 (C-3), 81.1, 81.9, 83.1 (C-4, C-5, C-6), 113.3 (C(CH3)2), 127.2 (2 C-Ar), 129.4 (2 C-Ar), 133.0 (C-Ar), 139.7 (C-Ar), 171.5 (C=O). IR (film) ν: 3279, 2990, 2933, 1735. ESI-HRMS: m/z calcd for C17H23NO6SNa [M+Na]+ 408.1087; found 408.1090.

3,6-anhydro-2,7-dideoxy-4,5-O-(1-methylethyldene)-7-benzosulfonylamino-D-altro-heptonic acid 54

Prepared starting from 53 (200 mg, 0.52 mmol) following procedure described for 21. Yield: 96% (185 mg). Colorless oil. Rf: 0.09 (EA/MeOH: 1/2). [α]D = +23.7° (c = 0.2, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.30 (s, 3H, C(CH3)2), 1.45 (s, 3H, C(CH3)2), 2.69 (dd, 1H, Jgem = 17.0, J2,3 = 6.0 Hz, H-2), 2.78 (dd, 1H, J2',3 = 6.0 Hz, H-2'), 2.91 (ddd, 1H, Jgem = 13.0, J6,7 = 8.0, J3,NH = 4.0 Hz, H-3), 3.08 (ddd, 1H, J6,7 = 5.0, J7,NH = 8.0 Hz, H-7'), 4.09 (ddd, 1H, J8,9 = 1.0 Hz, H-6), 4.14 (td, 1H, J5,6 = 4.0 Hz, H-3), 4.56 (dd, 1H, J4,5 = 6.0 Hz, H-5), 4.71 (dd, 1H, H-4), 5.12 (dd, 1H, NH), 7.48-7.62 (m, 3H, 3',4',5'-Ar), 7.84-7.89 (m, 2H, H-Ar). 13C NMR (62.9 MHz, CDCl3): δ = 25.1 (CH3), 26.3 (CH3), 34.1 (C-2), 42.8 (C-7), 76.5 (C-3), 81.1, 82.1, 83.1 (C-4, C-5, C-6), 113.4 (C(CH3)2), 127.2 (2 C-Ar), 129.4 (2 C-Ar), 133.0 (C-Ar), 139.6 (C-Ar), 175.4 (C=O). IR (film) ν: 3265, 2985, 2928, 1716. ESI-HRMS: m/z calcd for C16H22NO7SNa [M+Na]+ 394.0931; found 394.0929.

Nα-[2-C-[2,3-O-(1-methylethyldene)-5-benzosulfonylamino-α-D-ribofuranosyl]-carboxymethyl]-L-nitro-arginine methyl ester 55

Prepared starting from 54 (170 mg, 0.46 mmol) following procedure described for 39. Yield: 71% (191 mg). White solid. Rf: 0.37 (EA/MeOH: 9/1). [α]D = +7.0° (c = 0.5, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.27 (s, 3H, C(CH3)2), 1.44 (s, 3H, C(CH3)2), 1.63-1.83 (m, 3H, CH2 arg), 1.93 (m, 1H, CH2 arg), 2.54 (dd, 1H, Jgem = 15.5, J2,3 = 3.0 Hz, H-2), 2.75 (dd, 1H, J2,3 = 9.5 Hz, H-2'), 2.89 (m, 1H, H-7), 3.06 (ddd, 1H, Jgem = 13.5, J = 8.0, J = 4.5 Hz, H-7'), 3.31 (m, 1H, CH2 arg), 3.47 (m, 1H, CH2 arg), 3.78 (s, 3H, OCH3), 4.12 (m, 1H, H-6), 4.19 (m, 1H, H-3), 4.49 (d, 1H, J5,6 = 6.0 Hz, H-5), 4.57-4.65 (m, 2H, H-α, H-4), 6.17 (br s, 1H, NH), 7.26 (m, 1H, NH), 7.47-7.62 (m, 5H, 3' H-Ar, 2 NH), 7.83-7.88 (m, 2H, H-Ar), 8.59 (br s, 1H, NH). 13C NMR (62.9 MHz, CDCl3): δ = 25.0 (CH2 arg, CH3), 26.4 (CH3), 30.2 (CH2 arg), 36.8 (C-2), 40.8 (CH2 arg), 42.8 (C-7), 51.5 (C-α), 53.2 (OCH3), 76.6 (C-3), 81.6, 83.1, 83.3 (C-4, C-5, C-6), 113.3 (C(CH3)2), 127.1 (2 C-Ar), 129.5 (2 C-Ar), 133.0 (C-Ar), 140.1 (C-Ar), 159.6 (C=N), 172.2 (C=O), 173.5 (C=O). IR (film) ν: 3303, 2990, 2933, 2876, 1737, 1636, 1598, 1537. ESI-HRMS: m/z calcd for C25H34N6O10SNa [M+Na]+ 609.1949; found 609.1940.

Nα-[2-C-[2,3-O-(1-methylethyldene)-5-benzosulfonylamino-α-D-ribofuranosyl]-carboxymethyl]-L-arginine methyl ester 56

Prepared starting from 55 (150 mg, 0.26 mmol) following procedure described for 40. Yield: 70% (94 mg). White solid. Rf: 0.22 (H2O/MeOH: 1/1). HPLC (70/30): tR = 5.526 min. [α]D = +17.1° (c = 0.1, H2O). 1H NMR (250 MHz, D2O): δ = 1.37 (s, 3H, C(CH3)2), 1.52 (s, 3H, C(CH3)2), 1.59-1.95 (m, 4H, CH2 arg), 2.63 (dd, 1H, Jgem = 14.5, J2,3 = 7.5 Hz, H-2), 2.72 (dd, 1H, J2,3 = 6.5 Hz, H-2'), 3.06 (dd, 1H, Jgem = 14.0, J6,7 = 8.0 Hz, H-7), 3.14 (dd, 1H, J6,7 = 6.0 Hz, H-7'), 3.23 (t, 2H, J = 7.0 Hz, CH2 arg), 4.12 (dd, 1H, H-6), 4.20-4.27 (m, 2H, H-3, H-α), 4.74 (d, 1H, J4,5 = 6.0 Hz, H-5), 4.84 (dd, 1H, J4,5 = 4.0 Hz, H-4), 7.66-7.80 (m, 3H, H-Ar), 7.91-7.95 (m, 2H, H-Ar). 13C NMR (62.9 MHz, D2O): δ = 23.6, 24.4, 25.0 (2 CH3, CH2 arg), 28.8 (CH2 arg), 35.2 (C-2), 40.7 (CH2 arg), 41.8 (C-7), 54.6 (C-α), 76.6 (C-3), 80.7, 82.1, 82.4 (C-4, C-5, C-6), 113.1 (C(CH3)2), 126.6 (2 C-Ar), 129.6 (2 C-Ar), 133.6 (C-Ar), 138.3 (C-Ar), 156.7 (C=N), 171.7 (C=O), 178.5 (C=O). IR (pellets) ν: 3331, 3134, 2988, 2928, 2860, 1632, 1575, 1446. ESI-HRMS: m/z calcd for C22H33N6O8SNa [M+Na]+ 550.1942; found 550.1957.
3,6-Anhydro-2,7-dideoxy-4,5-O-(1-methylthiylidene)-7-[(4-bromomethyl)benzoylsulfonylamino]-D-altro-heptonic acid methyl ester 57

To a solution of 52 (165 mg, 0.6 mmol, 1.0 eq.) in MeOH (10 mL) was added Pd/C (10%) (17 mg, 10% w/w). After stirring under H2 atmosphere (15 psi) until completion of the reaction, the mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo. The crude amine was solubilized in CH2Cl2 (1.5 mL) under argon and pyridine (165 µL, 2.1 mmol, 3.5 eq.) and (4-bromomethylbenzyl)sulfonyl chloride (178 mg, 0.66 mmol, 1.1 eq.) were added dropwise. After stirring at room temperature for 30 min, the mixture was diluted with CH2Cl2 (10 mL) and washed with 1N HCl (2x5 mL). The organic layer was dried over MgSO4, filtered and the solvent was evaporated under reduced pressure. The compound was purified by flash chromatography (silica gel, cyH/EA) to provide 57 (174 mg, 61%). Colorless oil. Rf: 0.43 (ch/EA: 1/1). [α]D = +18.5° (c = 0.2, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.29 (s, 3H, C(CH3)2), 1.45 (s, 3H, C(CH3)), 2.64 (dd, 1H, Jgem = 15.5, J2,3 = 6.5 Hz, H-2), 2.74 (dd, 1H, J2',3' = 8.0 Hz, H-2'), 2.90 (ddd, 1H, Jgem = 13.0, J6,7 = 8.5, J7,NH = 4.0 Hz, H-7), 3.08 (ddd, 1H, J6,7' = 5.0, J7,NH = 8.0 Hz, H-7'), 3.71 (s, 3H, OCH3), 4.06 (ddd, 1H, J5,6 = 1.0 Hz, H-6), 4.12 (td, 1H, J3,4 = 4.0 Hz, H-3), 4.53 (dd, 1H, J4,5 = 6.0 Hz, H-5), 4.62 (s, 2H, CH2-Br), 4.70 (dd, 1H, H-4), 4.81 (dd, 1H, NH), 7.52-7.58 (m, 2H, H-Ar), 7.84-7.89 (m, 2H, H-Ar). 13C NMR (62.9 MHz, CDCl3): δ = 24.9 (CH3), 26.1 (CH3), 33.9 (C-2), 42.6 (C-7), 44.8 (CH2-Br), 51.8 (OCH3), 76.3 (C-3), 80.9, 81.7, 82.9 (C-4, C-5, C-6), 113.2 (C(CH3)2), 127.5 (2-C-Ar), 129.2 (2-C-Ar), 139.5 (C-Ar), 142.4 (C-Ar), 171.3 (C=O). IR (film) ν: 3274, 2985, 2924, 2848, 1735, 1440. ESI-HRMS: m/z calcd for C18H24BrNO7SNa [M+Na]+ 500.0349; found 500.0317.

3,6-Anhydro-2,7-dideoxy-4,5-O-(1-methylthiylidene)-7-[(4-azidomethyl)benzoylsulfonylamino]-D-altro-heptonic acid methyl ester 58

To a stirred solution of compound 57 (120 mg, 0.25 mmol, 1 eq.) in DMF (2 mL) was added sodium azide (97 mg, 1.5 mmol, 6 eq.) and the mixture was stirred for 15 hours at 60°C. The solvent was removed in vacuo and the residue was diluted with CH2Cl2 (10 mL), washed with H2O (5 mL) and brine (5 mL). The compound was purified by flash chromatography (silica gel, cyH/EA) to provide 58 (110 mg, quantitative yield). White solid. Rf: 0.21 (cH/EA: 1/3). [α]D = +21.7° (c = 0.25, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.29 (s, 3H, C(CH3)2), 1.45 (s, 3H, C(CH3)), 2.65 (dd, 1H, Jgem = 16.5, J2,3 = 7.0 Hz, H-2), 2.73 (dd, 1H, J2',3' = 6.5 Hz, H-2'), 2.88 (ddd, 1H, Jgem = 13.0, J6,7 = 9.0, J7,NH = 3.5 Hz, H-7), 3.08 (ddd, 1H, J6,7' = 5.0, J7,NH = 8.0 Hz, H-7'), 3.71 (s, 3H, OCH3), 4.03-4.14 (m, 2H, H-6, H-3), 4.46 (s, 2H, CH2-N3), 4.53 (dd, 1H, J4,5 = 6.0 Hz, J5,6 = 1.5 Hz, H-5), 4.69 (dd, 1H, J4',5' = 4.0 Hz, H-4), 4.84 (dd, 1H, NH), 7.49 (d, 2H, J = 8.0 Hz, 2 H-Ar), 7.89 (d, 2H, 2 H-Ar). 13C NMR (62.9 MHz, CDCl3): δ = 25.1 (CH3), 26.3 (CH3), 34.0 (C-2), 42.7 (C-7), 52.0 (OCH3), 54.1 (CH2-N3), 76.5 (C-3), 81.1, 81.9, 83.1 (C-4, C-5, C-6), 113.4 (C(CH3)2), 127.8 (2-C-Ar), 128.8 (2-C-Ar), 139.5 (C-Ar), 140.9 (C-Ar), 171.5 (C=O). IR (pellets) ν: 3274, 2980, 2933, 2104, 1735. ESI-HRMS: m/z calcd for C18H24NaO6SNa [M+Na]+ 463.1258; found 463.1207.

3,6-Anhydro-2,7-dideoxy-4,5-O-(1-methylthiylidene)-7-[(4-azidomethyl)benzoylsulfonylamino]-D-altro-heptonic acid 59

Prepared starting from 58 (100 mg, 0.23 mmol) following procedure described for 30. Yield: quantitative yield (98 mg). Colorless oil. Rf: 0.21 (ch/EA/1): 0.21 (ch/EA/1). [α]D = +15.7° (c = 0.3, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.29 (s, 3H, C(CH3)2), 1.45 (s, 3H, C(CH3)), 2.63-2.80 (m, 2H, H-2), 2.91 (m, 1H, H-3), 3.07 (m, 1H, H-7'), 4.04-4.17 (m, 2H, H-6, H-3), 4.45 (s, 2H, CH2-N3), 4.56 (d, 1H, J4,5 = 6.0 Hz, H-5), 4.70 (dd, 1H, J3,4 = 4.0 Hz, H-4), 5.39 (dd, 1H, NH), 7.47 (d, 2H, J = 8.0 Hz, 2 H-Ar), 7.87 (d, 2H, 2 H-Ar). 13C NMR (62.9 MHz, CDCl3): δ = 24.9 (CH3), 26.1 (CH3), 34.1 (C-2), 42.6 (C-7), 53.9 (CH2-Br), 76.4 (C-3), 81.0, 82.0, 82.9 (C-4, C-
N³-[2-C-[2,3-O-(1-methylethyldiene)-5-][(4-azidomethyl)benzo]sulfonylamino]-α-D-ribofuranosyl[carbonylmethyl]|]-L-arginine methyl ester 60

To a solution of H-L-Arg-OMe.2HCl (55 mg, 0.21 mmol, 1.1 eq.) and 59 (80 mg, 0.19 mmol, 1 eq.) in DMF (10 mL) were added HATU (108 mg, 0.29 mmol, 1.5 eq.) and DIEA (105 µL, 0.61 mmol, 3.2 eq.) at 0°C under argon atmosphere. The reaction mixture was stirred overnight at room temperature and the solvent was evaporated. This crude product was solubilized in THF (3 mL) and water (1 mL) and LiOH (30 mg, 0.57 mmol, 3 eq.) was added. The mixture was stirred until completion of the reaction monitored by tlc. Amberlite® IR-120 was added until pH = 3-4 and the mixture was filtered off. The solvent were evaporated and the product was purified by column chromatography (C-18 silica gel, H 2O/MeOH) and lyophilized to provide 60 (95 mg, 86%). White solid. Rp; 0.18 (H₂O/MeOH: 1/1). [α]D= +31.0° (c = 0.1, MeOH). 1H NMR (250 MHz, MeOH-d₄): δ = 1.29 (s, 3H, C(CH₃)₂), 1.43 (s, 3H, C(CH₃)₂), 1.57-1.70 (m, 2H, CH₂ arg), 1.75 (s, 1H, CH₂ arg), 1.87 (m, 1H, CH₂ arg), 2.53 (dd, 1H, J₁-α = 8.0 Hz, H-2'), 2.63 (dd, 1H, J₂-α = 8.0 Hz, H-2), 2.87-3.04 (m, 2H, 2H-7), 3.17-3.23 (m, 2H, CH₂ arg), 4.02 (dd, 1H, J₆,₇ = 8.0, J₆,₇ = 6.0 Hz, H-6), 4.17 (m, 1H, H-3), 4.28 (dd, 1H, J = 7.5, J = 5.5 Hz, H-a), 4.50 (s, 2H, CH₂-N₃), 4.62-4.69 (m, 2H, 2H-4, 2H-5), 7.55 (d app., 2H, 2H-4, 2H-5), 7.75 (d app., 2H, 2H-4, 2H-5). 13C NMR (100 MHz, DMSO-d₆): δ = 23.9 (CH₃), 24.8 (CH₂ arg), 36.1 (C-2), 41.0 (CH₂ arg), 42.6 (C-7), 53.6, 54.2 (CH₂-N₃, C-α), 77.0 (C-3), 81.6, 83.2, 83.4 (C-4, C-5, C-6), 112.5 (C(CH₃)₂), 127.3 (2-C-Arg), 128.7 (2-C-Arg), 141.1 (C-Arg), 157.4 (C=O), 177.2 (C=O). IR (pellets) ν: 3364, 3184, 2981, 2938, 1648, 1598. ESI-HRMS: m/z calcd for C₂₅H₃₄N₈O₈SNa [M+Na]+ 605.2113; found 605.1130.

N³-[2-C-[2,3-O-(1-methylethyldiene)-5-][(4-aminomethyl)benzo]sulfonylamino]-α-D-ribofuranosyl[carbonylmethyl]|]-L-arginine methyl ester 61

To a solution of 60 (80 mg, 0.14 mmol) in MeOH (5 mL), was added Pd/C (10%) (8 mg, 10% w/w). After stirring under H₂ atmosphere (15 psi) until completion of the reaction, the mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo. The crude was purified by column chromatography (C-18 silica gel, H₂O/MeOH) and lyophilized to provide 61 (65 mg, 87%). White solid. Rp; 0.38 (H₂O/CH₃CN/TFA: 7/3/0.1%). HPLC (70/30): tR = 3.681 min. [α]D= +6.9° (c = 0.1, H₂O). 1H NMR (400 MHz, DMSO-d₆): δ = 1.25 (s, 3H, C(CH₃)₂), 1.38 (s, 3H, C(CH₃)₂), 1.45-1.61 (m, 3H, CH₂ arg), 1.73 (m, 1H, CH₂ arg), 2.47-2.53 (m, 2H, 2-H-2), 2.68 (m, 1H, H-7), 2.82 (m, 1H, H-7'), 3.07-3.11 (m, 2H, CH₂ arg), 3.87 (t, 1H, J₆,₇ = 7.0 Hz, H-6), 4.10-4.17 (m, 3H, 2H-3, 2H₂-NH₂), 4.20 (td, 1H, J = 8.0, J = 5.0 Hz, H-a), 4.63 (d, 1H, J₆,₇ = 6.0 Hz, H-5), 4.66 (dd, 1H, J₆,₇ = 3.5 Hz, H-4), 6.99 & 7.20 (br s, 4H, 4NH), 7.59 (t, 1H, J = 5.5 Hz, NH), 7.67 (d, 2H, J = 8.0 Hz, 2H-4'), 7.84-7.90 (m, 3H, 2H-4', NH), 8.18 (d, 1H, J₆,₇ = 8.0 Hz, NH), 8.28 (br s, 3H, CH₂-NH₂). 13C NMR (100 MHz, DMSO-d₆): δ = 25.2 (CH₃), 25.5 (CH₂ arg), 26.6 (CH₃), 28.8 (CH₂ arg), 35.3 (C-2), 40.7 (CH₂ arg), 42.2 (C-7), 42.9 (CH₂-NH₂), 51.8 (C-α), 77.0 (C-3), 81.3 (C-4), 82.1 (C-6), 83.0 (C-5), 111.8 (C(CH₃)₂), 127.3 (2-C-Arg), 130.1 (2-C-Arg), 139.0 (C-Arg), 140.7 (C-Arg), 157.1 (C=N), 158.5 (TFA), 170.0 (C=O amide), 173.9 (C=O acid). IR (pellets) ν: 3393, 3189, 2990, 2928, 1676, 1546. ESI-HRMS: m/z calcd for C₂₅H₃₄N₈O₈SNa [M+Na]+ 557.2388; found 557.2399.

3,6-Anhydro-2,7-dideoxy-4,5-O-(1-methylethyldiene)-7-[(4-methoxycarbonylmethyl)benzo]sulfonylamino]-d-altro-heptonic acid 62
To a solution of 52 (192 mg, 0.71 mmol) in THF (5 mL) and water (2 mL) was added LiOH (51 mg, 2.13 mmol, 3 eq.) and the mixture was stirred until completion of the reaction monitored by tlc. THF was removed under reduced pressure, CH2Cl2 (20 mL) was added and the pH was adjusted to 2-3 with aqueous 1N HCl. The organic layer was washed with brine and dried over MgSO4, filtered and concentrated under reduced pressure. This carboxylic acid was solubilized in MeOH (10 mL) and Pd/C (10%) (20 mg, 10% w/w) was added. After stirring under H2 atmosphere (15 psi) until completion of the reaction, the mixture was filtered through a pad of celite and the filtrate was concentrated in vacuo. The crude amine was solubilized in pyridine (1 mL) and this solution was added dropwise under argon atmosphere to a stirred solution of methyl 2-[4-(chlorosulfonyl)phenyl]acetate (212 mg, 0.85 mmol, 1.2 eq.) in pyridine (2 mL). After stirring at room temperature until completion of the reaction, the solvent was removed under reduced pressure. The residue was diluted with CH2Cl2 (15 mL) and the pH was adjusted to 3-4 with 1N HCl. The layers were separated and the organic layer was washed with H2O (2x5 mL). The organic layer was dried over MgSO4, filtered and the solvent was evaporated under reduced pressure. The compound was purified by flash chromatography (silica gel, cycH/EA) to provide 62 (95 mg, 30%). Colorless oil. Rf: 0.48 (EA). [α]D= +13.1° (c = 0.1, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.34 (s, 3H, C(CH3)2), 1.47 (s, 3H, C(CH3)2), 2.77 (dd, 1H, J2,3 = 6.0 Hz, H-2'), 2.91 (m, 1H, H-7'), 3.09 (m, 1H, H-7'), 3.70 (s, 2H, C2-CO2CH3), 3.71 (s, 3H, CO2C(CH3)2), 4.01-4.18 (m, 2H, H-6, H-3), 4.57 (dd, 1H, J4,5 = 6.0, J5,6 = 1.0 Hz, H-5), 4.71 (dd, 1H, J3,4 = 4.0 Hz, H-4), 5.25 (dd, 1H, NH), 7.43 (d, 2H, J = 8.0 Hz, H-Ar), 7.81 (d, 2H, H-Ar), 9.53 (br s, 1H, COOH). 13C NMR (62.9 MHz, CDCl3): δ = 24.9 (C-H), 25.7 (C-H), 35.6 (C-2), 39.0 (CH2-CH2-CH3), 42.7 (C-7), 52.3 (CO2CH3), 76.4 (C-3), 80.9, 82.0, 83.0 (C-4, C-5, C-6), 113.2 (C(CH3)2), 127.3 (2 C-Ar), 130.2 (2 C-Ar), 138.4 (C-Ar), 139.1 (C-Ar), 171.1 (C=O), 175.3 (C=O). IR (film) ν: 3270, 2985, 2938, 1730, 1714. ESI-HRMS: m/z calcd for C19H25NO9SNa [M+Na]+ 466.1142; found 466.1190.

N\textsuperscript{ω}-(2-C-[2,3-O-(1-methylthylidene)-5-][(4-carboxymethyl)benzo)sulfonylamino]-α-d-ribofuranosyl[carbonylmethyl]]]-L-arginine methyl ester 63

Prepared starting from 62 (71mg, 0.16 mmol) following procedure described for 60. Yield: 54% (50 mg). Colorless oil. Rf: 0.75 (H2O/CH3CN/TFA: 7/3/0.1%). HPLC (70/30): tR = 4.383 min. [α]D= +5.0° (c = 0.2, H2O). 1H NMR (250 MHz, DMSO-d6): δ = 1.22 (s, 3H, C(CH3)2), 1.36 (s, 3H, C(CH3)2), 1.43-1.62 (m, 3H, CH2 arg), 1.70 (m, 1H, CH2 arg), 2.64-2.93 (m, 4H, 2 H-2, 2 H-7), 3.03-3.12 (m, 2H, CH2 arg), 3.68 (s, 2H, CH2-COOH), 3.84 (t, 1H, J6,7 = 7.0, J6,7' = 7.0 Hz, H-6), 4.08-4.22 (m, 2H, H-3, H-α), 4.56-4.64 (m, 2H, H-4, H-5), 7.08 (br s, 2H, NH), 7.46 (d, 2H, J = 8.0 Hz, H-2 Ar), 7.73 (d, 2H, J = 8.0 Hz, 2 H-Ar), 8.12 (m, 1H, NH), 12.52 (br s, 2H, 2 COOH). 13C NMR (62.9 MHz, DMSO-d6): δ = 25.3 (CH3), 25.7 (CH2 arg), 26.8 (CH3), 29.1 (CH2 arg), 35.6 (C-2), 40.8 (CH2 arg), 41.2 (C-7), 43.2 (CH2-COOH), 52.0 (C-α), 77.2 (C-3), 81.5, 82.3, 83.3 (C-4, C-5, C-6), 112.0 (C(CH3)2), 127.1 (2 C-Ar), 131.0 (2 C-Ar), 139.4 (C-2 Ar), 140.6 (C-Ar), 157.4 (C=), 159.1 (TFA), 170.2, 172.8, 174.1 (3 C=O). IR (pellets) ν: 3374, 3213, 2985, 2938, 1718, 1664, 1648, 1544. ESI-HRMS: m/z calcd for C24H35N5O10SNa [M+Na]+ 608.2026; found 608.1997.

3,6-Anhydro-2,7-deoxy-4,5-O-(1-methylthylidene)-7-[(4-iodo)benzo)sulfonylamino]-D-altro-heptonic acid 64

Prepared starting from 52 (100 mg, 0.37 mmol) and (4-iodo-benzo)sulfonyl chloride (135 mg, 0.44 mmol, 1.2 eq.) following procedure described for 47. Yield: 82% (155 mg). Colorless oil. Rf: 0.25 (CH3/EA: 7/3). [α]D= +18.5° (c = 0.8, CHCl3). 1H NMR (250 MHz, CDCl3): δ = 1.32 (s, 3H, C(CH3)2), 1.47 (s, 3H, C(CH3)2), 2.67 (dd, 1H, Jgem = 16.0, J2,3 = 7.0 Hz, H-2'), 2.76
(dd, 1H, J_{2',3} = 7.0 Hz, H-2'), 2.91 (ddd, 1H, J_{gem} = 13.0, J_{6,7} = 9.0, J_{7,NH} = 4.0 Hz, H-7), 3.09 (ddd, 1H, J_{6,7} = 5.0, J_{7,NH} = 8.0 Hz, H-7'), 3.73 (s, 3H, OCH_{3}), 4.08 (ddd, 1H, J_{5,6} = 1.0 Hz, H-6), 4.15 (td, 1H, J_{3,4} = 4.0 Hz, H-3), 4.55 (dd, 1H, J_{4,5} = 6.0 Hz, H-5), 4.72 (dd, 1H, H-4), 5.01 (dd, 1H, NH), 5.78-7.63 (m, 3H, H-Arg), 7.88-7.93 (m, 2H, H-Arg). \textsuperscript{13}C NMR (62.9 MHz, CDCl_{3}): \delta = 25.1 (CH_{3}), 26.3 (CH_{3}), 34.0 (C-2), 42.7 (C-7), 52.0 (OCH_{3}), 76.5 (C-3), 81.1, 81.9, 83.1 (C-4, C-5, C-6), 100.3 (C-Ar-I), 113.3 (C(CH_{3})_2), 128.6 (2 C-Ar), 138.6 (2 C-Ar), 139.4 (C-Ar), 171.5 (C=O). IR (film) v: 3279, 2985, 2938, 1735, 1572. ESI-HRMS: m/z calcd for C_{17}H_{22}NO_{7}SNa [M+Na]^+ 534.0054; found 534.0056.

3,6-Anhydro-2,7-deoxy-4,5-O-(1-methylethylidene)-7-[(4-(1-trimethylsilylacetylenyl)benzo[sulfonamido]-d-altro-heptonic acid 65

To a stirred solution of 64 (137 mg, 0.25 mmol, 1 eq.), PdCl_{2}(PPh_{3})_{2} (18 mg, 0.025 mmol, 0.1 eq.) and Cul (3 mg, 0.015 mmol, 0.06 eq.) in Et_{3}N (1 mL) was added ethynyltrimethylsilane (42 \mu L, 0.3 mmol, 1.2 eq.). The mixture was stirred at 80 \degree C until completion of the reaction. The solvent was removed under reduced pressure and the crude was purified by flash chromatography (silica gel, cC H/EA: 7/3). [\alpha]_{D} = +20.2\degree (c = 0.2, CHCl_{3}). 1H NMR (250 MHz, CDCl_{3}: \delta = 0.29 (s, 9H, C(CH_{3})_{2}), 1.32 (s, 3H, C(CH_{3})_{3}), 1.47 (s, 3H, C(CH_{3})_{2}), 2.67 (dd, 1H, J_{gem} = 17.0, J_{3,3} = 7.0 Hz, H-2), 2.76 (dd, 1H, J_{2,3} = 7.0 Hz, H-2'), 2.89 (ddd, 1H, J_{gem} = 12.0, J_{6,6} = 9.0, J_{7,NH} = 3.5 Hz, H-7), 3.08 (ddd, 1H, J_{6,6} = 5.0, J_{5,NH} = 8.0 Hz, H-7'), 3.74 (s, 3H, OCH_{3}), 4.07 (ddd, 1H, J_{5,6} = 1.0 Hz, H-6), 4.13 (td, 1H, J_{3,4} = 4.0 Hz, H-3), 4.54 (dd, 1H, J_{4,5} = 6.0 Hz, H-5), 4.72 (dd, 1H, H-4), 4.87 (dd, 1H, NH), 7.61 (d app, 2H, J = 8.5 Hz, H-Arg), 7.82 (d app, 2H, H-Arg). \textsuperscript{13}C NMR (62.9 MHz, CDCl_{3}): \delta = 0.0 (C(CH_{3})_{3}), 25.2 (CH_{3}), 26.4 (CH_{3}), 34.1 (C-2), 42.8 (C-7), 52.1 (OCH_{3}), 76.6 (C-3), 81.2, 82.0, 83.2 (C-4, C-5, C-6), 98.9, 103.3 (C=O), 113.4 (C(CH_{3})_{2}), 127.1 (C-Ar), 127.2 (C-Ar), 128.3 (C-Ar), 132.7 (C-Ar), 132.8 (C-Ar), 139.1 (C-Ar), 171.5 (C=O). IR (film) v: 3279, 2995, 2957, 2900, 2156, 1735, 1588. ESI-HRMS: m/z calcd for C_{18}H_{21}NO_{7}SNa [M+Na]^+ 504.1483; found 504.1479.

3,6-Anhydro-2,7-deoxy-4,5-O-(1-methylethylidene)-7-[[4-(1-acetylenyl)benzo[sulfonamido]-d-altro-heptonic acid 66

Prepared starting from 65 (80 mg, 0.17 mmol) following procedure described for 21. Yield: 82% (55 mg). White foam. \textit{Rf}: 0.48 (EA). [\alpha]_{D} = +27.5\degree (c = 0.1, CHCl_{3}). 1H NMR (250 MHz, CDCl_{3}): \delta = 1.30 (s, 3H, C(CH_{3})_{2}), 1.46 (s, 3H, C(CH_{3})_{2}), 2.70 (dd, 1H, J_{gem} = 17.0, J_{3,3} = 7.0 Hz, H-2), 2.78 (dd, 1H, J_{2,3} = 6.0 Hz, H-2'), 2.90 (ddd, 1H, J_{gem} = 13.0, J_{6,6} = 8.5, J_{7,NH} = 4.0 Hz, H-7), 3.08 (ddd, 1H, J_{6,6} = 5.0, J_{7,NH} = 8.0 Hz, H-7'), 3.26 (s, 1H, H-C(=C)), 4.09 (m, 1H, H-6), 4.15 (m, 1H, H-3), 4.55 (dd, 1H, J_{4,5} = 6.0, J_{5,6} = 1.0 Hz, H-5), 4.71 (dd, 1H, J_{3,4} = 4.0 Hz, H-4), 5.20 (dd, 1H, H-7), 7.59-7.63 (m, 2H, H-Arg), 7.79-7.83 (m, 2H, H-Arg). \textsuperscript{13}C NMR (62.9 MHz, CDCl_{3}): \delta = 24.9 (CH_{3}), 26.1 (CH_{3}), 34.0 (C-2), 42.6 (C-7), 76.3 (C-3), 80.8, 80.9, 81.9, 83.0 (C-4, C-5, C-6, C=C), 113.3 (C(CH_{3})_{2}), 127.0 (3 C-Ar), 132.8 (2 C-Ar), 139.5 (C-Ar), 175.4 (C=O). IR (film) v: 3270, 2990, 2928, 1711. ESI-HRMS: m/z calcd for C_{18}H_{21}NO_{7}SNa [M+Na]^+ 418.0931; found 418.0941.

N\textsubscript{\textalpha}^-[2-C-[2,3-O-(1-methylethylidene)-5-[(4-acetylenyl)benzo]sulfonamido]-\alpha-D-ribo furanosyl[carbonylmethyl]]-\textalpha-L-arginine methyl ester 67

Prepared starting from 66 (45 mg, 0.11 mmol) following procedure described for 60. Yield: 67% (42 mg). White solid. \textit{Rf}: 0.12 (H_{2}O/MeOH: 1/1). [\alpha]_{D} = +10.6\degree (c = 0.3, H_{2}O). 1H NMR (400 MHz, D_{2}O): \delta = 1.23 (s, 3H, C(CH_{3})_{2}), 1.39 (s, 3H, C(CH_{3})_{2}), 1.46-1.54 (m, 2H, CH_{2} arg), 1.62 (m, 1H, CH_{2} arg), 1.77 (m, 1H, CH_{2} arg), 2.49 (dd, 1H, J_{gem} = 15.0, J_{2,3} = 7.0 Hz, H-2), 2.58 (dd, 1H, J_{2,3} = 7.0 Hz, H-2'), 2.86 (dd, 1H, J_{gem} = 13.5, J_{6,6} = 7.0 Hz, H-7), 2.91 (dd, 1H, J_{6,6} = 7.0 Hz, H-7'), 3.07 (t, 2H, J = 7.0 Hz, CH_{2} arg), 3.95 (t, 1H, H-6), 4.07 (td, 1H, J_{3,4} = 4.0 Hz, H-4).
Hz, H-3), 4.11 (dd, 1H, J\textsubscript{H,H,A1} = 8.0, J\textsubscript{H,H,A1'} = 4.5 Hz, H-α), 4.57 (d, 1H, J\textsubscript{4,5} = 6.0 Hz, H-5), 4.67 (dd, 1H, H-4), 7.62 (d app., 2H, J = 8.0 Hz, H-Ar), 7.72 (d app., 2H, H-Ar). \(^{13}\)C NMR (100 MHz, D\textsubscript{2}O): δ = 23.5 (C\textsubscript{H3}), 24.4 (CH\textsubscript{2} arg), 24.9 (CH\textsubscript{3}), 28.8 (CH\textsubscript{2} arg), 35.3 (C-2), 40.6 (CH\textsubscript{2} arg), 42.7 (C-7), 54.5 (C-α), 76.7 (C-3), 80.6 (C-4), 81.9 (C=C), 82.6, 82.7 (C-5, C-6), 112.9 (C(CH\textsubscript{3})), 125.8 (C-Ar), 126.2 (2 C-Ar), 132.8 (2 C-Ar), 140.4 (C-Ar), 156.6 (C=N), 171.7 (C=O), 178.5 (C=O). IR (pellets) ν: 3350, 3289, 3184, 2990, 2938, 2872, 1648, 1593. ESI-HRMS: m/z calcd for C\textsubscript{24}H\textsubscript{33}N\textsubscript{5}O\textsubscript{8}SNa ([M+Na]+ 574.1942; found 574.1960.

**Compound 69**

To a solution of alcyne 67 (33 mg, 0.04 mmol, 1 eq.) and azido sugar 68 (15 mg, 0.04 mmol, 1 eq.) in a water/tBuOH mixture (0.5 mL/0.5 mL) were added sodium ascorbate (2.4 mg, 0.008 mmol, 0.2 eq.) and Cu(OAc)\textsubscript{2} (1.2 mg, 0.004 mmol, 0.1 eq.). The solution turned progressively pale green and the mixture was stirred at room temperature until completion of the reaction (the blue color reappeared). Chelex\textsuperscript{®} resin (100 mg) was then added to the solution and the suspension was stirred until the solution became colorless. The resin was filtered off and the solvent were removed under reduced pressure. The crude was purified by column chromatography (C-18 silica gel, H\textsubscript{2}O/MeOH) and lyophilized to provide 69 (37 mg, 77%). White solid. Rf; 0.29 (H\textsubscript{2}O/MeOH: 1/1). HPLC (70/30): t\textsubscript{R} = 4.180 min. [α]D= -6.7° (c = 0.1, H\textsubscript{2}O). \(^{1}H\) NMR (400 MHz, D\textsubscript{2}O): δ = 1.31 (s, 3H, C(C\textsubscript{H3})\textsubscript{2}), 1.46 (s, 3H, C(C\textsubscript{H3})\textsubscript{2}), 1.53-1.61 (m, 2H, C\textsubscript{H}\textsubscript{2} arg), 1.69 (m, 1H, C\textsubscript{H}\textsubscript{2} arg), 1.84 (m, 1H, C\textsubscript{H}\textsubscript{2} arg), 2.59 (dd, 1H, J\textsubscript{gem} = 15.0, J\textsubscript{2,3} = 8.0 Hz, H-2), 2.66 (dd, 1H, J\textsubscript{2,3} = 6.5 Hz, H-2'), 3.08-3.17 (m, 3H, C\textsubscript{H}\textsubscript{2} arg, H-7'), 3.26 (m, 1H, H-7), 4.08 (dd, 1H, J\textsubscript{6,7} = 8.5, J\textsubscript{6,7'} = 6.5 Hz, H-6), 4.14-4.25 (m, 3H, H-3, H-α, H-9), 4.33 (m, 1H, H-9), 4.47 (d, 1H, J\textsubscript{10,11} = 8.0 Hz, H-10), 4.61 (dd, 2H, J\textsubscript{HF} = 47.5, J\textsubscript{14,15} = 2.0 Hz, H-15), 4.68 (d, 1H, J\textsubscript{4,5} = 6.0 Hz, H-5), 4.73-4.77 (m, 3H, H-4, 2 H-8), 7.94 (d app., 2H, J = 8.0 Hz, H-Ar), 7.99 (d app., 2H, H-Ar), 8.49 (H-triazole). \(^{13}\)C NMR (62.9 MHz, D\textsubscript{2}O): δ = 23.9 (CH\textsubscript{3}), 24.6 (CH\textsubscript{2} arg), 25.3 (CH\textsubscript{3}), 29.1 (CH\textsubscript{2} arg), 35.6 (C-2), 40.9 (CH\textsubscript{2} arg), 42.2 (C-7), 50.9 (C-8), 54.8 (C-α), 68.5 (d, J\textsubscript{C,F} = 7.0 Hz, C-13), 68.7 (C-9), 73.1 (C-11), 74.5 (d, J\textsubscript{C,F} = 18.0 Hz, C-14), 75.7 (C-12), 77.0 (C-3), 81.0, 82.4, 82.7 (C-4, C-5, C-6), 82.1 (d, J\textsubscript{C,F} = 168.0 Hz, C-15), 102.9 (C-10), 113.3 (C(CH\textsubscript{3})\textsubscript{2}), 124.4 (C-Ar), 126.7 (2 C-Ar), 127.8 (2 C-Ar), 134.7 (C-triazole), 138.5 (C-Ar), 146.0 (C-triazole), 156.9 (C=N), 172.0 (C=O), 178.7 (C=O). ESI-HRMS (235.2 MHz, D\textsubscript{2}O): δ = -235.16 (td, J\textsubscript{HF} = 47.0, J\textsubscript{HF} = 27.0 Hz). IR (ATR) ν: 3346, 3236, 19F NMR (235.2 MHz, D\textsubscript{2}O): δ = -235.16 (td, J\textsubscript{HF} = 47.0, J\textsubscript{HF} = 27.0 Hz). IR (ATR) ν: 3346, 3236, 1655, 1505, 1150. ESI-HRMS: m/z calcd for C\textsubscript{32}H\textsubscript{47}FN\textsubscript{8}O\textsubscript{13}SNa [M+Na]+ 825.2860; found 825.2845.
Molecular modeling

The structures of the investigated compounds were prepared according to the CORINA\(^1\) (for the 3D conformers) and MOPAC\(^2\) softwares (for the atomic charges). The chemicals were firstly docked using the GOLD software.\(^3\) The protein target was the 3D structure of the b1 domain of NRP-1 as solved by X-ray when bound with the small molecule EG00229\(^4\) (pdb code 3I97). Previous to the docking process, all hydrogen atoms were added to the PDB protein chain A considered at pH 7. In the GOLD settings, the protein binding site was defined according to the position of the EG00229 ligand within the complex providing a list of interacting amino acid residues with an extra range of 10 Å from each ligand atom. For each docking run, 50 different ligand starting poses were used and optimized according to the Goldscore docking function. Molecular dynamics simulations were performed using the NAMD software.\(^5\) All protein/ligand complexes resulting from the best GOLD docking poses were checked for their stability using short 10 ns molecular dynamics. For this purpose, each protein 3D model was first solvated with an 80 Å\(^3\) box of TIP3P explicit water molecules. Next, ions were added for ensuring the electrostatic neutrality of the whole protein+solvent systems. The initial states for dynamics were generated from the GOLD models after 64,000 steps of conjugate gradients minimization followed by an equilibration stage of 1 ns. The simulations were carried out in the isobaric-isothermal ensemble, maintaining the pressure and the temperature at 1 atm and 300 K respectively by using Langevin dynamics and the Langevin piston approaches. The equations of motion were integrated with a 1fs time step. Long-range interactions were treated using the particle-mesh Ewald approach with an 11 Å cut-off (switching distance 9 Å) for the real space calculation. The calculation of forces and motion equations was repeated to generate the trajectories corresponding to the requested simulation times. A conformation of the whole molecular system was recorded every 10ps, generating for each 10 ns runs a conformational sample of 1,000 frames which were analyzed using the VMD graphic software.\(^6\)
Protein production, purification and biocrystallography

Recombinant pET-15b (Novagen) vector producing the human NRP-1-b1 domain protein were previously constructed using the NdeI and BamHI restriction sites. The recombinant N-terminal His-fusion protein containing a bovine thrombin protease site were produced in E. coli cells strain BL21 (DE3) Rosetta 2 (Novagen). Growth was performed in 2 L of Luria Broth medium under agitation at 37°C and induced to an OD of 0.8 by addition of 0.2 mM Isopropyl-β-D-Thiogalactopyranoside (Euromedex) and shifted at 20°C under the night. Cell harvesting was carried out by centrifugation (4 000 g, 30 min, 4°C). For purification, the frozen pellet was resuspended in 30 ml of lysis buffer (TrisHCl 50 mM pH 8, NaCl 300 mM, Imidazole 20 mM, β-Mercaptoethanol 5 mM) for sonication. After a centrifugation of 30 min at 4°C, 37 000 g, the supernatant was injected on a HiTrap TALON crude 5 ml column (GE Healthcare) and eluted by an imidazole gradient. The eluate was injected on a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare) in GF buffer (TrisHCl 20 mM pH 8, NaCl 50 mM, Tris 2-CarboxylEthyl Phosphine 0.5 mM) and the adequate fractions were pooled and concentrated using an Amicon Ultra-15 (Merck Millipore, 10 kDa cut-off) to a concentration of about 40 mg/ml.

A crystallization screening of NRP-1-b1 fragment including a 6-His tag at the N-terminus was carried out in presence of compounds 27, 40, 50 and 56. The protein was diluted to a concentration of 7 mg/ml and 10 mM ligand prior to crystallization with the hanging drop method at 20°C. Crystals were obtained by mixing 2μl of protein solution with 1.5 μl of precipitating solution from the well: 25% PEG 550 mme, 5% PEG 20 000, 60 mM MgCl2, Na-Bicine 100 mM pH 8.5. Diffraction data were collected at BM30A beamline at ESRF (Grenoble, France) at a wavelength of 0.97967 Å using a ADSC Quantum 315r detector. The diffraction intensities were indexed, integrated, scaled and merged with XDS and XSCALE. The structure, including modelled protein atoms, was refined with PHENIX starting from the coordinates of the 1KEX PDB entry. The crystal structure has been deposited at the Protein Data Bank with code 5C7G.
Biological Assay

**General procedure for receptor binding assays:** NRP-1 and KDR were obtained from R&D Systems (Lille, France), as recombinant chimeric proteins. The binding of new derivatives to recombinant NRP-1 protein was determined using a competition assay initially described by Tirand et al. (see details in SI). The surface of microplates (Dutscher) was coated with either NRP-1 (2 mg/mL) or KDR (2 mg/mL) in PBS, overnight at room temperature. The plates were blocked with PBS containing 0.5% bovine serum albumin (blocking buffer) during 1 h at 37°C, to prevent non-specific interactions. Binding of compounds to NRP-1 was assessed using 5 ng/mL of biotinylated VEGF-A165 (R&D Systems) in blocking buffer containing 2 mg/mL heparin. Biotinylated VEGF-A165 was added to the coated wells, in competition, or not, with an excess of compounds or non labelled VEGF-A165 (R&D Systems), as a positive control. After a 2-h incubation at room temperature, the plates were washed and the amount of bound biotinylated VEGF-A165 stained with streptavidin horseradish peroxidase conjugate (R&D Systems). After 20 min at room temperature, reaction was stopped by the addition of Stop Solution (R&D Systems). Optical densities were measured at 450 nm. Results were expressed as relative absorbance to wells containing only blocking buffer. Three wells per condition were used.

**General procedure for HUVECs culture and treatments:** Briefly, HUVECs were collected from umbilical cords as previously described by Jaffe et al (see details in SI). HUVECs were cultured and used until passage 3 in EndoGro medium (Merck-Millipore, France) supplemented with 10% calf serum, 2 mM l-glutamine (GibcoBRL), 100 U/ml penicillin (GibcoBRL, France), 100 μg/ml streptomycin (GibcoBRL, France), 2.5 μg/ml amphothericin B (GibcoBRL, France). For all experiments, tuftsin (Bachem, Switzerland), (TKPR) the natural ligand of NRP-1, was used as reference control. For angiogenesis and migration assays, bevacizumab (Roche, France) was used as positive control at the concentration of 200 μg/mL.

**General procedure for NRP1 signaling pathways:** For analysis of pAKT and pERK1/2 expression, western blotting was realized as previously described (see details in SI). Briefly, protein aliquots (40 μg) were denatured in the Laemmli buffer, containing β-mercaptoethanol and before to be resolved in SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred onto PVDF membranes (Amersham Biosciences, Orsay, France). After blocking the PVDF membrane with Tris base-buffered saline prepared with 0.1% (v/v) Tween-20 containing 5% (w/v) bovine serum albumin, the following primary antibodies against human AKT (#9272, Cell Signaling, 1:1000 dilution), phosphorylated AKT at ser473 (pAKT) (#9271, Cell Signaling, 1:1000 dilution), ERK1/2 (#9102, Cell Signaling, 1:1000 dilution), phosphorylated ERK1/2 at Thr202/Tyr204 (pERK1/2) (#9106, Cell Signaling, 1:1000 dilution) were incubated overnight at 4°C. Subsequently, immuno-reactive proteins were visualized using the enhanced chemiluminescence procedure (Pierce, USA). Quantification of relative band densities was performed using densitometer (LAS Imager FujiFilm) and AKT and ERK were used as control.

**General procedure for cell viability assays:** Roche’s WST-1 cell proliferation reagent is a simple, colorimetric assay designed to measure the relative proliferation rates of cells in culture (see details in SI). The assay principle is based on the conversion of the tetrazolium salt WST-1 into a colored dye by mitochondrial dehydrogenase enzymes. The soluble salt is released into the media. Within a given time period, the reaction produces a color change which is directly proportional to the amount of mitochondrial dehydrogenase in a given culture. As a result, the assay actually measures the net metabolic activity of cells. To perform the assay, the ready-to-
use WST-1 reagent is simply added directly into the media of cells cultured in 96 well plates. The cultures are then given 30 minutes to reduce the reagent into the dye form. At the set time, 0.5 μmol/L WST-1 were added into each well. WST-1 reagent was used according to manufacturer instructions. The absorbance of the resulting solution was measured at 450 nm using wells without cells as blank (Multiskan Ascent microplate photometer, Thermo Scientific, France).

**Angiogenesis assay:** HUVECs were plated (90,000 cells/cm²) onto 24-well plate ibidi precoated with Matrigel™ Basement Membrane Matrix (BD Biosciences, France, see details in SI). After 1 h, compounds were added on HUVECs which were cultured during overnight before being fixed with 4% paraformaldehyde. Photomicrographs of whole culture surface were taken (Nikon AZ100, Digital Sight DS-Qi1Mc camera, Nikon, France). The number of junctions and segments as well as the network length was quantified with Angiogenesis Analyzer for ImageJ.14

**Invasion assay:** The invasion ability of cells was analyzed using culture permeable support (8 µm pore size, Falcon) coated with 100 µL Matrigel™ (BD Biosciences). HUVECs were pretreated with the different compounds during 24h and plated at a density of 4.105 cells in 500 µL serum free medium, inoculated in the upper chamber; while 700 µL of medium containing 10% FBS was used as the chemoattractant was placed in the lower chamber. After incubating the cells for 24h à 37°C, the non-invading cells remaining on the upper side of the filter were gently removed with cotton swab. The invading cells on the lower chamber were fixed with 4% paraformaldehyde (PFA) for 10 min and stained with crystal violet for 30 min. Crystal violet was solubilized with 4% acetic acid and absorbance at 540 nm was measured. All results were given as mean ± standard error of the mean (SEM) was used. Unpaired t test was employed to determine the statistical significance with a limit set to p<0.05 using GraphPad Prism 5 (GraphPad Software, US) versus non treated cells.

Table S1. Crystallographic summary
<table>
<thead>
<tr>
<th>Diffraction data</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P 4_1 2_1 2</td>
</tr>
<tr>
<td>Unit cell $a, c$ (Å)</td>
<td>62.280   85.960*</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.97967</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>1.45</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>30 507</td>
</tr>
<tr>
<td>Average redundancy</td>
<td>12.2</td>
</tr>
<tr>
<td>$R_{\text{mean}}$(%)</td>
<td>5.1</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Refinement</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$R$-factor (%)</td>
<td>14.1</td>
</tr>
<tr>
<td>$R$-free (%)</td>
<td>17.2</td>
</tr>
<tr>
<td># Protein atoms</td>
<td>2431</td>
</tr>
<tr>
<td># water atoms</td>
<td>294</td>
</tr>
<tr>
<td>Wilson $B$-factor (Å²)</td>
<td>26.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RMSD from target</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bond lengths (Å)</td>
<td>0008</td>
</tr>
<tr>
<td>Bond angles (deg)</td>
<td>1.26</td>
</tr>
</tbody>
</table>

* The $c$ unit cell axis is significantly shorter (85.96 vs. 88.375 Å) compared to the published free protein fragment structure$^9$
Ribbon view of the NRP-1 b1 dimer in the tetragonal crystal, where bicine interacts with residues of a symmetry related protein molecule. Symmetry code: \(-X+\frac{1}{2}, Y-\frac{1}{2}, -Z+\frac{1}{4}\). Some residues interacting with bicine and the Na\(^+\) ion are shown.

The binding of bulky ligands in the cleft is not compatible with the formation of the present tetragonal crystals. The binding cleft is located too close to a symmetry related protein. The b1 domain of human NRP-1 bound with bulky molecule EG00229 crystallized actually in a different space group with two molecules in the asymmetric unit.\(^4\)

The bicine molecule was used as pH buffer at a concentration of 100mM in the conditions yielding crystals. The initial structure of the free protein\(^9\) which was crystallized at pH 6 using MES buffer has only water molecules in the binding site.
Figure S2. a) Stereo view (wall eyes) of the electron density showing the bicine molecule in the binding site. The map shown is $\sigma_A$-weighted $2mF_o-DF_c$ map contoured at 1.2$\sigma$.
b) Stereoview (wall eyes) of the bicine molecule within the binding site surface. The protein-only surface is shown at an electron density level of 0.01 e/Å$^3$ and colored according to atom types (oxygen: red, nitrogen: blue, carbon: grey, hydrogen: white). The water molecules are shown as red spheres and the Na$^+$ ion as a purple sphere. The image was created with MoProViewer.$^{15}$ The Na$^+$ ion is also stabilized in this position distant from Asp320 due to a second electrostatic interaction with the symmetry related carboxylate of Asp289 ($d = 4.1$ Å)
**Figure S3**: Effect of the different compounds (c = 50 µM) on HUVECs ability to migrate through a basement membrane matrix (invasion assay). The results are presented as mean ± standard error of the mean of the percentage of invading cells with non treated cells used as reference, n=6 p<0.05 was considered to be statistically significant. tuf, tuftsin; beva, bevacizumab.

**References**

54. Molecular Networks GmbH, Erlangen, Germany