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Total synthesis of the collagen glycosylated cross-link β-D-galactopyranosyl-*O*-pyridinoline and of its unnatural epimer β-D-galactopyranosyl-*O*-*epi*pyridinoline

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Abstract—A short parallel synthesis of β-D-galactopyranosyl-*O*-pyridinoline (Gal-PYD), a collagen glycoconjugated cross-link, and of Gal-*epi*PYD, a (5*S*)-epimer, useful as internal standard in the analytical evaluation of the natural isomer in human tissue, is reported.

1. Introduction

Glucosyl-galactosyl pyridinoline (Glc-Gal-PYD) **1** and galactosyl pyridinoline (Gal-PYD) **2** are two glycoconjugated cross-links of collagen (Fig. 1), the first formed by the glycosylation of pyridinoline (PYD) **3** mainly present in synovial tissue, the second formed from PYD mainly present in bone.[1] Recently,[2] we reported the first chemical synthesis of Glc-Gal-PYD **1**, which is currently under extensive investigation as a possible marker of the variations in joint-tissue remodelling and of the progression of articular cartilage destruction in osteoarthritis.[1,3–5] In fact, according to Gineyts et al.,[3] Glc-Gal-PYD **1** is strongly associated with the presence of osteoarthritis at the tibiofemoral and patellofemoral joints in men, and its urinary levels are considered more specific markers of joint progressive destruction in various diseases, including rheumatoid arthritis.[3–5]

Gal-PYD **2** has been detected, after alkaline hydrolysis of different tissues[6] but, until now, it has not been available by synthesis. Its potential utility in the diagnosis and therapy management of metabolism of bone or osteoporosis has also not been checked.

Intrigued by the possibility of exploring the potential biological relevance of Gal-PYD **2**, we report herein its synthesis, as point of efforts directed towards providing access to all known reducible and non-reducible collagen cross-links.[7] Moreover, we also report the parallel synthesis of Gal-*epi*PYD **4**, with an unnatural (5*S*)-hydroxylysine side chain, confident that it could be differentiated by HPLC from its natural isomer and consequently could be used as internal standard in the analytical measurement of Gal-PYD **2** in bone or in other biological media.

Herein, we report the different possibilities of synthesising diastereomerically pure Gal-PYD **2** and Gal-*epi*PYD **4**, together with their differentiation by HPLC. The final short protocol proposed starts from a diastereomeric mixture of *tert*-butyl (2*S*,5*R*)- and *tert*-butyl (2*S*,5*S*)-6-azido-2-benzyloxycarbonylamino-5-hydroxyhexanoate **5** and **6**, two protected synthons of natural (5*R*)-hydroxylysine and of its unnatural (5*S*)-epimer, respectively.[8]

2. Results and discussion

On the basis of our previous work,[2] acquired during the synthesis of Glc-Gal-PYD **1**, the synthesis of Gal-PYD **2** and Gal-*epi*PYD **4**, required the solution of two crucial synthetic problems as the stereoselective β-glycosylation of the hydroxylysine side chain and the subsequent assembling of its 1,4,5-trisubstituted 3-hydroxypyridinium ring. With our previous results in mind,[2,9] we first prepared a series of glycosylated congeners **7**, starting from the known azide **5**,[8] and then, through the reaction sequence depicted in Scheme 1, Gal-PYD **2**. With a parallel sequence of reactions, starting from one of the glycosylated azides **11**

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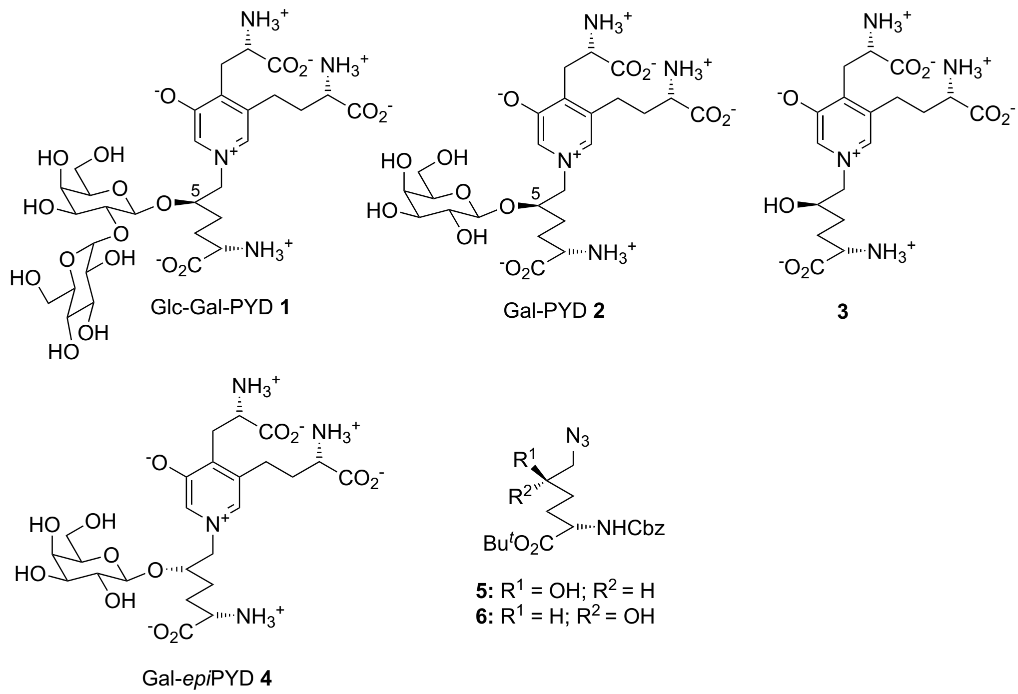
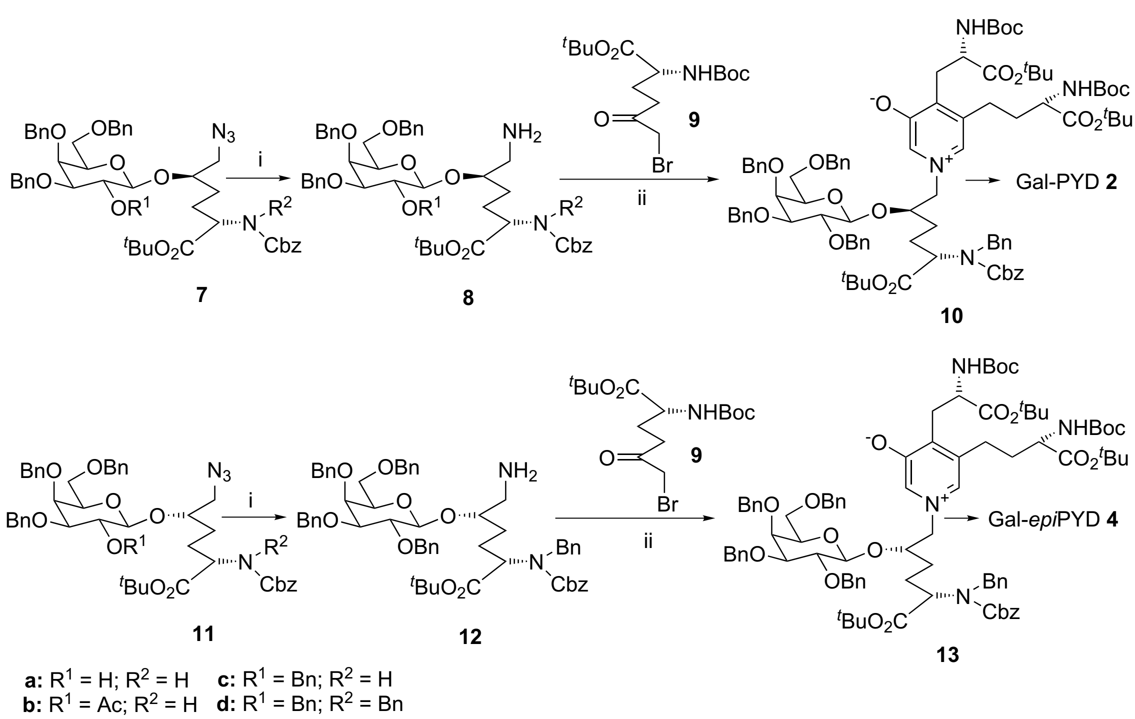


Figure 1. Pyridinoline and glycosylated pyridinolines.



Scheme 1. Synthesis of protected galactopyridinolines. Reagents and conditions: (i) $SnCl_2$, PhSH, Et_3N, THF, rt, 2 h, 83–86%; (ii) Na_2CO_3, MeCN, rt, 6 h, then Na_2CO_3, O_2, MeOH, rt, 7 d, 51–61%.

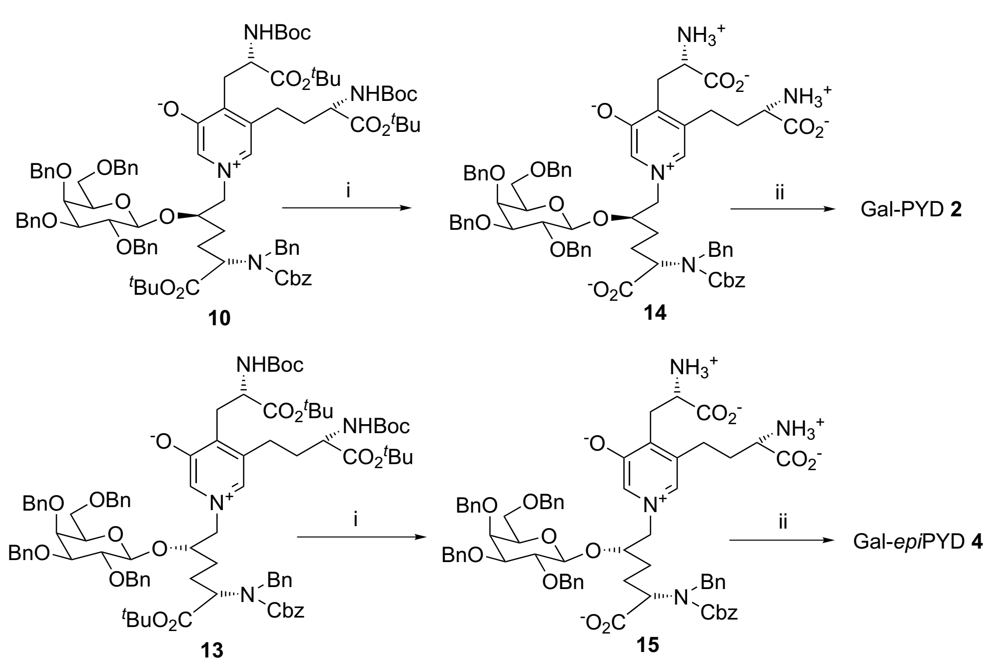
derived[2] from the epimeric azide **6**, we prepared Gal-5*epi*-PYD **4** (Scheme 1).

As shown in Scheme 1, azide **7a** was reduced to the corresponding amine **8a** by reaction with a tin(II) complex formed by treatment of $SnCl_2$ with appropriate amounts of thiophenol and triethylamine in THF.[10] The obtained glycosylated amine **8a** was then reacted with an excess of bromoketone **9** (1:2.5), in CH_3CN containing Na_2CO_3, to promote the initial dialkylation of the amino group of **8a** and to start the sequence of 'one-pot reactions' leading to the 4,5-disubstituted 3-hydroxypyridinium compound **10** (inner condensation of the initially formed amino diketone, followed by air oxidation of the cyclic dienol formed).[11] Moreover, the reaction afforded poor results and only trace amounts of a fluorescent compound, having the molecular mass of **10**, could be detected in the reaction mixture (isolated by TLC). We considered that this result could be due to the concurrent interaction of the free 2-hydroxyl group with bromoketone **9**. In fact, we obtained similar results starting from the glycosylated hydroxylysine **8b**, obtained by acetylation of **7a** to **7b** and successive reduction of the azide group, since the 2-acetate undergoes hydrolysis under the basic conditions of the reaction. At this point we decided to protect the 2-hydroxy group as a benzyl derivative and to start the synthesis with the compound **8c**. This protection should not prolong the synthesis since, at the end, a contemporaneous regeneration of all groups protecting the galactose portion of the molecule could still be possible. Moreover, when we tried to obtain compound **8c** by selective benzylation of the 2-hydroxy group of **7a** and subsequent chemical reduction, we observed, under different reaction conditions, that a concurrent benzylation of the free amidic hydrogen, with the formation of the dibenzylated azide **7d** (TLC and MS evidences) was always operative. Thus we decided to start the synthesis from the completely benzylated amine **8d**. This was obtained by benzylation of **7a**, with benzyl bromide and sodium hydride in the presence of tetrabutylammonium iodide, and successive chemical reduction of the azide group. The glycosylated amine **8d** was then reacted with an excess of bromoketone derivative **9** (1:2.5 molar ratio) in CH_3CN containing Na_2CO_3 to prepare, in a 'one-pot reaction', glycoconjugate **10**. The initial dialkylation showed the expected course and was complete after 6–8 h. At this point, according to our protocol,[2,11] the CH_3CN was replaced by MeOH and the resulting mixture vigorously shaken under a slight pressure of oxygen (1.3 atm) at room temperature for 70 h. Standard work-up and chromatography afforded the glycoconjugate compound **10** in satisfactory yields (61%), as a glass that showed the expected physico-chemical properties, elemental analysis and molecular ion, together with appropriate NMR evidences, which was consistent with the presence of the pyridinium ring, three carboxylic groups and an intact β-bonded galactosidic ring. Treatment of glycoconjugate **10** with aqueous trifluoracetic acid (Scheme 2) regenerated all the carboxylic groups and the Boc protected amino groups, affording benzylated compound **14**, which in turn, via catalytic reduction, afforded the desired Gal-PYD **2** (Scheme 2).

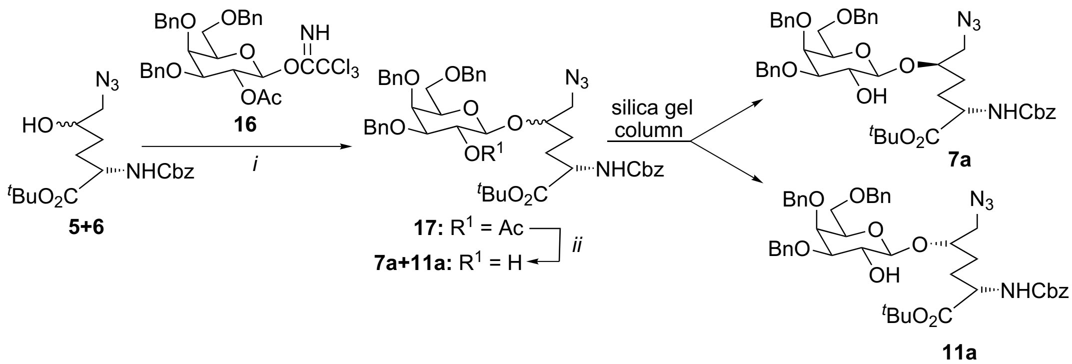
A parallel sequence of reactions, starting from the epimeric azide **11a**, afforded the epimeric compounds **12** and **13** (Scheme 1). Hydrolysis of compound **13** (Scheme 2) afforded amino acid **15** and finally the desired Gal-*epi*PYD **4**.

Thus, we reached our synthetic goal but however we did not consider it completely satisfactory due to the laborious preparation[8,9] of the starting compounds **7a** and **11a**, which reduces the relative simplicity of the total syntheses of Gal-PYD **2** and Gal-*epi*PYD **4**. In fact, the preparation of glycoconjugated azides **7a** and **11a** (starting compounds of the syntheses) also suffers from the difficulty of the preparation of the starting material, that is, the diastereomerically pure (5*R*)- and (5*S*)-masked hydroxylysines **5** and **6**, which at best are obtainable in diastereomeric pure form after a laborious three step separation[8,12,13] of their diastereomeric mixture.

Thus, we considered the possibility of simplifying the preparation of glycoconjugated azides **7a** and **11a**, avoiding the initial separation of the masked hydroxylysines **5** and **6**.



Scheme 2. Deprotection to obtain the galactopyridinolines. Reagents and conditions: (i) TFA, rt, 1 h; (ii) H_2, $PdCl_2$, MeOH–H_2O–AcOH, rt, 12 h, 88–89% over two steps.



Scheme 3. Shortened parallel preparation of the starting materials. Reagents and conditions: (i) ${}^{t}BuMe_2SiSO_3CF_3$, molecular sieves 3 Å, Et_2O, rt, 1 h, 51%; (ii) Cs_2CO_3, MeOH, rt, 6 h, 39% (**7a**) and 41% (**11a**).

With the glycosylated azides (5*R*)-**7a** and (5*S*)-**11a** in hand, we found that it was possible to separate them by simple rapid column chromatography. This allowed their preparation to be shortened, which could start from a diastereomeric mixture of the hydroxyazides, **5** and **6**, thus avoiding their initial laborious separation. Moreover, we were able to additionally shorten the synthesis of glycosylated azides (5*R*)-**7a** and (5*S*)-**11a** using as a galactosyl donor, in the glycosidation of **5** and **6**, the tribenzyl-2-acetylgalactosyl-1-trichloracetimidate **16** in place of the corresponding 2-chloroacetate used previously,[9] done before experiencing the lability of the 2-acetate to basic medium (Scheme 3). Acetate **16** can be easily prepared in 6 steps from commercial galactose[14] while the preparation of the tribenzyl-2-chloroacetylgalactosyl-1-trichloracetamidate requires 8 steps from the same parent sugar.[9]

Thus a convenient and relatively short parallel synthesis has been achieved of the unreported glycoconjugated Gal-PYD **2** and Gal-*epi*PYD **4**, preparing the starting azides according to the sequence of reaction reported in Scheme 3 and pursuing the synthesis according to the reaction sequence reported in Schemes 1 and 2.

With the galactosylated pyridinolines in the hand, we also found the analytical HPLC conditions (see Section 4) required to separate the native and the unnatural isomer which then is a suitable inner standard for the analysis of Gal-PYD **2**.

3. Conclusion

In conclusion a short, simple synthesis of Gal-PYD **2** and Gal-*epi*PYD **4** both of interest for studies in the biological relevance of GalPYD **2** levels in bone and in other biological tissues has been achieved.

The results of the present work have also allowed us to simplify and shorten the preparation of galactosyl hydroxylysine and galactosyl *epi*hydroxylysine, two compounds of biological interest. The shortening of the preparation of azides **7a** and **11a** represents a formal shortening of our previously reported synthesis of these glycosylated amino acids.[9]

4. Experimental

4.1. General methods

Nuclear magnetic resonance spectra were recorded at 298 K on Bruker AM-500 spectrometer operating at 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C. Chemical shifts are reported in parts for million (ppm, δ units) and are referenced to residual $CHCl_3$ ($\delta_H = 7.26$ ppm) and to $CDCl_3$ ($\delta_C = 77.0$ ppm) for solutions in $CDCl_3$ or to internal CH_3OD ($\delta_H = 3.34$ ppm and $\delta_C = 49.5$ ppm) for solutions in D_2O. ^{1}H NMR data are tabulated in the following order: number of protons, multiplicity (s, singlet; d, doublet; br s, broad singlet; m, multiplet), coupling constant(s) in Hertz, assignment of proton(s). The ^{1}H and ^{13}C resonances were assigned by ^{1}H decoupling, ^{1}H–^{1}H COSY and ^{1}H–^{13}C correlation experiments. The nomenclature of the single positions is given as follows (Fig. 2).

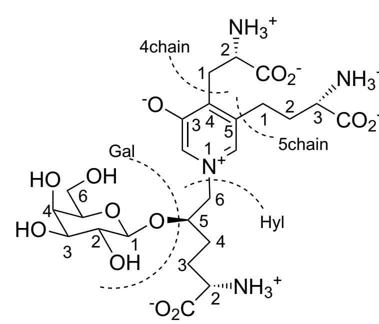


Figure 2. Carbon numeration used in this work.

Optical rotations were taken on a Perkin–Elmer 241 polarimeter and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Mass spectra were obtained using a Finnigan LCQdeca (ThermoQuest) ion trap mass spectrometer fitted with an electrospray source (ESI). The spectra were collected in continuous flow mode by connecting the infusion pump directly to the ESI source. Solutions of compounds were infused at a flow rate of 0.5 mL/min. The spray voltage was set at 5.0 kV in the positive and at 4.5 kV in the negative ion mode with a capillary temperature of 220 °C. Full-scan mass spectra were recorded by scanning a *m*/*z* range of 100–2000. All reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck Silica Gel plates (60 F_{254}) using UV light, 50% sulfuric acid, anisaldehyde/H_2SO_4/EtOH solution or 0.2% ninhydrin in ethanol and heat as developing agent. E. Merck 230–400 mesh silica gel was used for flash column chromatography.[15] Work-up refers to washing with water, drying over Na_2SO_4 and evaporation of the solvent.

4.2. *tert*-Butyl (2*S*,5*R*)-6-azido-2-benzyloxycarbonylamino-5-(3,4,6-tri-*O*-benzyl-2-*O*-acetyl-β-D-galactopyranosyl)hexanoate 7b

The *tert*-butyl (2*S*,5*R*)-6-azido-2-benzyloxycarbonylamino-5-(3,4,6-tri-*O*-benzyl-2-hydroxy-β-D-galactopyranosyl)hexanoate **7a** (330 mg; 0.41 mmol) was dissolved in pyridine (1 mL) and treated with Ac_2O (0.9 mL) at room temperature for 3 h. The addition of methanol followed by dilution with ethyl acetate and washing with a solution of citric acid afforded, after the usual work-up, the 2-acetylated compound **7b** (270 mg; 78%): an oil, showing in TLC $R_f = 0.30$ (eluting with hexane/AcOEt; 70:30; v/v); $[\alpha]_D^{20} = +9.3$ (*c* 1, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ: 7.1–7.2 (aromatic protons), 5.36 (2H, overlapping, d, *J* 7.5, N*H* and dd, *J* = 10.1, 7.9, H_{Gal}-2), 5.2–4.4 (8H, benzylic protons), 4.43 (H_{Gal}-1, overlapped with benzylic protons), 4.24 (1H, m, H_{Hyl}-2), 3.94 (1H, d, *J* = 2.8, H_{Gal}-4), 3.67 (1H, m, H_{Hyl}-5), 3.63 (2H, m, H_{Gal}-6), 3,58 (1H, dd, *J* = 6.6, <1, H_{Gal}-5), 3.50 (1H, dd, *J* = 10.1, 2.8, H_{Gal}-3), 3.40 (2H, m, H_{Hyl}-6), 2.02 (3H, s, C*H*$_3$CO), 1.88 (1H, m, H_{Hyl}-3$_a$), 1.71 (0.5H, m, H_{Hyl}-3$_b$), 1.62 (2.5H, m, H_{Hyl}-4 and H_{Hyl}-3$_b$), 1.48 [3H, s, C(C*H*$_3$)$_3$]; ^{13}C NMR ($CDCl_3$) δ: 171.1 (*C*OO^{t}Bu), 169.3 (CH$_3$*C*OO), 155.7 (N*C*OOR), 139–127 (Aromatics), 101.6 (C_{Gal}-1), 82.4 [*C*(CH$_3$)$_3$], 80.3 (C_{Gal}-3), 78.3 (C_{Hyl}-5), 74.4 (O*C*H$_2$Ph), 73.7 (C_{Gal}-5), 73.6 (O*C*H$_2$Ph), 72.4 (C_{Gal}-4), 72.0 (O*C*H$_2$Ph), 71.4 (C_{Gal}-2), 68.7 (C_{Gal}-6), 66.9 (O*C*H$_2$Ph), 54.5 (C_{Hyl}-6), 54.1 (C_{Hyl}-2), 28.3 (C_{Hyl}-3), 27.9 (C_{Hyl}-4) 27.9 [C(*C*H$_3$)$_3$], 20.9 (*C*H$_3$CO); ESI-MS (positive) *m*/*z*: 875.4 (M+Na$^+$). Anal. Calcd for $C_{47}H_{56}N_4O_{11}$: C, 66.18; H, 6.62; N, 6.57. Found: C, 66.29; H, 6.48; N, 6.61.

4.3. *tert*-Butyl (2*S*,5*R*)-6-amino-2-benzyloxycarbonylamino-5-(3,4,6-tri-*O*-benzyl-2-*O*-acetyl-β-D-galactopyranosyl)hexanoate 8b

Anhydrous $SnCl_2$ (79 mg; 0.42 mmol) was dissolved under stirring into anhydrous THF (1.0 mL) at 25 °C, after which PhSH was added (174 μL; 1.69 mmol) followed by Et_3N (174 μL; 1.27 mmol). After 20 min, the azide **7b** (240 mg; 0.28 mmol), dissolved in anhydrous THF (1.0 mL), was added and stirring continued for 2 h. At this time, the solvent was evaporated under reduced pressure and the residue was recovered with dichloromethane and washed with aqueous NaOH (1 M). After the usual work-up, the residue (250 mg) was chromatographed (eluting with CH_2Cl_2/MeOH; 100:4; v/v) to afford the desired amine **8d** (200 mg; 86% yield); $[\alpha]_D^{20} = +5.2$ (*c* 1, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ: 7.1–7.2 (aromatic protons), 5.36 (2H, overlapping, d, *J* 8.3, N*H*, dd, *J* = 10.1, 7.9, H_{Gal}-2), 5.1–4.4 (8H, benzylic protons), 4.49 (H_{Gal}-1, overlapped with benzylic protons), 4.28 (1H, ddd, *J* = 12.8, 7.8, 4.5, H_{Hyl}-2), 3.98 (1H, d, *J* = 2.7, H_{Gal}-4), 3.75 (1H, m, H_{Hyl}-5), 3.64 (2H, m, H_{Gal}-6), 3.59 (1H, dd, *J* = 11.4, 4.9, H_{Gal}-5), 3.54 (1H, dd, *J* = 10.1, 2.7, H_{Gal}-3), 3.32 (1H, dd, *J* = 13.0, 4.0, H_{Hyl}-6$_a$), 3.20 (1H, dd, *J* = 13.0, 6.5, H_{Hyl}-6$_b$), 2.06 (3H, s, C*H*$_3$CO), 1.92 and 1.81 (2 × 1H, 2m, H_{Hyl}-3), 1.56 (2H, m, H_{Hyl}-4), 1.42 [3H, s, C(C*H*$_3$)$_3$]; ^{13}C NMR ($CDCl_3$) δ: 171.4 (*C*OO^{t}Bu), 169.6 (CH$_3$*C*OO), 155.9 (N*C*OOR), 139–127 (aromatics), 100.7 (C_{Gal}-1), 82.1 [*C*(CH$_3$)$_3$], 80.4 (C_{Gal}-3), 76.6 (C_{Hyl}-5), 74.5 (O*C*H$_2$Ph), 73.5 (O*C*H$_2$Ph), 73.5 (C_{Gal}-5), 72.6 (C_{Gal}-4), 72.0 (O*C*H$_2$Ph), 71.3 (C_{Gal}-2), 68.5 (C_{Gal}-6), 66.7 (O*C*H$_2$Ph), 54.7 (C_{Hyl}-6), 53.8 (C_{Hyl}-2), 28.2 (C_{Hyl}-4), 28.2 (C_{Hyl}-3) 27.9 [C(*C*H$_3$)$_3$], 21.0 (*C*H$_3$CO); ESI-MS (positive) *m*/*z*: 875.4 (M+Na$^+$). Anal. Calcd for $C_{47}H_{58}N_2O_{11}$: C, 68.26; H, 7.07; N, 3.39. Found: C, 68.39; H, 7.20; N, 3.31.

4.4. *tert*-Butyl (2*S*,5*R*)-6-azido-2-[benzyl(benzyloxycarbonyl)amino]-5-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)hexanoate 7d

In a dried round bottom flask, a solution of galactosyl azide **7a** (338 mg; 0.417 mmol) in anhydrous THF (4 mL) was prepared and cooled to 0 °C. NaH (64 mg; 60% in mineral oil; 1.73 mmol) was added and the reaction was stirred at the same temperature for 10 min. Benzyl bromide (205 μL; 1.73 mmol) and tetrabutylammonium iodide (127 mg; 0.345 mmol) were then added and the reaction allowed to warm to room temperature and left under stirring overnight.

The reaction was quenched by adding an NaH_2PO_4 aqueous solution (1.2 M, 5 mL), diluted with AcOEt (20 mL) and worked-up. The flash chromatography purification of the crude material, (eluting with hexane/AcOEt; 80:20; v/v), gave the title compound **7d** (289 mg; 70% yield), in pure form, as an amorphous solid: $[\alpha]_D^{20} = -2.4$ (*c* 1, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, *T* = 323 K) δ: 7.1–7.2 (30H, aromatic protons), 5.23–4.48 (12H, benzylic protons), 4.32 (H_{Gal}-1, overlapping with benzylic protons), 4.16 (1H, b, H_{Hyl}-2), 3.89 (1H, dd, *J* 3.1, 3.0, H_{Gal}-4), 3.80 (1H, dd, *J* = 9.7, 7.6, H_{Gal}-2), 3.64 (2H, m, H_{Gal}-6), 3.57 (1H, m, H_{Hyl}-5), 3.51 (1H, dd, *J* 6.4, 3.0, H_{Gal}-5), 3.48 (1H, dd, *J* = 9.7, 3.0, H_{Gal}-3), 3.3 and 3.2 (2 × 1H, 2m, H_{Hyl}-6), 2.1 and 1.8 (2 × 1H, 2m, 2 conformers in the ratio 6:4, H_{Hyl}-3), 1.5 (2H, 2m, 2 conformers in the ratio 6:4, H_{Hyl}-4), 1.35 [3H, s, C(C*H*$_3$)$_3$]; ^{13}C NMR ($CDCl_3$, *T* = 323 K) δ: 169.9 (*C*OOR), 156.7 (N*C*OOR), 139–127 (aromatics), 103.4 (C_{Gal}-1), 82.4 (C_{Gal}-3), 81.6 [*C*(CH$_3$)$_3$], 79.6 (C_{Gal}-2), 78.1 (C_{Hyl}-5), 75.2 (O*C*H$_2$Ph), 74.5 (O*C*H$_2$Ph), 73.9 (C_{Gal}-4), 73.6 (C_{Gal}-5), 73.6 (O*C*H$_2$Ph), 73.1 (O*C*H$_2$Ph), 69.0 (C_{Gal}-6), 67.5 (O*C*H$_2$Ph), 60.9 (C_{Hyl}-2), 54.5 (C_{Hyl}-6), 50.5 (N*C*H$_2$Ph), 29.4 (C_{Hyl}-4), 27.9 [C(*C*H$_3$)$_3$], 26.1 (C_{Hyl}-3). ESI-MS (positive) *m*/*z*: 1008.3 (M+NH$_4^+$), 10013.5 (M+Na$^+$). Anal. Calcd for $C_{59}H_{66}N_4O_{10}$: C, 71.49; H, 6.71; N, 5.65. Found: C, 71.35; H, 6.59; N, 5.51.

4.5. *tert*-Butyl (2*S*,5*R*)-6-amino-2-[benzyl(benzyloxycarbonyl)amino]-5-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)hexanoate 8d

To a solution of anhydrous $SnCl_2$ (73 mg; 0.385 mmol) in anhydrous THF (3 mL), PhSH (150 μL; 1.36 mmol) and

Et_3N (150 μL; 1.08 mmol) were added and left under stirring for 5 min. A solution of the azide **7d** (227 mg; 0.229 mmol in 1 mL of dry THF) was then added. The reaction was stirred at room temperature for 2 h, after which the solvent was evaporated and the crude residue purified by flash chromatography. By-products were eluted using CH_2Cl_2/MeOH (98:2; v/v) and the glycosylated amine **8d** using CH_2Cl_2/MeOH (92:8; v/v) (168 mg; 76% yield). The compound, obtained as a glassy material, showed: $[\alpha]_D^{20} = -0.7$ (*c* 1, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 323 K) δ: 7.2–7.1 (30H, aromatic protons), 5.2–4.2 (12H, benzylic protons), 4.31 (H_{Gal}-1, overlapping with benzylic protons), 4.05 (1H, m, H_{Hyl}-2), 3.86 (1H, br s, H_{Gal}-4), 3.77 (1-H, m, H_{Gal}-2), 3.66 (1H, m, H_{Gal}-6$_a$), 3.6 (m, H_{Hyl}-5, partially overlapped), 3.57–3.52 (3H, overlapping H_{Gal}-5, H_{Gal}-6$_b$ and H_{Gal}-3), 2.8 (2H, m, H_{Hyl}-6), 2.1 and 1.8 (2 × 1H, 2m, H_{Hyl}-3), 1.5 (2H, m, H_{Hyl}-4), 1.34 [3H, s, C(C*H*$_3$)$_3$]; ^{13}C NMR ($CDCl_3$, $T = 323$ K) δ: 169.8 (*C*OOR), 156.5 (N*C*OOR), 139–127 (aromatics), 103.4 (C_{Gal}-1), 82.4 (C_{Gal}-3), 81.5 [*C*(CH$_3$)$_3$], 80.2 (C_{Gal}-5), 79.3 (C_{Gal}-2), 75.1 (O*C*H$_2$Ph), 74.7 (O*C*H$_2$Ph), 73.8 (C_{Gal}-4), 73.5 (O*C*H$_2$Ph) 73.5 (C_{Gal}-5), 73.18 (O*C*H$_2$Ph), 68.6 (C_{Gal}-6), 67.5 (O*C*H$_2$Ph), 60.9 (C_{Hyl}-2), 50.6 (N*C*H$_2$Ph), 44.8 (C_{Hyl}-6), 30.4 (C_{Hyl}-4), 27.9 [C(*C*H$_3$)$_3$], 25.6 (C_{Hyl}-3). ESI-MS (positive) *m*/*z*: 965.5 (M+H$^+$). Anal. Calcd for $C_{59}H_{68}N_2O_{10}$: C, 73.42; H, 7.10; N, 2.90. Found: C, 72.85; H, 7.25; N, 3.07.

4.6. Completely protected β-D-galactopyranosyl-*O*-pyridinoline 10

To a solution of glycosylated amine **8d** (168 mg; 0.174 mmol) in CH_3CN (15 mL) containing Na_2CO_3 (368 mg; 3.48 mmol), the protected bromoketone **6** (165 mg; 0.435 mmol) was added and the mixture was stirred under nitrogen for 6 h. At this time, the disappearance of both the starting glycosylated amine **8d** and of the initially formed monoalkylated product was observed (TLC; CH_2Cl_2/MeOH; 100:5; v/v; $R_f = 0.3$ and 0.7, respectively). The solvent was then evaporated under reduced pressure after which the crude residue was recovered with MeOH (30 mL) and vigorously shaken, under a slight pressure of oxygen (1.3 atm), at room temperature for 7 days. After this period of time, the mixture was diluted with CH_2Cl_2 (50 mL) and filtered on a pad of Celite. Evaporation of the solvent afforded a crude residue, which was purified by flash chromatography on silica. Elution with CH_2Cl_2/MeOH (100:4; v/v) afforded the protected glycosylated pyridinoline **10** (136 mg; 61% yield) as a resinous material: $[\alpha]_D^{20} = -0.7$ (*c* 1, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, $T = 318$ K) δ: 7.5–7.0 (30H, aromatic protons), 5.20–4.44 (12H, benzylic protons), 4.2 (overlapping, H_{Gal}-1, H_{Hyl}-2 and H_{4Ch}-2), 4.07 (1H, b, H_{5Ch}-3), 3.83 (1H, br s, H_{Gal}-4), 3.69 (1H, m, H_{Gal}-2), 3.47–3.37 (5H, overlapping, H_{Hyl}-5, H_{Gal}-5, H_{Gal}-6 H_{Gal}-3), 3.25 (1H, dd, *J* 11.4, 11.2, H_{4Ch}-1$_a$), 2.92 (1H, dd, $J = 12.6$, <1, H_{4Ch}-1$_b$), 2.55 (2H, m, H_{5Ch}-1), 2.1–1.5 (overlapping, H_{Hyl}-3, H_{5Ch}-2, H_{Hyl}-4), 1.48, 1.47, 1.45, 1.41 and 1.33 [5 × 3H, 5s, 5 × C(C*H*$_3$)$_3$]; ^{13}C NMR ($CDCl_3$, $T = 318$ K) δ: 171.3, 169.6, 169.6 (*C*OOR), 156.5, 155.1, 143.3, 136.4, 130.5 (pyridinium ring), 103.8 (C_{Gal}-1), 82.2 (C_{Gal}-3), 82.0, 81.7, 81.0 [3 × *C*(CH$_3$)$_3$], 79.2 (C_{Gal}-2), 79.1, 77.8 [2 × *C*(CH$_3$)$_3$], 75.2 (O*C*H$_2$Ph), 75.1 (O*C*H$_2$Ph), 74.6 (O*C*H$_2$Ph), 73.5 (O*C*H$_2$Ph), 73.4 (C_{Gal}-4), 73.3 (C_{Gal}-5), 72.9 (O*C*H$_2$Ph), 68.6 (C_{Gal}-6), 67.6 (O*C*H$_2$Ph), 32.9 (C_{5Ch}-2), 28.4 and 28.3 [3 × C(*C*H$_3$)$_3$], 28.3 (C_{4Ch}-1), 28.0, 28.0 and 27.8 [3 × C(*C*H$_3$)$_3$], 26.3 (C_{5Ch}-1), 25.5 and 25.1 (C_{Hyl}-3 and 4). ESI-MS (positive) *m*/*z*:1565.6 (20%; M+Na), 1543.6 (100%, M+H$^+$). Anal. Calcd for $C_{89}H_{114}N_4O_{19}$: C, 69.24; H, 7.44; N, 3.63. Found: C, 69.19; H, 7.35; N, 3.57.

4.7. Preparation of β-D-galactopyranosyl-*O*-pyridinoline Gal-PYD 2

Compound **10** (110 mg; 0.071 mmol) was dissolved in CF_3COOH/H_2O (2 mL; 95:5, v/v) and the resulting solution was stirred at room temperature for 1 h. The solvent was then evaporated under reduced pressure and the residue triturated with a 1:1 mixture of diisopropyl ether/hexane, affording the partially protected glycosylated pyridinoline **14** as its *tris*-trifluoroacetate salt as a powder. The ^{1}H NMR spectrum was recorded in order to verify the complete cleavage of the Boc groups and of the *tert*-butyl esters. The obtained compound was dissolved in 55 mL of a MeOH/H_2O/AcOH mixture (8:2:1; v/v/v). $PdCl_2$ (50 mg) was added and the reaction mixture was shaken overnight, at room temperature, under an atmospheric pressure of hydrogen. The solution was then filtrated, after which MeOH was evaporated under reduced pressure and the remaining solution diluted with water and loaded on a strong acidic resin column (2 mL; Dowex® 50WX8-200). The resin was washed with water and finally with a 1 M NH_3 solution in H_2O/MeOH (2:1; v/v) to recover the product. After evaporation of MeOH the solution was freeze-dried and the residue was dissolved in water and freeze-dried twice to afford the title compound **2** (37 mg; 88% yield over two steps) as a fluffy material which showed: $[\alpha]_D^{20} = -4.4$ (*c* 0.5, H_2O); ^{1}H NMR (D_2O) δ: 7.70 (2H, br s, pyridinium protons), 4.58 (1H, m, H_{Hyl}-6a), 4.38 (1H, d, $J = 7.7$, H_{Gal}-1), 4.32 (1H, m, H_{Hyl}-6b), 4.26 (1H, m, H_{Hyl}-5), 4.02 (1H, dd, $J = 6.6$, 4.6, H_{4Ch}-2), 3.86–3.75 (3H, overlapping, H_{Hyl}-2, H_{5Ch}-3 and H_{Gal}-4), 3.59 (1H, dd, $J = 9.8$, 3.5, H_{Gal}-3), 3.54–3.41 (4H, overlapping, H_{Gal}-2, H_{Gal}-5, H_{Gal}-6a and H_{Gal}-6b), 3.32-3.24 (2H, AB system, H_{4Ch}-1a and H_{4Ch}-1b), 2.96 (1H, m, H_{5Ch}-1a), 2.72 (1H, m, H_{5Ch}-1b), 2.21–2.08 (3H, overlapping, H_{5Ch}-2a, H_{5Ch}-2b and H_{Hyl}-3a), 2.00 (1H, m, H_{Hyl}-3b), 1.81 (1H, m, H_{Hyl}-4a), 1.69 (1H, m, H_{Hyl}-4b); ^{13}C NMR (D_2O, $T = 318$ K) δ: 172.4, 172.2, 171.7 (3 × *C*OOH), 163.3, 140.6, 137.4, 130.9, 128.2 (pyridinium ring), 101.0 (C_{Gal}-1), 75.8 (C_{Hyl}-5), 72.8 (C_{Gal}-5), 70.9 (C_{Gal}-3), 69.1 (C_{Gal}-2), 66.6 (C_{Gal}-4), 61.7 (C_{Hyl}-6), 58.9 (C_{Gal}-6), 52.7 (C_{Hyl}-2), 52.6 (C_{4Ch}-2), 52.5 (C_{5Ch}-3), 29.3 (C_{5Ch}-2), 26.5 (C_{4Ch}-1), 25.9 (C_{Hyl}-4), 24.3 (C_{Hyl}-3), 24.1 (C_{5Ch}-1). Anal. Calcd for $C_{24}H_{38}N_4O_{13}$: C, 48.81; H, 6.49; N, 9.49. Found: C, 48.89; H, 6.55; N, 9.56.

4.8. *tert*-Butyl (2*S*,5*S*)-6-azido-2-[benzyl(benzyloxycarbonyl)amino]-5-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)hexanoate 11d

Starting with the hydroxy azide **11a** (316 mg; 0.389 mmol) and following the procedure described above for compound **7d**, the protected azide **11d** was prepared (290 mg;

75% yield); $[\alpha]_D^{20} = +7.0$ (*c* 1, CH_2Cl_2); ^1H NMR ($CDCl_3$, $T = 323$ K) δ: 7.1–7.2 (30H, aromatic protons), 5.23 (12H, benzylic protons), 4.42 (H_{Gal}-1, overlapping with benzylic protons), 3.89 (1H, d, $J = 1.8$, H_{Gal}-4), 3.80 (1H, dd, $J = 9.7$, 7.7, H_{Gal}-2), 3.7–3.5 (5H, overlapping, H_{Gal}-6, H_{Hyl}-5, H_{Gal}-5 and H_{Gal}-3), 3.2 (2H, m, H_{Hyl}-6), 2.1–1.4 (4H, m, H_{Hyl}-3 and H_{Hyl}-4), 1.35 [3H, s, $C(CH_3)_3$]; ^{13}C NMR ($CDCl_3$, $T = 323$ K) δ: 170.1 (COOR), 156.6 (NCOOR), 138–127 (aromatics), 103.0 (C_{Gal}-1), 82.6 (C_{Gal}-3), 81.7 (C_{Hyl}-5), 81.4 [$C(CH_3)_3$], 79.5 (C_{Gal}-2), 75.4 (OCH_2Ph), 74.7 (OCH_2Ph), 73.9 (C_{Gal}-4), 73.5 (OCH_2Ph), 73.3 (C_{Gal}-5), 72.9 (OCH_2Ph), 68.8 (C_{Gal}-6), 67.4 (OCH_2Ph), 60.9 (C_{Hyl}-2), 54.3 (C_{Hyl}-6), 51.0 (NCH_2Ph), 29.9 (C_{Hyl}-4), 27.9 [$C(CH_3)_3$], 25.8 (C_{Hyl}-3). ESI-MS (positive) *m/z*: 1008.3 ($M+NH_4^+$), 10013.5 ($M+Na^+$). Anal. Calcd for $C_{59}H_{66}N_4O_{10}$: C, 71.49; H, 6.71; N, 5.65. Found: C, 71.55; H, 6.63; N, 5.51.

4.9. *tert*-Butyl (2*S*,5*S*)-6-amino-2-[benzyl(benzyloxycarbony)amino]-5-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)hexanoate 12

Starting with azide **11d** (330 mg; 0.332 mmol) and following the procedure described above for compound **8d**, title compound **12** was prepared (268 mg; 83% yield): $[\alpha]_D^{20} = +4.8$ (*c* 1, CH_2Cl_2); ^1H NMR ($CDCl_3$, $T = 318$ K) δ: 7.1–7.2 (30H, aromatic protons), 5.2–4.4 (12H, benzylic protons), 4.60 (H_{Hyl}-2, overlapped with benzylic protons), 4.43 (H_{Gal}-1, overlapped with benzylic protons), 3.93 (1H, br s, H_{Gal}-4), 3.80 (1H, dd, *J* 9.7, 7.8, H_{Gal}-2), 3,66 (1H, dd, $J = 8.5$, 7.9, H_{Gal}-6_a) 3.6–3.5 (3H, overlapped, H_{Gal}-6_b, H_{Gal}-5 and H_{Gal}-3), 3.45 (1H, b, H_{Hyl}-5), 3.2 (2H, m, H_{Hyl}-6), 2.1–1.4 (4H, m, H_{Hyl}-3 and H_{Hyl}-4), 1.33 [3H, s, $C(CH_3)_3$]; ^{13}C NMR ($CDCl_3$) δ: 170.1 (COOR), 156.7 (NCOOR), 138–127 (aromatics), 103.3 (C_{Gal}-1′), 82.8 (C_{Gal}-3′), 81.7 (C_{Hyl}-5), 81.3 [$C(CH_3)_3$], 79.7 (C_{Gal}-2′), 75.2 (OCH_2Ph), 74.7 (OCH_2Ph), 73.9 (C_{Gal}-4′), 73.5 (OCH_2Ph), 73.3 (C_{Gal}-5′), 73.2 (OCH_2Ph), 68.9 (C_{Gal}-6′), 67.5 (OCH_2Ph), 61.0 (C_{Hyl}-2), 54.3 (C_{Hyl}-6), 51.0 (NCH_2Ph), 30.0 (C_{Hyl}-4), 27. [$C(CH_3)_3$], 25.8 (C_{Hyl}-3). ESI-MS (positive) *m/z*: 965.5 ($M+H^+$). Anal. Calcd for $C_{59}H_{68}N_2O_{10}$: C, 73.42; H, 7.10; N, 2.90. Found: C, 73.74; H, 7.02; N, 2.77.

4.10. Completely protected β-D-galactopyranosyl-*O*-*epi*pyridinoline 13

Starting with amine **12** (251 mg, 0.260 mmol) and following the procedure described above for compound **10**, title compound **13** was prepared (205 mg, 51% yield): $[\alpha]_D^{20} = +12.2$ (*c* 1, CH_2Cl_2); ^1H NMR ($CDCl_3$ $T = 323$ K) δ: 7.5–7.0 (30H, aromatic protons), 5.2–4.5 (12H, benzylic protons), 4.2 (3H, overlapping, H_{Gal}-1, H_{4Ch}-2, H_{5Ch}-3), 3.90 (1H, br s, H_{Gal}-4), 3.74 (1H, dd, *J* 8.5, 8.5, H_{Gal}-2), 3.6–3.4 (5H, overlapping, H_{Hyl}-5, H_{Gal}-5, H_{Gal}-6, H_{Gal}-3), 3.25 and 2.82 (2 × 1H, 2m, H_{4Ch}-1), 2.50 (m, H_{5Ch}-1), 2.1–1.5 (overlapping, H_{Hyl}-4, H_{5Ch}-2, H_{Hyl}-3), 1.47 [s, 27H, $3 \times C(CH_3)_3$], 1.41 and 1.33 [2s, 2 × 9H, $2 \times C(CH_3)_3$]; ^{13}C NMR ($CDCl_3$) δ: 171.2, 169.8, 1698 (COOR), 156.1, 155.5, 143.8, 136.5, 130.1 (pyridinium ring), 102.5 (C_{Gal}-1), 82.6 (C_{Gal}-3), 82.0, 81.7, 81.0 [$3 \times C(CH_3)_3$], 79.2 (C_{Gal}-2), 79.1, 77.8 [$2 \times C(CH_3)_3$], 75.2 (OCH_2Ph), 74.6 (OCH_2Ph), 73.9 (C_{Gal}-4), 73.6 (C_{Gal}-5), 73.5 (OCH_2Ph), 72.9 (OCH_2Ph), 68.6 (C_{Gal}-5), 67.6 (OCH_2Ph), 32.9 (C_{5Ch}-2), 28.4 and 28.3 [$2 \times C(CH_3)_3$], 28.3 (C_{4Ch}-1), 28.0, 28.0 and 27.8 [$3 \times C(CH_3)_3$], 26.3 (C_{5Ch}-1) 25.5 and 25.1 (C_{Hyl}-3 and C_{5Ch}-4); ESI-MS (positive) *m/z*:1565.6 (20%; M+Na), 1543.6 (100%, $M+H^+$). Anal. Calcd for $C_{89}H_{114}N_4O_{19}$: C, 69.24; H, 7.44; N, 3.63. Found: C, 69.39; H, 7.31; N, 3.77.

4.11. β-D-Galactopyranosyl-*O*-*epi*pyridinoline 4

Starting with the completely protected compound **13** (90 mg, 0.058 mmol) and following the procedure described above for compound **2**, title compound **4** was prepared (30 mg, 88% yield over two steps): $[\alpha]_D^{20} = +3.8$ (*c* 0.5, H_2O); ^1H NMR (D_2O) δ: 7.81 (1H, br s, pyridinium proton), 7.77 (1H, br s, pyridinium proton), 4.58 (1H, m, H_{Hyl}-6a), 4.38–4.34 (2H, overlapping, H_{Hyl}-5 and H_{Hyl}-6b), 4.14 (1H, d, $J = 7.7$, H_{Gal}-1), 4.04 (1H, dd, $J = 6.6$, 4.9, H_{4Ch}-2), 3.89-3.71 (5H, overlapping, H_{Hyl}-2, H_{5Ch}-3, H_{Gal}-4, H_{Gal}-6a and H_{Gal}-6b), 3.60 (1H, m, H_{Gal}-5), 3.54 (1H, dd, $J = 10.1$, 3.1, H_{Gal}-3), 3.45 (1H, dd, $J = 10.1$, 7.7, H_{Gal}-2), 3.32 (1H, dd, $J = 14.1$, 6.6, H_{4Ch}-1a), 3.26 (1H, dd, $J = 14.1$, 4.9, H_{4Ch}-1b), 2.95 (1H, ddd, $J = 14.3$, 12.2, 5.2, H_{5Ch}-1a), 2.73 (1H, ddd, $J = 14.3$, 11.9, 5.2, H_{5Ch}-1b), 2.20-2.02 (4H, overlapping, H_{5Ch}-2a, H_{5Ch}-2b, H_{Hyl}-3a and H_{Hyl}-3b), 1.75 (1H, m, H_{Hyl}-4a), 1.63 (1H, m, H_{Hyl}-4b); ^{13}C NMR (D_2O, $T = 318$ K) δ: 172.3, 171.9, 171.2 (3 × COOH), 163.3, 140.7, 137.3, 130.0, 127.4 (pyridinium ring), 100.4 (C_{Gal}-1), 75.0 (C_{Hyl}-5), 73.0 (C_{Gal}-5), 70.6 (C_{Gal}-3), 68.6 (C_{Gal}-2), 66.4 (C_{Gal}-4), 60.7 (C_{Hyl}-6), 58.9 (C_{Gal}-6), 52.2, 52.1, 52.1 (C_{4Ch}-2, C_{5Ch}-3, C_{Hyl}-2), 28.9 (C_{5Ch}-2), 26.1 (C_{4Ch}-1), 25.5 (C_{Hyl}-4), 23.8, 23.6 (C_{5Ch}-1, C_{Hyl}-3). Anal. Calcd for $C_{24}H_{38}N_4O_{13}$: C, 48.81; H, 6.49; N, 9.49. Found: C, 48.73; H, 6.42; N, 9.43.

4.12. 3 *tert*-Butyl (2*S*,5*R*)- and (2*S*,5*S*)-6-azido-2-benzyloxycarbonylamino-5-(3,4,6-tri-*O*-benzyl-2-hydroxy-β-D-galactopyranosyl)hexanoate 7a and 11a

A mixture of *tert*-butyl (2*S*,5*R*)- and (2*S*,5*S*)-6-azido-2-benzyloxycarbonylamino-5-hydroxyhexanoate **5** and **6** (2.6 g; 6.9 mmol), *O*-(3,4,6-tri-*O*-benzyl-2-*O*-acetyl-α-D-galactopyranosyl) trichloroacetimidate **16** (5.4 g; 8.5 mmol) and powdered molecular sieves (3 Å, 0.8 g) in anhydrous diethyl ether (40 mL) was stirred for 15 min at 25 °C. After this time, *tert*-butyldimethylsilyl trifluoromethanesulfonate (250 μL; 1.08 mmol) was added and stirring was continued for 1 h under argon. The powdered molecular sieves were then filtered off and washed with AcOEt and the organic solution was worked-up. The residue was purified by flash chromatography (eluting with hexane/AcOEt; 75:25; v/v) to give an inseparable mixture of the crude diastereomeric title compounds **7b** and **11b** (2.98 g; 51% yield): an oil; $R_f = 0.30$ (hexane/AcOEt; 70:30; v/v). The ^1H NMR of the obtained mixture showed the presence of the previously obtained isomer **7b** and that of its (5*S*)-epimer **11b** in a 1:1 ratio.

The mixture of stereomers (2.00 g; 2.30 mmol) was then dissolved in methanol (40 mL), after which Cs_2CO_3 (2 g; 6.1 mmol) was added and the mixture was stirred for 6 h at room temperature. The solution was concentrated and the Cs_2CO_3 filtered. The solvent was then evaporated to afford a crude residue, which was chromatographed (eluting with hexane/AcOEt; 75:25; v/v) to afford first the amine **7a** (713 mg; 39% yield) and then the (5*S*)-epimer **11a** (750 mg; 41% yield).

4.13. Comparison of HPLC behavior of Gal-Pyd 2 and of Gal-*epi*Pyd 4

A sample of each compound was dissolved in water and analyzed using the best found chromatographic conditions to separate compound **2** from its epimer **4**: the HPLC column was a LiChrocart® 4-125, LiChrosphere RP-18 (5 μm); the mobile phase was a solution of water/CH_3CN (90:10; v/v) containing eptafluorobutyrric acid (0.02 M); the flow rate was 1 mL/min and the detection was performed by fluorescence ($\lambda_{ex} = 297$ nm; $\lambda_{emiss} = 380$ nm). The galactosyl-*O*-pyridinoline **2** was eluted after 24.8 min while galactosyl-*O*-*epi*-pyridinoline **3** was eluted after 27.5 min showing a satisfactory peak resolution ($R_s > 1$).

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