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Note

Xylosylated naphthoic acid–amino acid conjugates for investigation of glycosaminoglycan priming

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Abstract—Three different series of xylosylated naphthoic acid–amino acid conjugates containing one or two amino acid residues were synthesized for the investigation of glycosaminoglycan priming and potential use as anti-tumor drugs. All xylosylated naphthoic acid-conjugates inhibited the growth of normal lung fibroblasts to some extent, whereas the growth of tumor derived T24 carcinoma cells was not affected. There was no correlation between amino acid conjugation, retention time and the antiproliferative activity. Only one compound initiated the priming of glycosaminoglycans. Modification of the naphthalene ring with one or two amino acid residues did not have any effect on proteoglycan biosynthesis or glycosaminoglycan priming in T24 carcinoma cells.

Keywords: Glycosaminoglycan; Priming; Xylopyranoside; Amino acid; Naphthoic acid

Glycosaminoglycan (GAG) chains anchored to core proteins form proteoglycans (PG), a class of extracellular macromolecules having functions ranging from bulk construction material to involvements in cell–cell interactions. The biosynthesis of GAG chains start with the formation of a glycosidic bond between serine residues of the core protein and the unique xylose residue of the resulting GAG chain.[1] A specific linker tetrasaccharide, GlcA(β1-3)Gal(β1-3)Gal(β1-4)Xylβ is then assembled on which GAG chains are elongated and further modified through N-deacetylation/N-sulfation, O-sulfation and epimerization (Chart 1). Addition of GlcNAc or GalNAc to the linker defines whether heparan sulfate (HS) or chondroitin sulfate/dermatan sulfate (CS/DS) is formed.

Xylosides carrying hydrophobic aglycon can enter cells and serve as primers of GAG synthesis.[2] Depending on the structure of the aglycon, different GAG chains are formed. We have previously shown that the GAG-priming 2-(6-hydroxynaphthyl) β-D-xylopyranoside (**1**, Chart 1) selectively inhibits the proliferation of transformed or tumor-derived cells in vitro as well as in vivo.[3] Also, treatment with this xyloside reduced the average tumor load by 70–97% in a SCID mice model.[4] Toxicity studies of the 14 isomeric xylosylated dihydroxynaphthalenes revealed differences in anti-proliferative activity depending on the aglycon structure.[5,6]

Our results suggest that the priming of HS chains is required for selective growth inhibition,[3,4] and new xyloside analogues, which to a great extent prime HS as opposed to CD/DS, would thus be of great interest. It is still unclear what determines whether HS or CS/DS chains are attached to the core protein, but repetitive Ser-Gly sequences and a high proportion of Phe, Tyr or Trp promote the formation of HS.[7] Coupling of a Ser residue directly to the xylose has been investigated,[8] and whilst such xylosides prime GAG synthesis they

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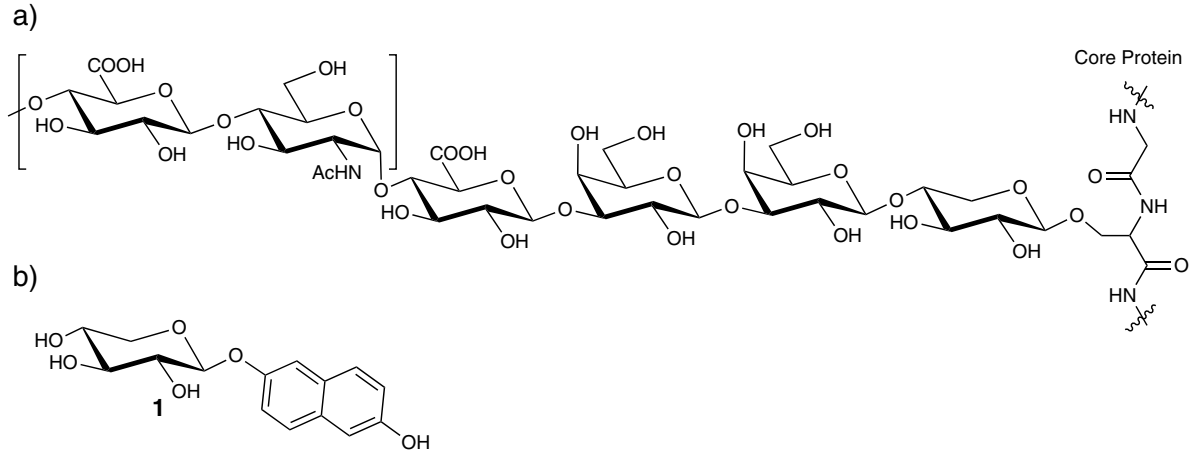


Chart 1. (a) Biosynthesis of glycosaminoglycan (GAG) chains start with the xylosylation of a serine residue after which a linker tetrasaccharide (GlcA(β1-3)Gal(β1-3)Gal(β1-4)Xylβ) is assembled. (b) Xylosides carrying hydrophobic aglycon, such as compound **1**, can enter cells and serve as primers of GAG chains.

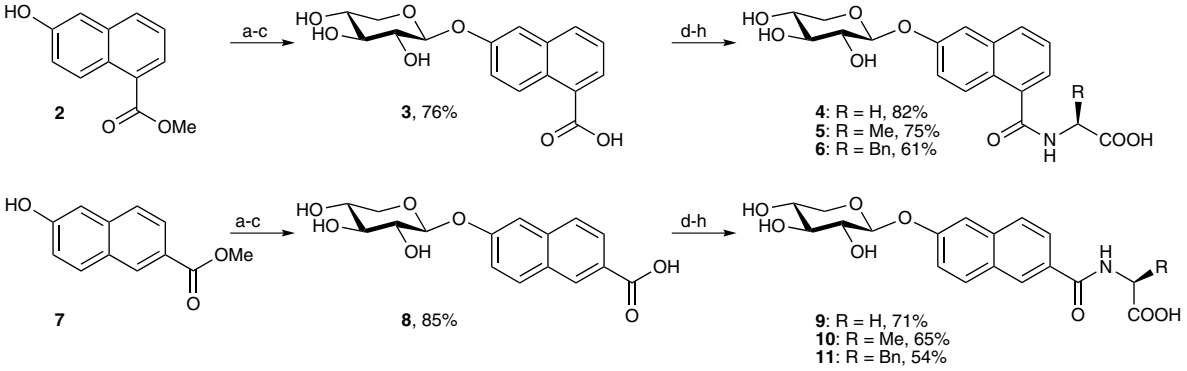
lack the naphthalenic moiety needed for increased HS priming.

We therefore looked into the synthesis of naphthoxyloside conjugates carrying different amino acid residues. In a first stage, we envisaged coupling amino acids with a carboxylic acid group on the naphthalene moiety of a naphthoxyloside. Since the naphthalene moiety would not carry two oxygen atoms, its inherent toxicity would most likely be low. This would enable us to study the effect of the amino acid on priming with minimal effect on cell proliferation.

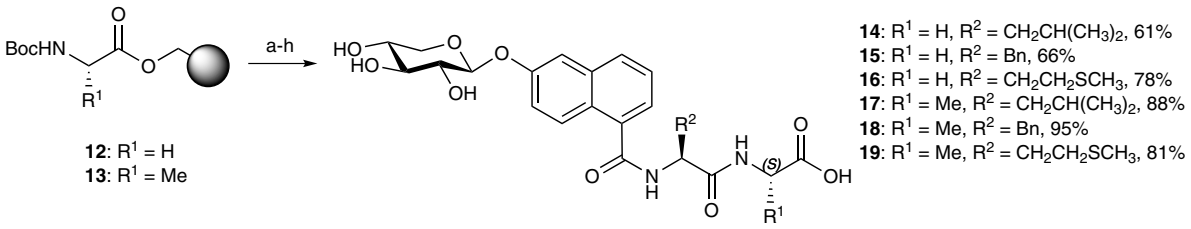
Our first attempts to xylosylate the free hydroxynaphthoic acids failed due to low solubility in CH_2Cl_2. Instead, the synthesis started from the known methyl 6-hydroxy-1-naphthoate (**2**)[9] and methyl 6-hydroxy-2-naphthoate (**7**).[10] To our surprise, xylosylation using trichloroacetimidate donor gave low yields (below 50%), and we therefore turned to the use of peracetylated xylose with the addition of triethylamine to suppress anomerization,[11] which gave excellent conversions. Selective deprotection of the carboxylic acid turned out to be difficult and instead we deprotected both the sugar moiety and the acid according to the two-step procedure of Grynkiewicz (Scheme 1).[12]

Selective protection of the sugar was then performed using Ac_2O in pyridine at room temperature, as heating in acetic anhydride gave substantial anhydride formation. A selection of ester-protected amino acids (glycine *t*-butyl ester hydrochloride, L-alanine methyl ester hydrochloride, L-phenylalanine methyl ester hydrochloride) were chosen for solution phase synthesis and couplings of the xylosylated hydroxynaphthoic acids proceeded smoothly. NaOMe–MeOH–CH_2Cl_2 was used to deprotect the sugar moiety, and full deprotection was then performed using NaOH. The conjugates were further purified using semi-preparative HPLC and lyophilized.

To synthesize a series of xylosylated naphthoic acid-dipeptide conjugates, we turned to solid-phase methodology, where preloaded resins allow for fast synthesis. The synthesis started from the commercially available Merrifield resins with Boc-protected glycine (0.5 mmol/g) and Boc-protected L-alanine (0.7 mmol/g). After deprotection of the amino group using TFA in CH_2Cl_2, a dipeptide was formed by peptide coupling with Boc-



Scheme 1. Synthesis of xylosylated naphthoic acid–amino acid conjugates. Reagents and conditions: (a) 1,2,3,4-tetra-*O*-acetyl-β-D-xylopyranose, $BF_3{\cdot}OEt_2$, CH_2Cl_2, rt; (b) NaOMe, MeOH, rt; (c) NaOH, MeOH, rt; (d) Ac_2O, pyridine, rt; (e) DMAP, protected amino acid, CH_2Cl_2, rt; (f) DIC in CH_2Cl_2, rt; (g) NaOMe, MeOH, CH_2Cl_2, rt; (h) NaOH (1 M, aq), MeOH, rt.



Scheme 2. Solid-phase synthesis of xylosylated naphthoic acid–amino acid conjugates containing two amino acid residues. Reagents and conditions: (a) TFA (80% in CH_2Cl_2 containing 0.4 M thiophenol), rt; (b) DMAP, protected amino acid, CH_2Cl_2, rt; (c) DIC in CH_2Cl_2, rt; (d) TFA (80% in CH_2Cl_2 containing 0.4 M thiophenol), rt; (e) DMAP, 6-(1-carboxynaphthyl) 2,3,4-tri-*O*-acetyl-β-D-xylopyranoside, CH_2Cl_2, rt; (f) DIC in CH_2Cl_2, rt; (g) NaOMe, MeOH, CH_2Cl_2, rt; (h) NaOH (1 M, aq), MeOH, rt.

protected amino acids (*N*-α-*t*-Boc-L-leucine, *N*-α-*t*-Boc-L-phenylalanine and *N*-α-*t*-Boc-L-methionine) using DIC/DMAP (Scheme 2). Unbound peptide was removed by washing and the terminal Boc-group was removed prior to coupling with the xylosylated hydroxynaphthoic acid. Cleavage from the resin and deprotection of the conjugates gave **14**–**19** in good yields with no noticeable epimerization.

It has been shown that gradient HPLC retention times, in contrary to isocratic retention times, can be treated as linear free-energy related parameters, that is, gradient HPLC retention times can be used to substitute log *P* values in biological evaluations.[13] The gradient HPLC retention times for the conjugates were measured using a C-18 column and a mobile phase of water (0.1% trifluoroacetic acid) with a gradient of acetonitrile from 1 min increasing by 1.2% per min up to 30 min. The retention times were measured for two separate runs per compound, and the calculated mean retention times are presented in Table 1.

For the determination of the antiproliferative activity, normal HFL-1 cells (human fetal lung fibroblasts) and T24 cells (human bladder carcinoma cells) were used. The xylosides were added to the growth medium at various concentrations and cell proliferation was recorded using the crystal violet method.[3] The inhibitory effect of the compounds is expressed as ED_{50} (μM) scored after 96 h of exposure relative to untreated cells (Table 1). As expected, the antiproliferative activities of naphthoxylosides carrying one or two amino acid residues were low in both normal lung fibroblasts and tumor derived T24 carcinoma cells. Interestingly, normal lung fibroblasts were at least two times more sensitive to these compounds with an ED 50 value ranging between 200 and 300 μM compared to T24 cells that grew well at these concentrations.

To test the GAG priming ability of the xylosides, T24 cells were incubated with 100 μM xyloside and [^{35}S]sulfate. GAG chains were then isolated from the medium and subsequently analyzed by size separation

Table 1. Retention times, GAG-priming and antiproliferative activity (ED_{50}, μM)

Compound	Retention time (min)	GAG-priming (T24 cells)[a]	Antiproliferative activity (T24 cells, ED_{50}, μM)	Antiproliferative activity (HFL-1 cells, ED_{50}, μM)
Series 1				
3	18.84 ± 0.09	0.75	>500	210
4	13.49 ± 0.00	0.62	>500	220
5	15.28 ± 0.01	0.60	>500	270
6	25.04 ± 0.06	0.93	>500	240
Series 2				
8	18.44 ± 0.01	0.63	>500	250
9	15.74 ± 0.02	0.63	>500	200
10	18.08 ± 0.00	0.66	>500	250
11	26.43 ± 0.10	1.43	>500	220
Series 3				
14	21.64 ± 0.00	0.47	>500	270
15	22.86 ± 0.01	0.75	>500	300
16	18.91 ± 0.02	0.43	>500	280
17	22.31 ± 0.00	0.45	>500	250
18	23.74 ± 0.01	0.36	>500	220
19	22.70 ± 0.03	0.54	>500	300

[a] The proportion of GAG-priming is given as the integrated value of [^{35}S]sulfate detected per min for the fractions containing GAG divided by the integrated value for fractions of untreated cells.

chromatography.[4] All treated cells secreted alkali sensitive proteoglycans to the extracellular space, and modifications of naphthalene ring with one or two amino acid residues did not affect proteoglycan biosynthesis or GAG priming ability. Only compound **11** initiated the synthesis of free GAG chains (Table 1). As a comparison, the GAG-priming capability of compound **1**, in T24 cells, was 7.2.[14]

The structure of the aglycon has been shown to play an important role for the priming ability, and for the structure of the GAG chains synthesized by the xylopyranosides.[15,16] The compounds used in this study may be internalized but not distributed to correct intracellular compartments to take part in GAG biosynthesis or may not be recognized by the GAG-priming enzymes. The results are similar to earlier studies using fluorescent analogs,[17] and it may be assumed that these compounds are unsuitable to function as GAG-primers. However, we note that compound **11**, which did initiate priming, is the most nonpolar (as indicated by HPLC retention times) of the investigated analogs and it is reasonable to assume that the polarity is important for the cellular transportation and targeting.

1. Experimental

1.1. General methods

CH_2Cl_2 for reactions was dried by passing through a column of Al_2O_3 (neutral, activity grade I). NMR-spectra were collected at 400 MHz (^1H) and 100 MHz (^{13}C) on a Bruker DRX 400 spectrometer. Chemical shifts are reported in ppm with the residual solvent peaks (^1H) and solvent signals (^{13}C) as reference. High-resolution mass-spectra were collected using a Micromass Q-Tof ESI or a FABMS JEOL SX-102. All the title compounds were further purified using reverse phase preparative HPLC using a Waters C18 symmetry column.

1.2. 6-(1-Carboxynaphthyl) β-D-xylopyranoside (3)

Compound **2**[9] (95 mg, 0.47 mmol) and 1,2,3,4-tetra-*O*-acetyl-β-D-xylopyranose (299 mg, 0.94 mmol) were dissolved in CH_2Cl_2 (10 mL) under N_2. Et_3N (0.07 mL, 0.47 mmol) followed by $BF_3{\cdot}OEt_2$ (0.295 mL, 2.34 mmol) were added. The soln was stirred at rt for 3 h and quenched by the addition of Et_3N, concentrated and chromatographed (SiO_2, 40:1 CH_2Cl_2–acetone). The intermediate (178.7 mg, 0.39 mmol) was dissolved in MeOH (10 mL). NaOMe (0.62 mL, 0.25 M) was added to the soln and stirred for 45 min at rt. The reaction was neutralized with solid CO_2, concentrated and chromatographed (SiO_2, 1:1 $CHCl_3$–MeOH). The intermediate (117.2 mg, 0.35 mmol) was dissolved in MeOH (15 mL). NaOH (7 mL, 1 M) was added and the soln was stirred for 19 h at rt. The reaction was neutralized with Amberlite IR-120 H^+, filtered and evaporated to dryness to give **3** as a white solid (112 mg, quant.). HRESIMS calcd for $C_{16}H_{16}O_7Na$ $[M+Na]^+$: 343.0794; found 343.0811.

1.3. 6-(2-Carboxynaphthyl) β-D-xylopyranoside (8)

Compound **8** was synthesized according to the procedure for **4** to give a white amorphous solid. HRESIMS calcd for $C_{16}H_{16}O_7Na$ $[M+Na]^+$: 343.0794; found 343.0806.

1.4. General procedure for the synthesis of compounds 4–6 and 9–11

Compounds **3** or **8** was dissolved in pyridine (15 mL). Ac_2O (0.71 mL, 7.5 mmol) was added, and the mixture was stirred for 4 h at rt. The soln was concentrated and the residue was dissolved in EtOAc and washed three times with HCl (10% aq) and the organic layer was dried with $MgSO_4$ and concentrated. The intermediate (10 mg, 0.022 mmol) and glycine *t*Bu-ester hydrochloride (4.9 mg, 0.029 mmol) were dissolved in CH_2Cl_2 (1.5 mL). DMAP (4.1 mg, 0.034 mmol) was added and the soln was stirred for 15 min. DIC (4.4 μL, 0.028 mmol) in CH_2Cl_2 (0.1 mL) was added and the soln was stirred under N_2 at rt for 3 h. The mixture was concentrated and chromatographed (SiO_2, 1:10 heptane–EtOAc). The intermediate (11.2 mg, 0.020 mmol) was dissolved in MeOH (2 mL). NaOH (0.40 mL, 1 M) was added and the mixture was stirred for 5 h. The reaction was quenched with Amberlite IR-120 H^+ and concentrated to give **4** as white amorphous solid.

1.5. General procedure for solid-phase dipeptide synthesis

Dipeptides were synthesized using dry CH_2Cl_2 as a solvent in a mechanically agitated reactor tube. Pre-loaded Merrifield resins with Boc-protected glycine (0.5 mmol/g, 0.020 mmol) and Boc-protected alanine (0.7 mmol/g, 0.021 mmol) were used. The resins were swelled for 10 min followed by Boc-deprotection. The deprotection was performed twice using 4:1 TFA–CH_2Cl_2 (2 mL). The resins were then washed with CH_2Cl_2. The second Boc-protected amino acid was added alongside with DMAP and CH_2Cl_2 and the tube was agitated for 15 min and DIC was added. The mixture was agitated for 3 h after which the resins were washed with CH_2Cl_2 and MeOH. The Boc-group on the second amino acid was removed as described earlier. The resins were swelled for 10 min, followed by the addition of **4** (10.7 mg, 0.024 mmol), DMAP and CH_2Cl_2 and agitated for 15 min. DIC was added and the tube was agitated for 3 h. The resins were then washed as previously described. Swelling in CH_2Cl_2, followed by cleav-

age from the resin using NaOMe (0.5 mmol in 2:3 CH_2Cl_2–MeOH, 2.5 mL) for 10 min. The reactions were quenched with Amberlite IR-120 H^+ and concentrated. Deprotection of the ester was conducted using NaOH for 5 h. The reactions were neutralized with Amberlite IR-120 H^+ followed by concentration and purification by preparative HPLC.

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Supplementary data

Supplementary data (complete experimental procedures, product characterization and spectral data) associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.04.006.

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