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Synthesis of multivalent lactose derivatives by 1,3-dipolar cycloadditions: selective galectin-1 inhibition

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Abstract—Acetylene derivatives of phenylalanine, phenethylamine and the multifunctional unnatural amino acids, phenyl-bis-alanine and phenyl-tris-alanine, were synthesized and functionalized with 2-azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside via regioselective copper(I)-mediated 1,3-dipolar cycloaddition to give a panel of mono-, di- and trivalent lactoside derivatives. Evaluation of the compounds as inhibitors against the tumour- and inflammation-related galectin-1, -3, -4N, -4C, -4, -7, -8N and -9N revealed a divalent compound with a K_d value as low as 3.2 μM for galectin-1, which corresponded to a relative potency of 30 per lactose unit as compared to the natural disaccharide ligand lactose. This divalent compound had at least one order of magnitude higher affinity for galectin-1 than for any of the other galectins investigated.
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1. Introduction

Galectins are defined by two criteria established in 1994:[1] 'affinity for β-galactosides and significant sequence similarity in the carbohydrate-binding site . . .', and now include about 14 proteins in mammals and many in other species.[2] Galectins are implicated in different biological events such as cancer[3–7] and the regulation of immunity and inflammation.[8–11] In several of these biological events, glycoconjugate binding is crucial, which makes the development of galectin inhibitors important.

The recognition of glycoconjugates by lectins is typically characterized by low affinity and the biological systems use multivalent interactions to circumvent these low affinities, a phenomenon referred to as 'the glycoside clustering effect'[12–14] Several mechanisms may operate to enhance the relative potency of multivalent ligands. These processes include the intramolecular chelate effect, the intermolecular aggregative process and statistical effects. The intramolecular chelate effect appears when multivalent ligands occupy multiple binding sites within a protein. In contrast, when multivalent ligands bind to multivalent receptors, large cross-linked aggregates may form.[15] The statistical effect comes from slower off rates due to higher local concentrations of the binding moiety (in our case lactose) around a single binding site.

Several research groups have exploited the idea of using multivalent compounds to find high affinity galectin ligands/inhibitors. Multivalent enhancement has been seen for galectin-1,[16,17] galectin-3,[14,16–18] galectin-4 (N-terminal)[19] and galectin-5.[17] In these studies, a large array of scaffolds have been examined for creating multivalent carbohydrate ligands including glycopolymers and glycodendrimers.[20–22]

To optimize and study the glycoside cluster effect between multivalent ligands and galectins, we have developed new scaffolds between the linked carbohydrate units based on polyfunctional unnatural amino acids like phenyl-bis-alanine (PBA) and phenyl-tris-alanine (PTA). These scaffolds have previously been used for the preparation of amino-alcohol ligands, cage

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molecules and dendrimers.[23–25] Typically, the unnatural PBA and PTA amino acids have been further functionalized by peptide couplings to furnish more complex structures. An important advantage with the choice of PBA and PTA as scaffolds is that the two orthogonal functionalities (amine and ester) enable derivatization with two different molecular entities (e.g., a galectin ligand and a fluorescent tag). A drawback of using standard peptide coupling reactions for PBA and PTA functionalizations is that efficiencies and yields may vary significantly with the choice of coupling partner. In particular, carbohydrate carboxylic acid or amino derivatives that carry unprotected hydroxyl groups are often difficult to couple directly via amide bond formations. To avoid these problems, we turned our attention to other methods of attachment of unprotected galectin ligands to PBA and PTA.

Inspired by a multitude of successful copper catalyzed 1,3-dipolar cycloaddition reactions between terminal alkynes and azides, under mild conditions, for the synthesis of intricate carbohydrate structures and the ready availability of 2-azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside, we set out to synthesize suitable alkyne precursors derived from the above PBA and PTA. Acetylene derivatives of phenylalanine and of phenethylamine were also prepared for comparative purposes. In the presence of copper(I), the acetylene derivatives were coupled with 1,4-regioselectivity[20,26,27] with 2-azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside. The ligands were evaluated as inhibitors in a competitive fluorescence polarization assay against galectin-1, -3 and -7. They were also evaluated against intact galectin-4, the C-terminal domain of galectin-4, as well as the N-terminal domains of galectin-4, -8 and -9.[28,29] In this paper, we report the synthesis of these ligands and the relative potency, as galectin inhibitors, of the multivalent ligands as compared with their monovalent homologues.

2. Results and discussion

2.1. Synthesis of acetylenes

Amine precursors phenethyl amine, L-phenylalanine and D,L-phenylalanine methyl esters, and PBA and PTA methyl esters **6** and **7** were coupled with either of the two types of acetylenic precursors (Table 1). In the first case, propiolic amides **1**,[30] **3**, **4** and **10** were formed by coupling propiolic acid with the corresponding amine using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in dichloromethane (method A), as this activating agent has in our hands been found to give particularly satisfactory results in the synthesis of propargylic amides. In the second case, the amine was treated with propargyl chloroformate in the presence of triethylamine and a catalytic amount of 4-dimethylaminopyridine (DMAP) in dichloromethane (method B), to give carbamates **2**, **5** and **8**. The divalent acetylene derivatives **7** and **8** were synthesized from the corresponding diamine **6** using method A and method B, respectively. Diamine **6** was synthesized in two steps from the corresponding protected phenyl-bis-didehydro-alanine derivative by a modification of the literature procedure,[23] which uses the chiral Rh(I)-Et-DuPhos catalyst to synthesize diastereomerically enriched (*S*,*S*)-PBA.[31] However, due to unreliably low stability of Rh(I)-Et-DuPhos and hence unpredictable reactivity, together with a predicted limited importance of stereoconfiguration of PBA and PTA for galectin binding, we opted for a cheaper and more robust unselective catalyst. The styrene double bonds were thus saturated by hydrogenation using Wilkinson's catalyst at 60 psi for 5 days followed by hydrogenolytic removal of the carboxybenzyloxy protective group to afford the amine. This methodology was analogously applied to the preparation of the triamine precursor for the synthesis of tris acetylene derivative **10** using method A.[25]

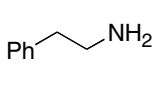
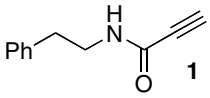
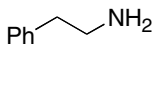
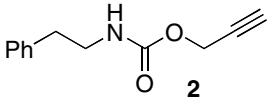
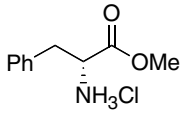
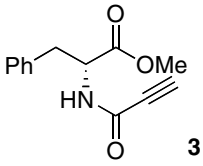
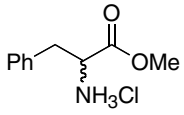
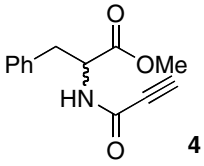
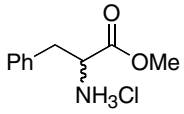
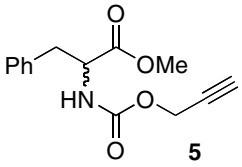
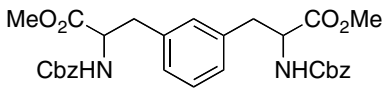
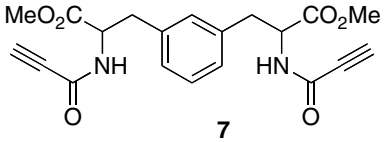
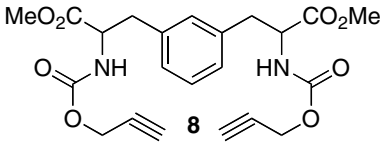
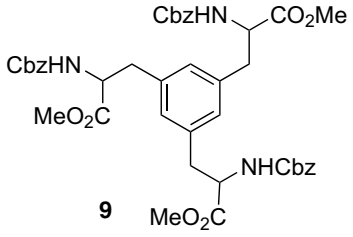
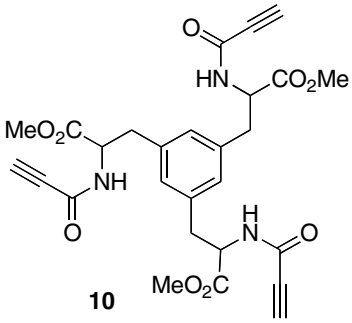
2.2. Synthesis of triazoles

The 1,3-dipolar cycloaddition between the acetylene derivatives **1**–**5**, **7**, **8** and **10** and 2-azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside **11**[32] was performed using *N*,*N*-diisopropylethylamine (DIPEA) as base and copper (I) iodide as mediator (Scheme 1). The 1,3-dipolar cycloaddition under these conditions has the advantage of forming triazoles at room temperature. Previous results have shown higher yields using toluene as compared to acetonitrile as solvent,[20] but due to solubility problems, acetonitrile was used for all reactions in this work except for the formation of **14**. The reaction times were between 2 and 7 days and yields varied between 53% and 80% for the formation of mono- and divalent structures **12**–**16** and **18**–**19**, whereas the yield for the trivalent **17** was 33%, which corresponds to 69% per cycloaddition.

2.3. Galectin binding

Dissociation constants for ligands **12**–**19** with galectin-1, -3, -4N, -4C, -4, -7, -8N and -9N were determined by competitive fluorescence polarization[28,29] in solution and compared to the methyl β-glycoside of lactose **20** as a reference compound. While galectin-3 could be evaluated at room temperature, the other galectins were studied at 4 °C to achieve interaction strengths high enough to assure accurate measurements. Importantly, the fluorescence polarization method measures in solution the affinity of ligands to the galectins, which typically function as soluble proteins in their biological environments. Other methods include solid phase assays and a direct comparison between these methods may be difficult due to matrix interference.[33] This is apparent in pre-

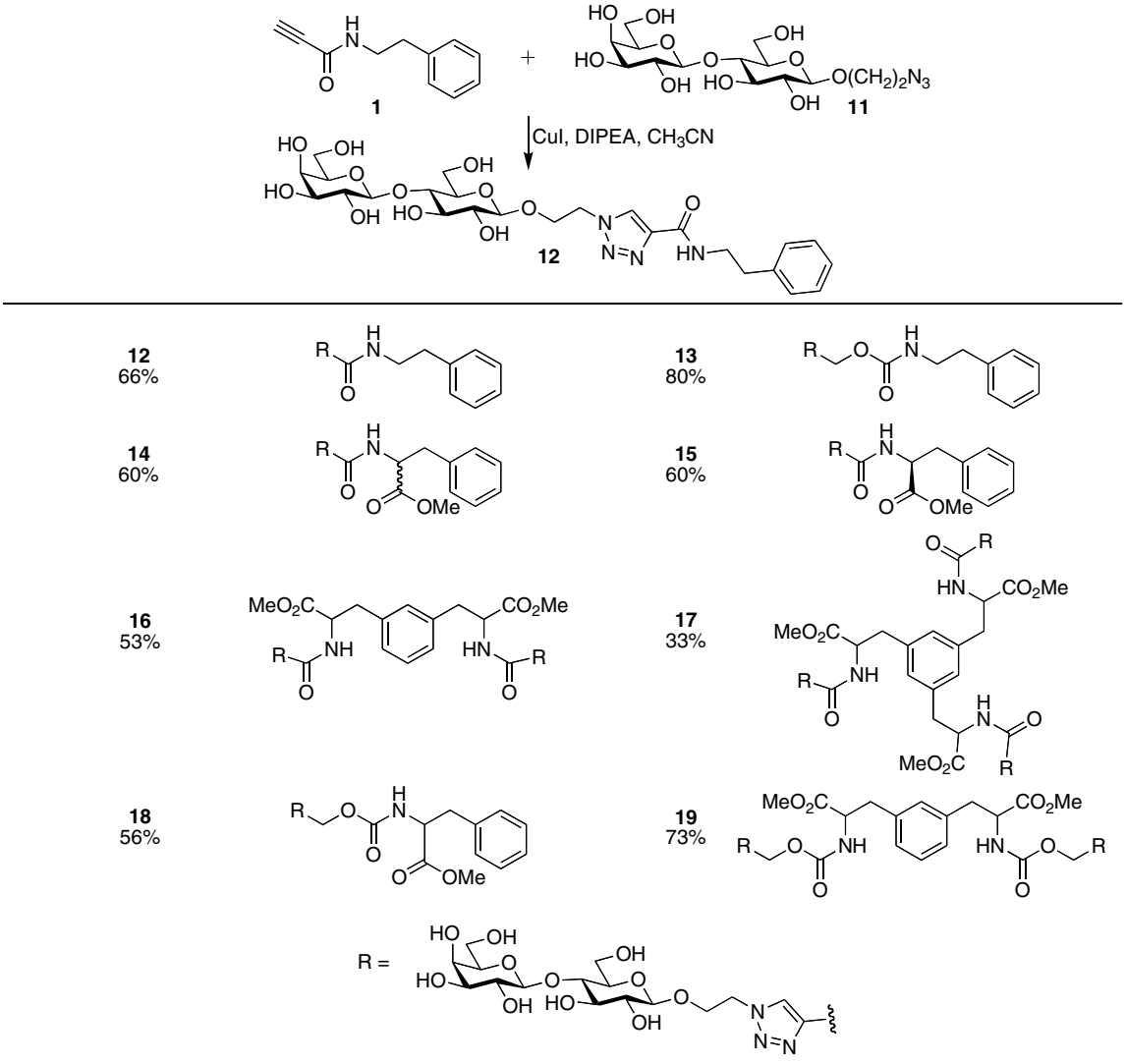
Table 1. Synthesis of acetylene derivatives

Amine	Method[a] (yield)	Product
	A (78%)	 **1**
	B (32%)	 **2**
	A (61%)	 **3**
	A (51%)	 **4**
	B (41%)	 **5**
 **6**	[i]H_2, Pd/C [ii]A (33%)	 **7**
6	[i]H_2, Pd/C [ii]B (38%)	 **8**
 **9**	[i]H_2, Pd/C [ii]A (20%)	 **10**

[a] Method A: amine precursor, EEDQ and propiolic acid in CH_2Cl_2. Method B: amine precursor, propargyl chloroformate, Et_3N and DMAP in CH_2Cl_2.

vious results where both a solid phase assay and a fluorescence titration on the same multivalent ligands were compared.[17] In this example for a divalent ligand, the solid phase assay gave a relative potency of 750 per lactose moiety, while a fluorescence titration on the same ligand gave a relative potency of 10.

It is apparent that the aglycon fragment has an effect on the affinity of the ligands for some of the galectins (Table 2). All monovalent ligands showed better affinity to galectin-1, -3 and -4N than lactoside **20**. In particular, **13** ($K_d = 24\ \mu M$) showed a pronounced affinity for galectin-1 with a relative potency of 8 as compared to



Scheme 1. Synthesis of 1,4-substituted 1,2,3-triazoles (**12**–**19**) via 1,3-dipolar cycloaddition reactions exemplified with **12**.

lactose ($K_d = 190$ μM). The longer linker in phenethylamine derived carbamate **13** results in a higher relative potency towards galectin-1 and -7 compared to the shorter linker in the phenethylamine derived amide **12**. This effect is not seen in galectin-3, -4, -4N, -4C, -8N or -9N. Comparing the affinity of the amide derivatives **14**, **15** and **12**, the presence or absence of the methyl ester side chain does not have any effect. However, comparing carbamate derivatives **13** and **18**, the derivative without the ester side chain gives better affinities in galectins-1, -3, -7 and -9N, whereas no effect was observed for other galectins. In general, the carbamate linker showed stronger affinity for galectin-1, which is not observed for other galectins. The diastereomerically enriched derivative **15** shows better affinity for galectins-1, -4N, -4C and -8N, than the diastereomeric mixture **14** does.

The literature on cluster effects between galectins and multivalent ligands invariably reports the cluster effects relative to lactose, which we believe may sometimes be misleading as effects from direct interactions between the galectins and lactose aglycon structures are not taken into account. A significant glycosidic cluster effect relative to methyl β-lactoside **20** can be seen with all multivalent ligands **16**, **17** and **19** for galectin-1 and to a lesser extent for galectin-3 and -4N (Table 3). However, when the cluster effects are calculated with respect to the corresponding monomers **14** or **18**, the cluster effects are much lower for galectin-1, almost unaffected for galectin-4N, and basically not observable for galectin-3. This clearly illustrates that direct interactions between lactose aglycons can be important and that cluster effects should be calculated with respect to a reference monomer instead of lactose when analyzing multivalent galectin inhibitors.

Nevertheless, the most pronounced relative potency effects per monomer unit are 7.7 for **19** and 7.2 for **16** (30 and 11 relative to methyl β-lactoside **20**), possibly due to **19** and **16** cross-linking two separate galectin-1

Table 2. Dissociation constants (μM) of **12**–**19** and relative potencies compared to methyl β-lactoside **20** as determined in a fluorescence polarization assay[a]

Compd	Valency	Galectin-1		Galectin-3		Galectin-4N		Galectin-4C	
		K_d	Rel	K_d	Rel	K_d	Rel	K_d	Rel
20	1	190	1	220	1	540	1	1200	1
12	1	80	2.4	66	3.3	230	2.3	2200	0.5
13	1	24	7.9	66	3.3	220	2.5	1600	0.8
14	1	120	1.6	75	2.9	460	1.2	2300	0.5
15	1	80	2.4	70	3.1	200	2.7	1100	1.1
16	2	8.3	23	30	7.3	110	4.9	710	1.7
17	3	7.4	26	17	13	22	25	360	3.3
18	1	49	3.9	86	2.6	220	2.5	1700	0.7
19	2	3.2	59	27	8.1	110	4.9	570	2.1
		Galectin-4		Galectin-7		Galectin-8N		Galectin-9N	
		K_d	Rel	K_d	Rel	K_d	Rel	K_d	Rel
20	1	1400	1	91	1	52	1	23	1
12	1	1300	1.1	380	0.2	63	0.8	51	0.5
13	1	1100	1.3	100	0.9	61	0.9	48	0.5
14	1	1700	0.8	210	0.4	94	0.6	97	0.2
15	1	1400	1	270	0.3	62	0.8	85	0.3
16	2	750	1.9	42	2.2	29	1.8	27	0.9
17	3	360	3.9	32	2.8	19	2.7	18	1.3
18	1	1300	1.1	240	0.4	58	0.9	110	0.2
19	2	630	2.2	96	0.9	35	1.5	31	0.7

[a] At 4 °C except for galectin-3 and 8N, which was evaluated at room temperature.

Table 3. Cluster effects of the di- and trivalent compounds **16**, **17** and **19** calculated relative to methyl β-lactoside **20** and to the corresponding monomers: Mon = compounds **14** or **18**[a]

Compd	Mon	Val	Galectin-1 Cluster effect		Galectin-3 Cluster effect		Galectin-4N Cluster effect		Galectin-4C Cluster effect	
			20	Mon	20	Mon	20	Mon	20	Mon
16	**14**	2	11	7.2	3.7	1.3	2.5	2.1	0.8	1.6
17	**14**	3	8.6	5.4	4.3	1.5	8.2	7.0	1.1	2.1
19	**18**	2	30	7.7	4.1	1.6	2.5	1.0	1.1	1.5
			Galectin-4 Cluster effect		Galectin-7 Cluster effect		Galectin-8N Cluster effect		Galectin-9N Cluster effect	
			20	Mon	20	Mon	20	Mon	20	Mon
16	**14**	2	0.9	1.1	1.1	2.5	0.9	1.6	0.4	1.8
17	**14**	3	1.3	1.6	0.9	2.2	0.9	1.6	0.4	1.8
19	**18**	2	1.1	1.0	0.5	1.3	0.7	0.8	0.4	1.8

[a] At 4 °C except for galectin-3 and 8N, which was evaluated at room temperature.

molecules leading to aggregation.[15] Furthermore, the carbamate derivative **19** displays higher affinity towards galectin-1 compared to the corresponding amide derivative **16**, which is consistent with the observations for the monovalent ligands **18** and **14**. Interestingly, trimer **17** has a less pronounced relative potency per lactose moiety of 8.6 for galectin-1. This suggests that trivalent ligands form aggregates less efficiently, which is in analogy with previous studies.[17] Unfortunately, the limited number of multivalent ligands evaluated, together with the relatively high flexibility of their aglycons, does not permit useful structure–activity relationship to be established with the aid of galectin crystal structures; it is clear that both the aglycon structure and level of multivalency of **12**–**19** influence the binding by galectins.

An unexpected cluster effect of 7 (8.2 relative to methyl β-lactoside **20**) was observed with trivalent **17** in galectin-4N, while no such effect was observed for galectin-4C or intact galectin-4. This may suggest that galectin-4N either can interact with more than one lactose unit of **17** or that galectin-4N form aggregates. It should be noted that the probe used in the fluorescence polarization competitive inhibitions involving intact galectin-4 has a strong preference for galectin-4C over galectin-4N. Hence, it is not surprising that the results for intact galectin-4 are similar to those with galectin-4C, as an inhibitor binding to the N-terminal of intact galectin-4 will not inhibit the assay reporting fluorescent probe that selectively binds the C-terminal of intact galectin-4. Ideally, intact galectin-4 should be

evaluated with an unfortunately not yet discovered fluorescent probe that binds efficiently to both N- and C-terminal domain.

3. Conclusions

The copper-mediated 1,3-dipolar cycloaddition proved to be a convenient mode of attachment for the synthesis of unprotected carbohydrate ligands to PBA and PTA. The inhibitor multivalency proved to have a significant influence on the affinity of the ligands against galectin-1 and -4N. Galectin-1 showed a preference for ligands with a carbamate linker and a moderate, but significant, glycoside cluster effect with all multivalent ligands **16**, **17** and **19** suggesting the formation of cross-linked aggregates. The strongest glycoside cluster effect of 7.7 (30 relative to methyl β-lactoside **20**) for galectin-1 was found for the divalent carbamate **19**, which compares favourably with the best multivalent lactoside inhibitor of galectin-1 reported.[17] Carbamate **19** had a K_d as low as 3.2 μM against galectin-1, which reflects a promising selectivity as this K_d is one order of magnitude or more lower than for any other galectin investigated. A glycosidic cluster effect of 7 (8.2 relative to methyl β-lactoside **20**) was also seen for **17** in galectin-4N. The results herein hold promise for the development of galectin-1 and 4N inhibitors and particularly useful research tools as an efficient inhibitor (e.g., **19** against galectin-1) would, via its ester functionality, allow for conjugation of different reporter structures such as fluorescent labels, enzymes, biotin or solid surfaces.

4. Experimental

4.1. General methods

All commercial chemicals were used without further purification. Thin layer chromatography (TLC) was carried out on $60F_{254}$ silica (Merck) and visualization was made by UV light followed by heating with aqueous sulfuric acid. Column chromatography was performed on silica gel (Amicon Matrex 35–70 μm, 60 Å). Reversed phase chromatography was performed on Waters Sep-Pack Vac 35cc C_{18} columns. NMR experiments were recorded with Bruker ARX 300 MHz or Bruker DRX 400 MHz spectrometers at ambient temperature. ^{1}H NMR assignments were derived from COSY experiments. Optical rotations were measured with a Perkin–Elmer 341 or a 241 polarimeter. High-resolution fast bombardment mass spectra HRMS (FAB) were recorded with a JEOL SX-120 instrument or HRMS (ESI) Micromass Q-TOF micro spectrometer. Melting points were measured on a Gallenkamp Melting Point Apparatus MFB-595 and are uncorrected. IR spectra were recorded on a Shimadzu FTIR-8300 instrument. Fluorescence polarization experiments and calculations were performed in general as described[28] and performed at 4 °C except for galectin-3 and -8N, which were done at ambient temperature. Each experiment was performed at least in duplicate. Concentrations and probes used for galectins-1, -3, -7, -8N, and 9N were as described.[28,29] For galectin-4 a probe with an A-tetra saccharide was used, which was synthesized in analogy with the methods used in Öberg et al.[34] (to be described elsewhere). The probe was used at 0.1 μM as for the other probes, and galectin-4N at 5 μM, galectin-4C at 0.5 μM, and intact galectin-4 at 0.5 μM. DL-Phenyl alanine methyl ester hydrochloride, 2-benzyloxycarbonylamino-3-[3-(2-benzyloxycarbonylamino-2-methoxycarbonyl-vinyl)-phenyl]-acrylic acid methyl ester and 2-benzyloxycarbonylamino-3-[3,5-bis-(2-benzyloxycarbonylamino-2-methoxycarbonyl-vinyl)-phenyl]-acrylic acid methyl ester were synthesized according to literature procedures.[35–37]

4.2. Propynoic acid phenethyl-amide (1)

Propiolic acid (0.1 mL, 1.6 mmol) and EEDQ (442 mg, 1.8 mmol) were added to a stirred solution of phenethylamine (0.2 mL, 1.6 mmol) in CH_2Cl_2 (10 mL) at rt. The mixture was stirred overnight and then washed with 2.5 M HCl (2 × 10 mL), and dried (Na_2SO_4). After filtration, 600 mg of silica was added to the filtrate and the solvent was removed under reduced pressure. The residue thus absorbed on silica was purified by column chromatography (5:1, petroleum ether/EtOAc) to give the title compound (219 mg, 78%), which was recrystallized from CH_2Cl_2/pentane to give **1** as thin white needles the melting point of which was 56–58 °C (lit. 53–54 °C) and the ^{1}H NMR data were in accordance with the literature data.[30]

4.3. Phenethyl-carbamic acid prop-2-ynyl ester (2)

Propargylchloroformate (0.5 mL, 5.1 mmol), Et_3N (0.6 mL, 4.6 mmol) and DMAP (57 mg, 0.4 mmol) were added to a stirred solution of phenethylamine (0.58 mL, 4.6 mmol) in CH_2Cl_2 (20 mL) at rt. The mixture was stirred for 2 days or until TLC analysis of the reaction mixture did not indicate any phenethylamine (ninhydrin staining). The mixture was then washed with HCl 1 M (2 × 20 mL) and dried (Na_2SO_4). After filtration, 2 g of silica was added to the filtrate and the solvent was removed under reduced pressure. The residue thus absorbed on silica was purified by column chromatography (5:1, petroleum ether/EtOAc) to give **2** as a clear oil (300 mg, 32%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.34–7.18 (m, 5H, ArH), 4.87 (br, 1H, NH partially exchanged), 4.68 (s, 2H, –OCH_2–), 3.47 (q,

2H, $J = 13.2$ Hz, $J = 6.5$ Hz, N–CH_2–), 2.83 (t, 2H, $J = 6.9$ Hz, Ar–CH_2–), 2.47 (t, 1H, $J = 2$ Hz, CCH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.5, 138.7, 128.9, 128.8, 126.7, 78.4, 74.7, 52.6, 42.4, 36.1; IR (NaCl film): 3375, 2937, 2142, 1715, 1534 cm^{-1}; FABMS *m/z* calcd for $[C_{12}H_{14}NO_2+H]^+$: 204.1025. Found: 204.1026.

4.4. 3-Phenyl-2-propynoylamino-propionic acid methyl ester (4)

The *S*-enantiomer of this compound has been prepared previously.[38] Compound **4** was therefore prepared analogously in racemic form (51%) mp 73.9–75.7 °C. The IR data of racemic **4** was in agreement with those of the enantiomerically enriched compound.

4.5. 3-Phenyl-2-prop-2-ynyloxycarbonylamino-propionic acid methyl ester (5)

Propargyl chloroformate (1 mL, 10 mmol), DMAP (244 mg, 2 mmol) and Et_3N (2.8 mL, 20 mmol) were added to a stirred solution of DL-phenylalanine methyl ester hydrochloride (2.2 g, 10 mmol) in CH_2Cl_2 (50 mL) at rt. The mixture was stirred for 2 days, washed with HCl 1 M (2 × 50 mL) and brine (50 mL) and dried (Na_2SO_4). After filtration, 6 g of silica was added to the filtrate and the solvent was removed under reduced pressure. The residue thus absorbed on silica was purified by column chromatography (4:1, petroleum ether/EtOAc) to give **5** as yellow oil (1.1 g, 41%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35–7.25 (m, 3H, ArH), 7.14 (d, 2H, Ar–H), 5.24 (br d, 1H, $J = 7.2$ Hz, NH partially exchanged), 4.67–4.62 (m, 3H, –OCH_2–, N–CH–C=O), 3.72 (s, 3H, OCH_3), 3.2–3.05 (m, 2H, ArCH_2–), 2.47 (t, 1H, $J = 2.4$ Hz, CCH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.9, 154.9, 135.7, 129.4, 128.8, 127.4, 78.1, 75.0, 55.0, 52.9, 52.5, 38.3; IR (NaCl film): 3337, 2959, 2115, 1726, 1518 cm^{-1}; FABMS *m/z* calcd for $[C_{14}H_{16}NO_4+H]^+$: 262.1079. Found: 262.1076.

4.6. 2-Benzyloxycarbonylamino-3-[3-(2-benzyloxycarbonylamino-2-methoxycarbonyl-ethyl)-phenyl]-propionic acid methyl ester (6)

2-Benzyloxycarbonylamino-3-[3-(2-benzyloxycarbonylamino-2-methoxycarbonyl-vinyl)-phenyl]-acrylic acid methyl ester (0.8 g, 1.5 mmol) was added to absolute EtOH (100 mL) and the obtained slurry was degassed on an ultrasonic bath for 15 min and then purged with argon for a further 15 min. Then Wilkinson's catalyst (163 mg, 0.2 mmol) was added and the mixture was hydrogenated at rt at 60 psi over 5 days. Silica (2.4 g) was then added to the solution and the solvent was removed under reduced pressure. The residue thus absorbed on silica was passed through a short plug of silica eluting with petroleum ether/EtOAc 1:1 (200 mL) to remove the catalyst and then the solvent was removed under reduced pressure to give **6** as a brownish oil in quantitative yield. ^{1}H NMR and MS data were in accordance with the literature data.[23]

4.7. 3-[3-(2-Methoxycarbonyl-2-propionylamino-ethyl)-phenyl]-2-propionylamino-propionic acid methyl ester (7)

10% Pd/C (100 mg) was added to a stirred solution of **6** (0.8 g, 1.5 mmol) in CH_3OH (50 mL) at rt. The slurry was hydrogenated at 1 atm overnight or until no starting material could be seen on TLC. The slurry was then filtered through a plug of Celite and the solvent was removed under reduced pressure to give the diamine as a brownish oil. The diamine was suspended in CH_2Cl_2 (50 mL). Propiolic acid (130 μL, 2.1 mmol) and EEDQ (0.6 g, 2.31 mmol) were then added to the solution and the resulting mixture was stirred for 2 days at rt and then washed with HCl 2 M (3 × 50 mL). The combined aqueous phases were extracted once with CH_2Cl_2 (50 mL) and the combined organic phases were dried (Na_2SO_4). After filtration, 600 mg of silica was added to the filtrate and the solvent was removed under reduced pressure. The residue thus absorbed on silica was purified by column chromatography (5:1, petroleum ether/EtOAc) to give **7** as a yellow syrup (88 mg, 33%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25–7.20 (m, 1H, ArH), 7.01–6.98 (m, 2H, Ar–H), 6.92 (d, 1H, $J = 11$ Hz, ArH), 6.65 (d, 1H, $J = 8$ Hz, NH partially exchanged), 6.53 (d, 1H, $J = 8$ Hz, NH, partially exchanged) 4.95–4.86 (m, 2H, N–CH–C=O), 3.74 (s, 6H, –OCH_3), 3.19–3.02 (m, 4H, Ar–CH_2–), 2.85 (d, 2H, $J = 6.8$ Hz, –CCH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.2, 151.7, 136.0, 130.5, 129.2, 128.5, 79.9, 74.6, 53.7, 53.0, 37.9; IR (NaCl film): 3255, 3019, 2109, 1736, 1638 cm^{-1}; FABMS *m/z* calcd for $[C_{20}H_{21}N_2O_6+H]^+$: 385.1400. Found: 385.1403.

4.8. 3-[3-(2-Methoxycarbonyl-2-prop-2-ynyloxycarbonylamino-ethyl)-phenyl]-2-prop-2-ynyloxycarbonylamino-propionic acid methyl ester (8)

Propargyl chloroformate (0.22 mL, 1.6 mmol), DMAP (20 mg, 0.2 mmol) and Et_3N (0.17 mL, 1.8 mmol) were added to a stirred solution of diamine (0.23 g, 0.8 mmol) in CH_2Cl_2 (50 mL). The mixture was stirred at rt for 2 days, washed with HCl 1 M (50 mL) and with brine (50 mL) and dried (Na_2SO_4). After filtration, 600 mg of silica was added to the filtrate and the solvent was evaporated under reduced pressure. The residue thus absorbed on silica was purified with column chromatography (10:1 → 2:1, petroleum ether/EtOAc) to give **8** as a yellow syrup (0.14 g, 38%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.22–7.17 (m, 1H, Ar–H), 6.98 (d, 2H, $J = 5.3$ Hz, Ar–H), 6.92 (t, 1H, $J = 22$ Hz, Ar–H), 5.47 (d, 1H, $J = 8$ Hz, NH partially exchanged), 5.34 (d, 1H, $J = 8$ Hz), 5.47–4.61 (m, 6H, N–CH–CO, O–CH_2–),

3.72 (d, 6H, $J = 4.2$ Hz, O–CH_3), 3.14–2.99 (m, 4H, Ar–CH_2–), 2.47 (q, 2H, $J = 4.8$ Hz, $J = 2.4$ Hz, CCH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.7, 154.9, 136.2, 130.5, 129.0, 128.3, 78.3, 75.0, 55.0, 53.0, 52.6, 38.3; IR (NaCl film): 3315, 2942, 2120, 1731, 1523 cm^{-1}; FABMS m/z calcd for $[C_{22}H_{25}N_2O_8+H]^+$: 445.1611. Found: 445.1608.

4.9. 2-Benzyloxycarbonylamino-3-[3,5-bis-(2-benzyloxycarbonylamino-2-methoxycarbonyl-ethyl)-phenyl]-propionic acid methyl ester (9)

2-Benzyloxycarbonylamino-3-[3,5-bis-(2-benzyloxycarbonylamino-2-methoxycarbonyl-vinyl)-phenyl]-acrylic acid methyl ester (0.8 g, 1 mmol) was added to a mixture of CH_3OH/EtOAc (1:1) and the obtained slurry was degassed on an ultrasonic bath for 15 min and then purged with argon for a further 15 min. Then Wilkinson's catalyst (100 mg, 0.1 mmol) was added and the mixture was hydrogenated at rt at 60 psi over 5 days. Silica (2.4 g) was then added to the solution and the solvent was removed under reduced pressure. The residue thus absorbed on silica was passed through a sort plug of silica eluting with petroleum ether/EtOAc (1:1, 200 mL) to remove the catalyst and then the solvent was removed under reduced pressure. The residue was coevaporated with toluene (3×5 mL) to give **9** as a brownish paste (0.77 g, 97%). ^{1}H NMR and MS data were in accordance with the literature data.[37]

4.10. 3-[3,5-Bis-(2-methoxycarbonyl-2-propynoylamino-ethyl)-phenyl]-2-propynoylamino-propionic acid methyl ester (10)

10% Pd/C (150 mg) was added to a stirred solution of **9** (0.75 g, 0.95 mmol) in CH_3OH (75 mL). The slurry was hydrogenated overnight or until no starting material could be seen on TLC (ninhydrin staining). The slurry was then filtered through a plug of Celite and the solvents were removed under reduced pressure to give the crude triamine intermediate as a brownish paste, which was suspended in CH_2Cl_2 (40 mL). Propiolic acid (204 μL, 3.3 mmol) and EEDQ (0.9 g, 3.6 mmol) were added to the solution and the resulting mixture was stirred for 2 days at rt and then washed with HCl 2 M (3×40 mL). The combined aqueous phases were extracted once with CH_2Cl_2 (50 mL) and the combined organic phases were dried (Na_2SO_4). After filtration, 900 mg of silica was added to the filtrate and the solvent was removed under reduced pressure. The residue thus absorbed on silica was purified by column chromatography (4:1 → 1:4, petroleum ether/EtOAc) to give **10** as a brown syrup (84 mg, 20%): ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.81–6.70 (m, 4H, Ar–H), 6.58–6.49 (m, 2H, NH partially exchanged), 4.95–4.84 (m, 3H, –N–CH–CO–), 3.73 (s, 9H, OCH_3), 3.17–2.96 (m, 6H, Ar–CH_2–), 2.86–2.82 (m, 3H, CCH); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.1, 151.7, 136.5, 129.6, 77.4, 74.6, 53.6, 53.0, 37.8; IR (NaCl): 3454, 2936, 2102, 1725, 1528 cm^{-1}; FABMS m/z calcd for $[C_{27}H_{28}N_3O_9+H]^+$: 538.1826. Found: 538.1821.

4.11. Lactosyl amide (12)

2-Azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside **11**[32] (32 mg, 77 μmol), **1** (37 mg, 0.21 mmol) and CuI (17 mg, 0.09 mmol) were dissolved in CH_3CN (2 mL). *N*,*N*-Diisopropylethylamine (14 μL, 77 μmol) was added and the reaction mixture was stirred over 6 days followed by concentration under reduced pressure. The residue was dissolved in H_2O and applied on to C-18 silica (5 g). Elution with a gradient of CH_3OH in H_2O and lyophilization gave **12** (30 mg, 66%): $[\alpha]_D^{20}$ +24; ^{1}H NMR (400 MHz, CH_3OD): δ 8.46 (s, 1H, H-triazole), 7.30–7.18 (m, 5H, Ar–H), 4.69 (t, 2H, $J = 5$ Hz, –CH_2–CH_2–N), 4.34 (d, 2H, $J = 7.8$ Hz, H-1′, H-1), 4.26–4.21 (m, 1H, O–CH_2–CH_2), 4.06–4.00 (m, 1H, O–CH_2–CH_2), 3.90 (dd, 1H, $J = 2.4$ Hz, $J = 12.1$ Hz, H-6), 3.84–3.81 (m, 2H), 3.78, (dd, 1H, $J = 7.4$ Hz, $J = 11.4$ Hz, H-6), 3.69, (dd, 1H, $J = 4.6$ Hz, $J = 11.4$ Hz, H-6), 3.63–3.48 (m, 7H), 3.44–3.40 (m, 1H, H-5), 3.27 (t, 1H, $J = 8.4$ Hz, H-2′), 2.91 (t, 2H, $J = 7.6$ Hz, $J = 7.2$ Hz, Ar–CH_2); ESIMS m/z calcd for $[C_{25}H_{36}N_4O_{12}+H]^+$: 585.2408. Found: 585.2295.

4.12. Lactosyl carbamate (13)

2-Azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside **11**[32] (32 mg, 77 μmol), **2** (49 mg, 0.24 mmol) and CuI (34 mg, 0.19 mmol) were dissolved in CH_3CN (2 mL). *N*,*N*-Diisopropylethylamine (14 μL, 77 μmol) was added and the reaction mixture was stirred over 5 days followed by concentration under reduced pressure. The residue was dissolved in H_2O and applied on to C-18 silica (5 g). Elution with a gradient of CH_3OH in H_2O and lyophilization gave **13** (25 mg, 80%): $[\alpha]_D^{20}$ +29; ^{1}H NMR (400 MHz, CH_3OD): δ 8.09 (s, 1H, triazole), 7.28–7.16 (m, 5H, Ar–H), 5.12 (s, 2H, O–CH_2–C), 4.65 (t, 2H, $J = 5$ Hz, –CH_2–CH_2–N), 4.34 (d, 1H, $J = 7.8$ Hz, H-1), 4.33 (d, 1H, $J = 7.6$ Hz, H-1′), 4.25–4.20 (m, 1H, O–CH_2–CH_2), 4.03–3.98 (m, 1H, O–CH_2–CH_2), 3.90 (dd, 1H, $J = 2.3$ Hz, $J = 12.1$ Hz, H-6), 3.85–3.70 (m, 4H), 3.58–3.46 (m, 5H), 3.42–3.40 (m, 1H, H-5), 3.35–3.30 (m, 2H, CH_2–NH–CO, partly hidden in solvent residual peak), 3.25 (t, 1H, $J = 8.4$ Hz, H-2), 2.77 (t, 2H, $J = 7.4$ Hz, Ar–CH_2); ESIMS m/z calcd for $[C_{26}H_{38}N_4O_{13}+H]^+$: 615.2514. Found: 615.2526.

4.13. Lactosyl methylester amide (14)

2-Azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside **11**[32] (33 mg, 81 μmol), **4** (40 mg, 0.17 mmol)

and CuI (21 mg, 0.11 mmol) were dissolved in toluene (3 mL). *N*,*N*-Diisopropylethylamine (15 μL, 81 μmol) was added and the reaction mixture was stirred for 2 days followed by concentration under reduced pressure. Purification by flash chromatography (4:1, CH_2Cl_2/CH_3OH) gave **14** (31 mg, 60%). ^{1}H NMR (300 MHz, CH_3OD): δ 8.47 (s, 1H, H-triazole), 7.30–7.20 (m, 5H, Ar–H), 4.69 (t, 2H, $J = 4.8$ Hz, –CH_2–CH_2–N), 4.60 (m, 1H, NH), 4.35 (br d, 2H, $J = 7.2$ Hz, H-1, H-1′), 4.27–4.20 (m, 1H, O–CH_2–CH_2), 4.06–4.99 (m, 1H, O–CH_2–CH_2), 3.90–3.66 (m, 8H), 3.60–3.45 (m, 5H), 3.43–3.37 (m, 1H, H-5), 3.28–3.21 (m, 3H, H-2, Ar–CH_2), 3.19–3.12 (m, 1H, NH–CH–CO); ESIMS *m*/*z* calcd for [$C_{27}H_{38}N_4O_{14}$+ Na]$^+$: 665.2282. Found: 665.2265.

4.14. Lactosyl methylester amide (15)

2-Azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside **11**[32] (30 mg, 73 μmol), **3** (24 mg, 0.11 mmol) and CuI (34 mg, 0.18 mmol) were dissolved in CH_3CN (2 mL). *N*,*N*-Diisopropylethylamine (14 μL, 77 μmol) was added and the reaction mixture was stirred over 3 days followed by concentration under reduced pressure. Purification by flash chromatography (4:1, CH_2Cl_2/CH_3OH) gave **15** (28 mg, 60%): $[\alpha]_D^{20}$ −31; ^{1}H NMR (400 MHz, CH_3OD): δ 8.47 (s, 1H, H-triazole), 7.29–7.18 (m, 5H, Ar–H), 4.68 (t, 2H, $J = 5$ Hz, –CH_2–CH_2–N), 4.59 (br s, 0.5H, N–H, partly exchanged), 4.35 (d, 1H, $J = 7.4$ Hz, H-1′), 4.35 (d, 1H, $J = 7.8$ Hz, H-1), 4.25–4.20 (m, 1H, O–CH_2–CH_2), 4.05–4.00 (m, 1H, O–CH_2–CH_2), 3.89 (dd, 1H, $J =$ 2.4 Hz, $J = 12.2$ Hz, H-6), 3.83–3.67 (m, 7H), 3.59–3.46 (m, 5H), 3.43–3.40 (m, 1H, H-5), 3.30–3.21 (m, 3H, H-2, Ar–CH_2), 3.16 (dd, 1H, $J = 8.5$ Hz, $J = 13.9$ Hz, NH–CH–CO); ESIMS *m*/*z* calcd for [$C_{27}H_{38}N_4O_{14}$+H]$^+$: 643.2463. Found: 643.2435.

4.15. Divalent lactosyl methylester amide (16)

2-Azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside **11**[32] (46 mg, 0.11 mmol), **7** (15 mg, 40 μmol) and CuI (26 mg, 0.14 mmol) were dissolved in CH_3CN (2 mL). *N*,*N*-Diisopropylethylamine (20 μL, 116 μmol) was added and the reaction mixture was stirred over 6 days followed by concentration under reduced pressure. The residue was dissolved in H_2O and applied on to C-18 silica (5 g). Elution with a gradient of CH_3OH in H_2O and lyophilization gave **16** (26 mg, 53%): ^{1}H NMR (400 MHz, CH_3OD): δ 8.48 (2s, 1H, triazole-H, from the diastereomers), 7.21–7.09 (m, 4H, Ar–H), 4.69 (t, 4H, $J = 4.9$ Hz, –CH_2–CH_2–N), 4.59 (s, 0.7H, NH), 4.36 (br d, 4H, $J = 7.6$ Hz, H-1′, H-1), 4.26–4.20 (m, 2H, O–CH_2–CH_2), 4.06–4.01 (m, 2H, O–CH_2–CH_2), 3.89 (br dd, 2H), 3.84–3.67 (m, 14H), 3.61–3.47 (m, 10H), 3.43–3.40 (m, 2H, H-5), 3.33–3.18 (m, 6H, H-2, Ar–CH_a, partly hidden in solvent residual peak), 3.11 (dd, 1H, $J = 8.2$ Hz, $J = 14.0$ Hz, –NH–CH–CO, partly hidden), 3.11 (dd, 1H, $J = 8.3$ Hz, $J = 13.7$ Hz, –NH–CH–CO); ESIMS *m*/*z* calcd for [$C_{48}H_{70}N_8O_{28}$+ H]$^+$: 1207.4378. Found: 1207.4337.

4.16. Trivalent lactosyl methylester carbamate (17)

2-Azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside **11**[32] (62 mg, 0.15 mmol), **10** (21 mg, 39 μmol) and CuI (30 mg, 0.15 mmol) were dissolved in CH_3CN (3 mL). *N*,*N*-Diisopropylethylamine (26 μL, 150 μmol) was added and the reaction mixture was stirred over 2 days followed by concentration under reduced pressure. The residue was dissolved in H_2O and applied on to C-18 silica (5 g). Elution with a gradient of CH_3OH in H_2O and lyophilization gave **17** (23 mg, 33%): ^{1}H NMR (400 MHz, D_2O): δ 8.46–8.44 (m, 3H, triazole), 7.09–7.01 (m, 3H, Ar–H), 4.77–4.73 (m, 6H, –CH_2–CH_2–N, partly hidden in solvent residual peak), 4.47–4.43 (m, 6H, H-1, H-1′), 4.32–4.29 (m, 3H, O–CH_2–CH_2), 4.16–4.13 (m, 3H, O–CH_2–CH_2), 3.94–3.90 (m, 6H), 3.83–3.53 (m, 39H), 3.32–3.08 (m, 9H, H-2, Ar–CH_a, NH–CH–CO); ESIMS *m*/*z* calcd for [$C_{69}H_{102}N_{12}O_{42}$+Na]$^+$: 1793.6112. Found: 1793.6088.

4.17. Lactosyl methylester carbamate (18)

2-Azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside **11**[32] (29 mg, 71 μmol), **5** (39 mg, 0.11mmol) and CuI (14 mg, 0.073 mmol) were dissolved in CH_3CN (2 mL). *N*,*N*-Diisopropylethylamine (14 μL, 77 μmol) was added and the reaction mixture was stirred over 2 days followed by concentration under reduced pressure. Purification by flash chromatography (4:1, CH_2Cl_2/CH_3OH) gave **18** (27 mg, 56%): ^{1}H NMR (400 MHz, CH_3OD): δ 8.06, 8.05 (2s, 1H, triazole-H, from the two diastereomers), 7.28–7.17 (m, 5H, Ar–H), 5.09 (s, 2H, O–CH_2–C), 4.63 (t, 2H, $J = 5$ Hz, –CH_2–CH_2–N), 4.60 (br s, 0.3H, NH, partly exchanged), 4.43 (dd, 1H, $J =$ 5.6 Hz, $J = 9.0$ Hz, CH–CO), 4.35 (d, 1H, $J = 7.5$ Hz, H-1′), 4.34 (d, 1H, $J = 7.9$ Hz, H-1), 4.25–4.19 (m, 1H, O–CH_2–CH_2), 4.03–3.97 (m, 1H, O–CH_2–CH_2), 3.90 (dd, 1H, $J = 2.3$ Hz, $J = 12.1$ Hz, H-6), 3.84–3.75 (m, 3H), 3.72–3.68 (m, 4H), 3.62–3.48 (m, 5H), 3.44–3.40 (m, 1H, H-5), 3.25 (t, 1H, $J = 8.2$ Hz, $J = 8.3$ Hz, H-2), 3.12 (dd, 1H, $J = 5.5$ Hz, $J = 13.8$ Hz, Ar–CH_a), 2.92 (dd, 1H, $J = 9.1$ Hz, $J = 13.8$ Hz, Ar–CH_b); ESIMS *m*/*z* calcd for [$C_{28}H_{40}N_4O_{15}$+H]$^+$: 673.2568. Found: 673.2535.

4.18. Divalent lactosyl methylester carbamate (19)

2-Azidoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside **11**[32] (74 mg, 0.18 mmol), **8** (28 mg, 64 μmol) and CuI (38 mg, 0.20 mmol) were dissolved in CH_3CN (2 mL). *N*,*N*-Diisopropylethylamine (31 μL, 178 μmol) was added and the reaction mixture was stirred over

2 days followed by concentration under reduced pressure. The residue was dissolved in H_2O and applied on to C-18 silica (5 g). Elution with a gradient of CH_3OH in H_2O and lyophilization gave **19** (59 mg, 73%): ^1H NMR (400 MHz, CH_3OD): δ 8.07 (br s, 2H, triazole), 7.19–7.04 (m, 4H, Ar–H), 5.09 (br s, 4H, O–CH_2–C), 4.63 (br t, 4H, $J = 4.2$ Hz, –CH_2–CH_2–N), 4.60 (s, 1.5H, NH), 4.45–4.43 (m, 2H, CH–CO), 4.36 (d, 2H, $J = 7.4$ Hz, H-1′), 4.35 (d, 2H, $J = 7.7$ Hz, H-1), 4.24–4.19 (m, 2H, O–CH_2–CH_2), 4.03–3.98 (m, 2H, O–CH_2–CH_2), 3.90 (br dd, 2H, $J = 2.3$ Hz, $J = 12.1$ Hz, H-6), 3.85–3.68 (m, 14H), 3.63–3.47 (m, 10H), 3.43–3.41 (m, 2H, H-5), 3.26 (br t, 2H, $J = 7.9$ Hz, H-2), 3.09 (br dd, 2H, Ar–CH_a), 2.91 (br dd, 2H, $J = 9.0$ Hz, $J = 13.4$ Hz, Ar–CH_b); ESIMS *m/z* calcd for $[C_{50}H_{74}N_8O_{30}+H]^+$: 1267.4589. Found: 1267.4578.

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Supplementary data

^1H NMR of **2**, **5**, **7**–**8**, **10** and **12**–**19**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.04.028.

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