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Targeted Delivery of Fluorescent High-Mannose-Type Oligosaccharide Cathepsin Inhibitor Conjugates

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Three fluorescent cathepsin inhibitor glycoconjugates have been designed, synthesized, and evaluated in terms of their cell internalization and cathepsin inhibitory properties. The conjugates are composed of a peptide epoxysuccinate, capable of covalent and irreversible binding to cysteine proteases, coupled to a fluorescent BODIPY dye and functionalized with a mono-, tri-, or heptamannoside. Mannose-receptor-dependent uptake of the probes in live dendritic cells is shown to depend on the type of carbohydrate attached. Where uptake of the monomannoside is poor and mannose-receptor-independent, the intracellular labeling of cathepsins by the probes equipped with a tri- or heptamannoside conjugate appeared concentration- and mannose-receptor-dependent.

Introduction

The targeted delivery of chemotherapeutics through the intermediacy of cell surface receptors represents an attractive means to selectively deliver cargo to target tissues or subcellular compartments. Conceptually different approaches have been developed over the years to selectively target therapeutics and diagnostics to specific cell types by receptor-mediated uptake of the deliverables. Various ligands have been used as a homing device, including antibodies and small synthetic molecules such as folic acid, peptides, and carbohydrates.[1] Lectins are carbohydrate-binding receptors, which occur both as membrane-bound and soluble proteins. They play a key role in a wide variety of cellular recognition and communication processes.[2] They are abundantly expressed on dendritic cells (DCs) and macrophages, cells that have evolved to survey their surroundings and detect pathogens and danger signals. Many of the lectins found on these cells are members of the C-type lectin family and these include the macrophage mannose receptor (MMR), Dectin-1, Dectin-2, and DC-SIGN.[3] These carbohydrate-binding receptors have been exploited in various antigen-targeting strategies to enable both the efficient uptake of antigens and simultaneous stimulation of the immune cells.[4] Peptidases play a key role in the processing of peptides and peptide antigens and as such play a pivotal role in the complex antigen presentation pathway. To probe the activity of cathepsins in living DCs we have previously reported the adaptation of the broad-spectrum cathepsin inhibitor DCG-04[5] to obtain targeted activity-based cathepsin probes. DCG-04 was originally developed by Bogyo and co-workers, in a seminal paper,[5a] which together with the first paper by Cravatt and co-workers on serine hydrolase probes,[6] shaped the field of activity-based protein profiling. Taking the natural product, the broad-spectrum cysteine protease inhibitor E-64, as a basis, Bogyo and co-workers appended both a biotin and—in a later contribution—a set of different fluorophores and showed that all these structures retain potency and (broad-spectrum) specificity against numerous mammalian cathepsin cysteine proteases.[5] From these studies, which yielded activity-based probes currently widely used by the chemical biology community, it became apparent that cathepsin cysteine proteases tolerate a wide variety of functional groups appended to the dipeptide epoxysuccinate core. We capitalized on this by appending, directly adjacent to a reporter fluorophore, a mannose cluster to allow for lectin-mediated uptake of the probe (**1**: UHG392; see Figure 1).[7,8] Our first-generation activity-based probe (ABP) **1** contains an artificial mannose cluster built up from a hexalysine oligopeptide with each lysine side chain modified to bear a monomannoside residue.[7]

We hypothesized that the nature of the mannose ligand may influence recognition by the cell surface lectins and consequently the uptake and routing of the conjugates. For example, it is known that the prevalent carbohydrate-binding lectins on DCs, DC-SIGN, and the MR bind oligomannosides better than monomannosides.[3,4] Glycan microarray studies have revealed that DC-SIGN strongly binds high-mannose-type structures[9] and available crystal structures of DC-SIGN bound to natural ligands show that a terminal branched trimannose structure, featuring α-(1,3) and α-(1,6) mannose branches on a core mannose residue, fits well in the carbohydrate-binding

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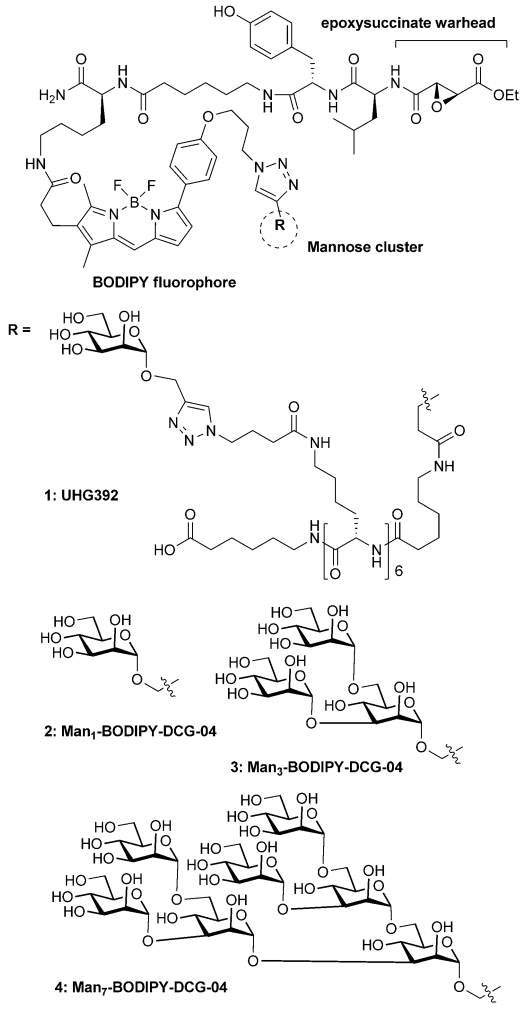
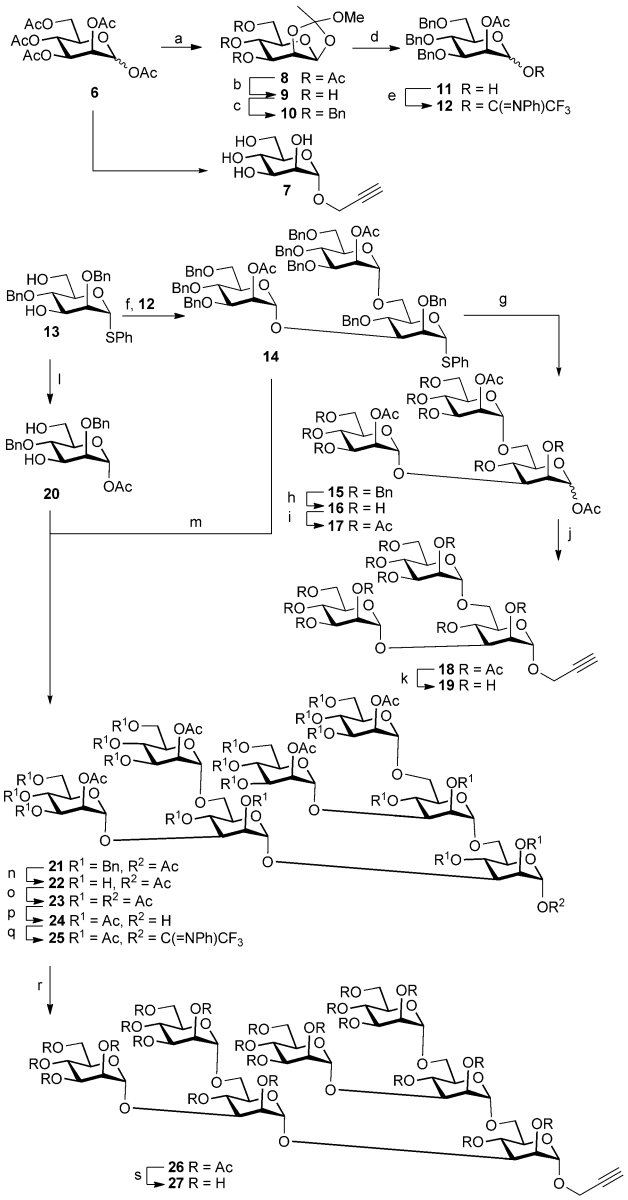


Figure 1. Structures of the DCG-04 mannose conjugate **1** previously studied and the mannosyl DCG-04 probes **2**, **3**, and **4** reported here.

site of this signaling receptor.[10] The MR also binds oligomannosides and a preference for the same type of branching has been reported.[11] We therefore designed and synthesized a set of BODIPY-DCG-04-oligomannose conjugates, bearing oligomannosides that feature natural glycosidic connections and contain the α-(1→3),α-(1→6)-branched trimannoside structure. We here describe the assembly of three BODIPY-DCG-04-mannose clusters (**2**, **3**, and **4**; Figure 1), bearing either a mono-, tri-, or heptamannoside targeting entity and their efficacy in the labeling of cathepsins in both cell lysates and live cells.

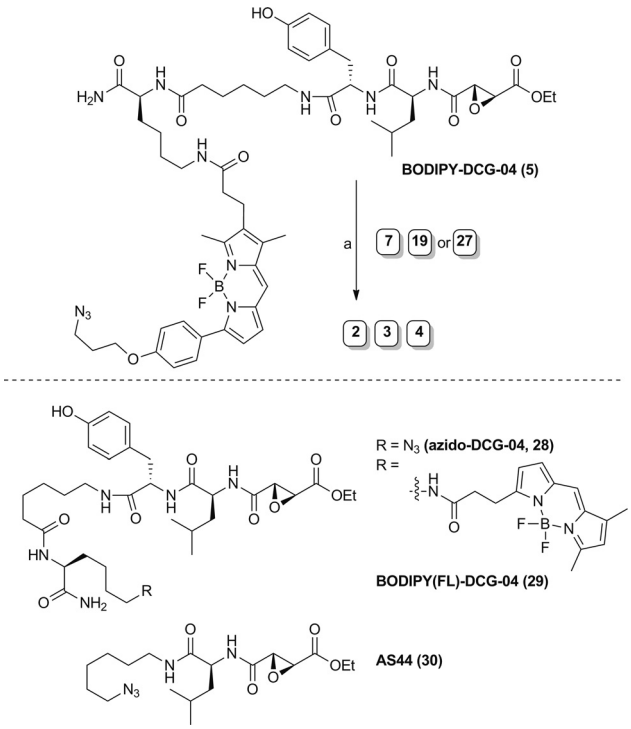
Results and Discussion

The three BODIPY-DCG-04 mannose conjugates **2**, **3**, and **4** were assembled by conjugation of the relevant propargyl mannosides (**7**, **19**, and **27**; Scheme 1) with azide-functionalized BODIPY-epoxysuccinate **5** (Scheme 2). α-Propargyl monomannoside **7** was synthesized following a literature procedure.[12] Oligomannosides **19** and **27** were synthesized in a convergent manner as depicted in Scheme 1. Starting from peracetylated mannose **6**, orthoester **8** was obtained via the intermediate



Scheme 1. Reagents and conditions: a) i. I_2, Et_3SiH, CH_2Cl_2, reflux; ii. MeOH, 2,6-lutidine, RT; b) K_2CO_3, MeOH, RT; c) NaH, BnBr, DMF, 0 °C to RT; d) DME/H_2O (10:1), *p*TsOH, 82% over 4 steps; e) Cs_2CO_3, acetone, $(CF_3)C(NPh)Cl$, 90%; f) **12**, DCM, TfOH, activated molecular sieves, −40 °C to 0 °C, 84%; g) NIS, AcOH, DCE/THF (1:1), 94%; h) i. Pd/C, H_2, EtOAc/*t*BuOH/H_2O (1:3:4), ii. Pd/C, H_2, H_2O; i) Ac_2O, pyridine, 0 °C to RT, 76% over 2 steps; j) propargyl alcohol, $BF_3{\cdot}Et_2O$, DCM, RT, 61%; k) MeOH, NaOMe, 50 °C, 68%; l) NIS, DCM/AcOH (1:1), RT, 40%; m) **14**, NIS, TfOH, activated molecular sieves, DCM, −40 °C to RT, 62%; n) i. Pd/C, H_2, EtOAc/MeOH/H_2O (5:4:1); ii. Pd/C, H_2, MeOH/H_2O (1:1); o) Ac_2O, pyridine, 0 °C to RT, quantitative yield over 2 steps; p) $H_2NNH_2{\cdot}AcOH$, DMF, 0 °C, 79%; q) $ClC(=NPh)CF_3$, Cs_2CO_3, acetone, quantitative; r) propargyl alcohol, TfOH, DCM, activated molecular sieves, −40 °C to 0 °C, 40%; s) i. NaOMe/MeOH; ii. 0.1 M NaOH (aq.), quantitative. DME = 1,2-dimethoxyethane, DMF = *N*,*N*-dimethylformamide, DCE = 1,2-dichloroethane, DCM = dichloromethane, Tf = trifluoromethylsulfonyl, NIS = *N*-iodosuccinimide.

formation of a mannosyl iodide, as reported by Adinolfi et al.[13] Intramolecular substitution of the iodide gave orthoester **8**, which was deacetylated and subsequently benzylated to give orthoester **10**. Acidic hydrolysis of **10** then yielded hemi-



Scheme 2. Assembly of the mannose-BODIPY-DCG-04 conjugates **2**, **3**, and **4** and structures of cathepsin binding probes **5** (azido-BODIPY-DCG-04), **28** (azido-DCG-04), **29** (green BODIPY(FL)-DCG-04), and **30** (AS44). Reagents and conditions: a) sodium ascorbate, $CuSO_4$, DMF/H_2O (1:1), Man_1-BODIPY-DCG-04 42%, Man_3-BODIPY-DCG-04 24%, Man_7-BODIPY-DCG-04 32%.

acetal **11**[14] in 82% yield over four steps. Treatment of this lactol with (*N*-phenyl)trifluoroacetamidoyl chloride in the presence of Cs_2CO_3 afforded (*N*-phenyl)trifluoroimidate donor **12** in 90% yield. The construction of key trisaccharide **14**, which was used as a precursor for both the propargyl trimannoside **19** and as a building block to construct heptasaccharide **27**, was accomplished by a double glycosylation of diol **13**[15] using donor **12** and a catalytic amount of TfOH. Next, trimer **14** was converted into the corresponding anomeric acetate **15** using NIS and AcOH. Removal of all benzyl groups from this trimer required a two-step sequence. The fully protected trisaccharide was first treated with Pd/C and H_2 in a mixture of EtOAc/*t*BuOH/H_2O (1:3:4) after which the solvent was replaced with water for the second reduction event to effect removal of all benzyl groups. Peracetylation of the crude trimer yielded **17** in 76% yield over the two steps. Propargyl alcohol was then condensed with the trimannosyl acetate under the agency of $BF_3{\cdot}Et_2O$ to provide the fully protected trimer **18**. Global deacetylation under Zemplén conditions yielded the propargyl trimannoside **19**.

The heptasaccharide **21** was assembled using trisaccharide donor **14** and acceptor **20**. The latter building block was obtained by treatment of thiomannoside **13** with NIS and AcOH. This led to the formation of the α-acetate **20** in 40% yield, along with its β-anomeric counterpart (37%) and a minor by-product that was characterized as 1,6-anhydro-2,4-di-*O*-benzyl-β-D-mannose (11%). Double condensation of diol acceptor **20** with trisaccharide donor **14** was achieved using the NIS/TfOH promotor couple to yield heptamer **21** as a single product in 62% yield. Hydrogenation of **21** with Pd/C and H_2 in EtOAc/MeOH/H_2O (5:4:1) was followed by a second hydrogenation in MeOH/H_2O (1:1) to give the debenzylated heptamer **22**, which was directly subjected to global acetylation. Attempts to introduce the propargyl moiety onto the peracetylated heptamer using $BF_3{\cdot}Et_2O$ did not lead to the desired product and therefore we switched to the use of a more potent glycosylating agent. To this end, the anomeric acetyl was chemoselectively deblocked using hydrazine acetate and the liberated alcohol was converted into the *N*-phenyl trifluoroacetimidate. Glycosylation of propargyl alcohol with donor **25** under mild acid catalysis yielded the peracetylated heptamannoside **26** in 40% yield. Deacetylation under standard Zemplén conditions led to the partial removal of the acetyl groups, necessitating an extra saponification step with aqueous 0.1 M NaOH to provide the target heptamer **27**.

The BODIPY-DCG-04-mannose conjugates were obtained through a Cu^I-catalyzed Huisgen 1,3-dipolar cycloaddition[16] of azido BODIPY-DCG-04 (**5**)[7,8] and the propargyl mannosides **7**, **19**, and **27** (Scheme 2). After HPLC purification the three target constructs were obtained in 42% (**2**), 24% (**3**), and 32% (**4**) yield, respectively.

To investigate labeling of cathepsins by activity-based probes **2**, **3**, and **4** we first evaluated their activity in cell lysates. To this end, mouse liver lysate was incubated with increasing concentrations of Man_1-BODIPY-DCG-04 (**2**), Man_3-BODIPY-DCG-04 (**3**), or Man_7-BODIPY-DCG-04 (**4**), after which the proteins in the lysates were resolved on SDS-PAGE (Figure 2A). All three mannosyl DCG-04 probes **2**, **3**, **4** label cathepsins in a concentration-dependent manner, as is evident from Figure 2A. A small difference in gel-shift is apparent for the three constructs and correlates to their molecular weights. A decrease in binding capacity was observed with increasing cluster size, suggesting that the steric bulk of the heptamannosyl cluster retards binding and cathepsin inactivation. The diminished binding efficacy of the larger mannosyl clusters, together with the difference in the gel-shift of the labeled proteins indicates that mannosidases present in the cell lysate do not (effectively) trim the probes when bound to the cathepsins or when unbound in the cell extract. Incubation of immature mouse dendritic cell (DC) lysate with the probes showed a similar concentration-dependent binding of cathepsin proteins (Figure 2B). In line with our previous findings, changing the pH of the buffer from pH 5.5 (the optimal pH for most cathepsin activity)[7] to pH 7 led to abrogation of cathepsin binding, showing that active enzymes are required for labeling. Next, a set of competition experiments was performed. The lysates were pre-incubated with different DCG-04 competitors: azido-DCG-04 **28**, green fluorescent probe BODIPY(FL)-DCG-04 **29**,[7] and AS44 **30**[8] (see Scheme 2 for the structures of the competitors), followed by incubation with the probes. As seen in Figure 2B labeling of the cathepsins with the red mannosyl BODIPY-DCG-04 conjugates was effectively prevented, leading to either disappearance of the fluorescent bands in the competition experiment with nonfluorescent azido-DCG-04 and

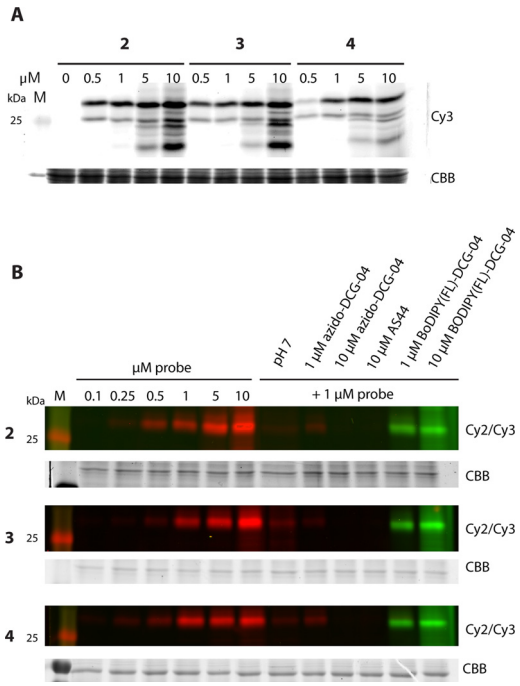


Figure 2. Cathepsin labeling experiments in mouse liver and dendritic cell lysate. Mouse liver lysate (10 μg total protein, (A)) or immature mouse dendritic cell (DC) lysate (8 μg total protein, (B)) was incubated (1 h, 37 °C) with an increasing concentration of probe **2**, **3**, or **4** at pH 5.5 or 1 μM at pH 7. Alternatively, lysates were incubated (1 h, 37 °C) with azido-DCG-04 (1 or 10 μM), AS44 (10 μM), or BODIPY(FL)-DCG-04 (1 or 10 μM), before treatment with probe **2**, **3**, or **4** (1 μM, 1 h, 37 °C). Proteins were resolved on 12.5% SDS-PAGE, followed by fluorescence scanning (Cy2 (green): BODIPY(FL), Cy3 (red): BODIPY(TMR)) and total protein staining with Coomassie brilliant blue (CBB). M: dual-color protein molecular weight marker.

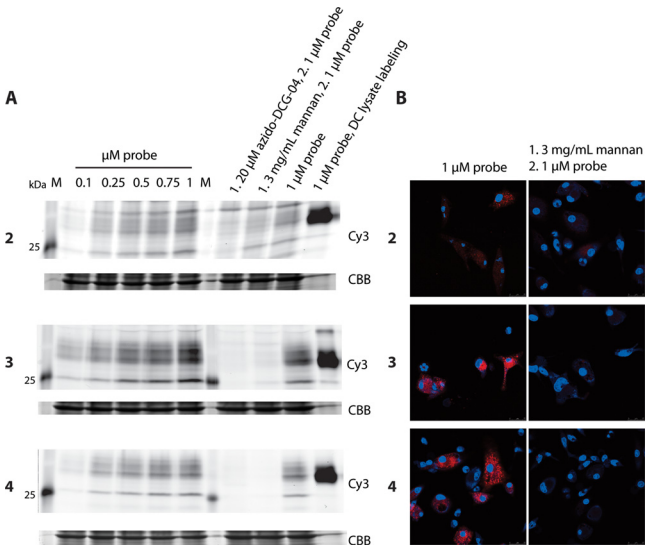


Figure 3. Uptake and cathepsin binding of the probes in live dendritic cells. A) DCs were treated with varying concentrations of **2**, **3**, or **4** (2 h, 37 °C) or pre-incubated (1 h, 37 °C) with azido-DCG-04 (20 μM) or mannan (3 mg mL^{-1}), followed by addition of **2**, **3**, or **4** (1 μM, 2 h, 37 °C), washed with PBS, lysed, and resolved on 12.5% SDS-PAGE. In-gel fluorescence of BODIPY (Cy3) and total protein stain (CBB) are shown. B) Representative confocal microscope images of DCs treated with 1 μM of probes **2, 3**, or **4** (left panels) or with mannan (3 mg mL^{-1}, right panels) for 1 h, followed by treatment with the probes. After treatment, cells were washed with PBS, fixed with 4% formaldehyde, nuclei stained with Draq5, and imaged using the Cy3 (λ_{ex} = 532 nm) settings for BODIPY (red) and Cy5 (λ_{ex} = 635 nm) settings for Draq5 as a nuclear stain (blue).

AS44, or the appearance of green fluorescent bands in the experiment with the green DCG-04 probe **29**.

Next, the probes were tested for uptake and binding of cathepsins in living DCs (Figure 3). In line with the results obtained with the cell lysates, a concentration-dependent labeling pattern was observed (Figure 3 A, left panel). The most efficient and selective labeling was achieved with the trimannosyl probe **3**, where the monomannosyl compound **2** showed the highest background fluorescence. Also in these experiments the heptamannoside probe **4** labeled the target cathepsins somewhat less efficiently than its trimannoside counterpart **3**. Competition experiments with the nonfluorescent cell-permeable azido-DCG-04 probe **28** indicated that, also in living DCs, active cathepsins are labeled by the probes. To test whether uptake of the probes was carbohydrate receptor mediated the DCs were incubated with mannan (3 mg mL^{-1}) prior to exposure to the probes. In doing so, labeling by the tri- and heptamannosyl probes was effectively blocked, showing that uptake of these ABPs is receptor-dependent. The receptor-mediated uptake and labeling was confirmed by confocal microscopy. Figure 3 B shows the clear uptake of Man_3-BODIPY-DCG-04 **3** and Man_7-BODIPY-DCG-04 **4** in DCs but little uptake of Man_1-BODIPY-DCG-04 **2** (Figure 3 B, left panels). Pre-incubation of the cells with mannan prevented uptake of probes **3** and **4**. Combined, the results show that Man_3-BODIPY-DCG-04 **3** and Man_7-BODIPY-DCG-04 **4** are taken up through the intermediacy of a carbohydrate binding receptor, where Man_1-BODIPY-DCG-04 **2** can be internalized (at least in part) through a receptor-independent pathway. Receptor-mediated internalization is clearly more efficient. Although it has previously been reported that the mannose receptor can bind monomannosides,[3b] in the case at hand it appears that this is not enough for effective internalization of the conjugate. With respect to our first-generation probe (**1**) it appears that DCG-04 labeling with the trimannoside probe is equally efficient. We have, however observed a difference in processing of the probes. Where probe **1** seems to be processed by mannosidases in living cells (as judged from the minimal difference in gel-shift for the labeled catepsins, indicating only a small shift in molecular size), the current probes are more resistant to the endo/lysosomal action of mannosidases.

Conclusion

We have reported on the assembly of three fluorescent cathepsin probes functionalized with different mannosides to investigate the role of these carbohydrate appendages on inhibition efficacy and internalization efficiency. The size of the mannose oligosaccharides proved to influence the amount of inhibition, with the largest heptamannoside showing least effective cathepsin labeling in cell lysates at low inhibitor concentrations. For effective uptake in live cells it is shown that the tri- and heptamannoside outcompete the monoman-

nosyl probe. The live cell experiments corroborated the more effective inhibition of the smaller trisaccharide inhibitors over its larger heptasaccharide counterpart.

Experimental Section

2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-D-mannopyranoside (11): To a solution of peracetylated mannose **6** (114.5 g, 293 mmol) in DCM (750 mL) was added iodine (104.2 g, 411 mmol) and triethylsilane (66 mL, 410 mmol). The reaction mixture was heated to reflux. After 4 h TLC showed complete conversion of the starting material and mixture was cooled to RT. To the reaction mixture was added 2,6-lutidine (140 mL), MeOH (71 mL) and the reaction mixture was stirred overnight at RT. The reaction mixture was concentrated in vacuo, dissolved in EtOAc, washed with water (1×), 10% $Na_2S_2O_3$ (aq.) (2×), H_2O (3×), and brine (2×), dried over Mg_2SO_4, filtered, and concentrated in vacuo. The residue was dissolved in MeOH (500 mL), and to the solution was added K_2CO_3 (6.6 g, 48 mmol) and stirred for 4 h at RT. The reaction mixture was concentrated in vacuo and co-evaporated with toluene (3×). The product was dissolved in DMF (1.0 L) and to the solution was added BnBr (158 mL, 1.32 mol). The reaction mixture was cooled to 0 °C and to the cooled solution was added NaH (60% m m^{-1}) (31.7 g, 1.32 mol) in small portions over 6 h. The reaction mixture was gradually warmed to RT and was stirred overnight at RT. The reaction mixture was cooled to 0 °C and quenched with MeOH. The solvent was removed in vacuo and the concentrate was dissolved in Et_2O, washed with H_2O (4×) and brine (2×), dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude was dissolved in DME/H_2O (10:1) (1.5 L) and the solution was cooled to 0 °C. To the cooled solution was added *p*TsOH (75 mmol, 14.25 g), after 3 h at 0 °C the reaction was quenched with sat. $NaHCO_3$ (aq.). Brine was added and the organic layer was separated. The product was extracted with DCM (3×) and the combined organic layers were dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography yielded mannose **11** as a colorless oil (118.4 g, 240 mmol, 82% yield over 4 steps as an α/β mixture 10:1). Spectroscopic data were in accordance with literature.[18]

2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-1-*O*-(*N*-phenyltrifluoroacetimidoyl)-α/β-D-mannopyranoside (12): To a solution of mannose **11** (24.6 g, 50 mmol) in acetone (200 mL) was added *N*-(*p*-anisyl)-2,2,2-trifluoroacetimidoyl chloride (10.4 mL, 68.8 mmol) and the reaction mixture was cooled to 0 °C. To the cooled solution was added Cs_2CO_3 (20.7 g, 55 mmol) and the reaction mixture was allowed to warm to RT. After 6 h the mixture was filtered over celite and the filtrate was concentrated in vacuo. Purification by column chromatography yielded imidate donor **12** as a yellow oil (29.8 g, 44.9 mmol, 90% yield as an 25:1 α/β mixture). $[\alpha]_d^{22}$ +26.4° (*c* = 1.0, DCM). FTIR: (neat): $\tilde{\nu}$ = 111.48, 1162.48, 1207.57, 1310.77, 1364.72, 1453.91, 1489.32, 1597.42, 1716.21, 1749.15, 2867.10, 3031.71 cm^{-1}. Spectroscopic data are reported for the major (α) isomer: ^{1}H NMR (400 MHz, $CDCl_3$, *T* = 328 K): δ = 7.38–7.01 (m, 18H), 6.79 (d, *J* = 7.4 Hz, 2H), 6.20 (s, 1H), 5.47 (dd, *J* = 3.2, 2.0 Hz, 1H), 4.87 (d, *J* = 10.9 Hz, 1H), 4.72 (d, *J* = 11.2 Hz, 1H), 4.64 (d, *J* = 12.0 Hz, 1H), 4.57 (dd, *J* = 11.3 Hz, 2H), 4.51 (d, *J* = 12.0 Hz, 1H), 4.01 (ddd, *J* = 8.3, 3.1, 1.3 Hz, 1H), 3.99–3.89 (m, 2H), 3.78 (dd, *J* = 7.8, 3.7 Hz, 1H), 3.72 (dd, *J* = 11.2, 1.7 Hz, 1H), 2.11 ppm (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 169.9, 143.4, 138.5, 138.4, 137.8, 128.9, 128.5, 128.5, 128.3, 128.0, 127.8, 127.8, 127.7, 124.6, 119.6, 77.7, 75.4, 74.5, 74.0, 73.6, 72.4, 68.9, 67.7, 20.9 ppm.

Phenyl 2,4-*O*-dibenzyl-3-*O*-(2-*O*-acetyl-3,4,6-*O*-tribenzyl-α-D-mannopyranosyl)-6-*O*-(2-*O*-acetyl-3,4,6-*O*-tribenzyl-α-D-mannopyranosyl)-1-thio-α-D-mannopyranoside (14): Trifluoro imidate donor **12** (19.91 g, 30 mmol) and acceptor **13** (4.53 g, 10 mmol) were dissolved in DCM (200 mL) and stirred over activated molecular sieves (3 Å) at RT for 30 min. The solution was cooled to −40 °C and to the cooled solution was added TfOH (0.18 mL, 2 mmol) and the reaction mixture was gradually allowed to warm to 0 °C. At 0 °C the reaction was quenched with triethylamine and the mixture was filtered over celite and rinsed with DCM. The organic phase was washed with H_2O and the aqueous phase was extracted with DCM (4×). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography yielded trimer **14** as a colorless oil (11.7 g, 8.4 mmol, 84% yield). Spectroscopic data were in accordance with literature.[19]

Acetyl 2,4-*O*-dibenzyl-3-*O*-(2-*O*-acetyl-3,4,6-*O*-tribenzyl-α-D-mannopyranosyl)-6-*O*-(2-*O*-acetyl-3,4,6-*O*-tribenzyl-α-D-mannopyranosyl)-α/β-D-mannopyranoside (15): To a suspension of NIS (1.25 g, 5.55 mmol) in DCE/THF (1:1) (27 mL) was added acetic acid (21.2 mL, 370 mmol). To the NIS mixture was added a solution of trimer **14** (5.17 g, 3.7 mmol) in DCE/THF (1:1) (5 mL) and the reaction mixture was stirred overnight at RT. The reaction mixture was diluted with EtOAc, washed with 2× 10% $Na_2S_2O_3$ (aq.) (1×), H_2O (1×), sat. aq. $NaHCO_3$ (3×), H_2O (3×), and brine (2×), dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography yielded trimer 15 as a colorless oil (4.67 g, 3.5 mmol, 94%). $[\alpha]_d^{22}$ = +50.0° (*c* = 1.0, DCM). FTIR (neat): $\tilde{\nu}$ = 975.53, 1026.80, 1048.27, 1090.94, 1231.12, 1368.81, 1453.91, 1496.72, 1743.42, 2870.34, 3031.12 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) (α/β mixture, 1:0.4): δ = 7.41–7.08 (m, 56H), 6.18 (d, *J* = 2.0 Hz, 1H), 5.59 (s, 0.4H), 5.50 (ddd, *J* = 8.3, 3.3, 1.9 Hz, 2.8H), 5.20 (d, *J* = 1.8 Hz, 1.4H), 4.96 (d, *J* = 1.9 Hz, 1H), 4.94 (d, *J* = 1.8 Hz, 0.4H), 4.89 (s, 1H), 4.86 (d, *J* = 2.8 Hz, 1.4H), 4.85–4.81 (m, 1.4H), 4.79–4.71 (m, 1.4H), 4.72 (d, *J* = 1.7 Hz, 1H), 4.70 (d, *J* = 2.0 Hz, 0.4H), 4.67 (s, 0.4H), 4.66–4.63 (m, 2.4H), 4.62–4.60 (m, *J* = 1.9 Hz, 2H), 4.57 (d, *J* = 2.3 Hz, 0.4H), 4.54 (s, 0.4H), 4.51 (s, 0.4H), 4.50–4.38 (m, 10H), 4.12 (dd, *J* = 9.6, 3.1 Hz, 1H), 4.02 (ddd, *J* = 8.1, 5.2, 2.7 Hz, 2H), 4.00–3.83 (m, 8.4H), 3.84–3.78 (m, 1H), 3.78–3.65 (m, 6H), 3.64 (s, 0.4H), 3.62 (t, *J* = 2.0 Hz, 0.8H), 3.59 (s, 0.4H), 3.57 (s, 0.4H), 3.51 (ddd, *J* = 8.8, 4.4, 2.0 Hz, 0.4H), 2.35 (s, 1H), 2.15 (s, 3H), 2.14 (s, 1.2H), 2.09 (s, 3H), 2.07 (s, 1.2H), 1.99 (s, 1.2H), 1.95 ppm (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.3, 170.3, 170.2, 170.1, 169.0, 168.9, 138.6, 138.6, 138.5, 138.3, 138.1, 137.9, 137.8, 137.8, 137.6, 129.1, 128.6, 128.5, 128.5, 128.5, 128.4, 128.4, 128.3, 128.3, 128.2, 128.1, 127.9, 127.9, 127.9, 127.9, 127.8, 127.7, 127.7, 127.7, 127.6, 127.6, 125.4, 100.1, 99.7, 98.5, 98.3, 92.8, 90.8, 79.7, 78.1, 78.0, 77.7, 76.5, 76.3, 75.3, 75.2, 75.1, 75.0, 74.7, 74.4, 74.3, 74.2, 74.1, 74.1, 74.0, 73.6, 73.5, 73.5, 73.4, 72.4, 72.2, 72.1, 72.0, 72.0, 71.7, 71.6, 71.5, 71.3, 69.2, 69.0, 68.8, 68.7, 68.6, 68.6, 68.4, 68.3, 66.4, 66.3, 21.3, 21.1, 21.0 ppm. HRMS: $[M+H]^+$ calcd for $C_{80}H_{87}O_{19}$ 1351.58361, found 1351.58399.

Acetyl 2,4-*O*-di-acetyl-3-*O*-(2,3,4,6-*O*-tetra-acetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-α/β-D-mannopyranoside (17): Trimer **15** (3.38 g, 2.5 mmol) was dissolved in an EtOAc/*t*BuOH/H_2O (1:3:4) mixture (50 mL) and the solution was purged with argon. To the solution was added cat. Pd/C (10%) and the mixture was stirred at RT under H_2 (g) atmosphere. After TLC showed complete conversion to a single spot, the Pd/C removed by filtration through a pad of celite which was rinsed with MeOH, and the filtrate was concentrated in vacuo. Next, the crude was taken up in H_2O (50 mL) and purged with argon. To the solution was added cat. Pd/C (10%) and the mixture was stirred at RT under H_2 (g) atmosphere overnight. The mixture was filtered through a pad of celite which was rinsed with H_2O, and the filtrate

was concentrated in vacuo. The ^{1}H NMR spectrum of the crude showed complete removal of aromatic signals and the crude was co-evaporated with 1,4-dioxane (3×). The crude was dissolved in pyridine (25 mL) and the solution was cooled to 0 °C. To the cooled solution was added acetic anhydride (2.5 mL) and the reaction mixture was gradually allowed to warm to RT. After complete conversion, the reaction mixture was cooled to 0 °C, quenched with MeOH, and concentrated in vacuo. The crude product was dissolved in EtOAc and washed with 1 M HCl (aq.) (1×), sat. $NaHCO_3$ (aq.) (1×), H_2O (3×), and brine (2×), dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography yielded per-*O*-acetylated trimer **17** as a colorless oil (1.81 g, 1.9 mmol, 76% yield). $[\alpha]_d^{22} = +40.4°$ ($c = 1.0$, DCM). FTIR (neat): $\tilde{\nu} = 975.27$, 1039.52, 1139.46, 1212.30, 1368.79, 1433.73, 1741.84, 2925.08 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) (α/β mixture, 1:0.25): $\delta =$ 6.05 (d, $J = 1.9$ Hz, 1 H), 5.81 (s, 0.25 H), 5.48 (d, $J = 3.2$ Hz, 0.25 H), 5.27 (dd, $J = 8.8$, 2.7 Hz, 4.4 H), 5.04 (d, $J = 2.6$ Hz, 2 H), 5.02 (s, 0.4 H), 4.80 (s, 1.2 H), 4.35–4.20 (m, 3.4 H), 4.19–4.00 (m, 7.2 H), 3.90 (dq, $J = 10.0$, 3.1 Hz, 1 H), 3.75 (dd, $J = 11.0$, 5.5 Hz, 1 H), 3.57 (dd, $J =$ 10.9, 3.2 Hz, 1 H), 2.27 (s, 0.8 H), 2.25 (s, 2.6 H), 2.18 (s, 4 H), 2.16 (d, $J = 2.1$ Hz, 12 H), 2.11 (s, 7 H), 2.07 (s, 5.5 H), 2.05 (s, 3 H), 2.00 (s, 4 H), 1.99 ppm (s, 4.5 H). ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.7$, 170.6, 170.3, 170.1, 170.0, 170.0, 169.9, 169.8, 169.6, 99.2, 97.6, 90.4, 74.7, 71.5, 69.9, 69.6, 69.5, 69.3, 69.1, 68.5, 68.2, 67.9, 66.9, 65.8, 62.4, 62.3, 60.4, 20.9, 20.9, 20.8, 20.7, 20.7 ppm. HRMS: $[M+H]^+$ calcd for $C_{40}H_{55}O_{27}$ 967.29252, found 967.29269.

Propargyl 2,4-*O*-diacetyl-3-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (18): To a solution of per-*O*-acetylated trimer **17** (29 mg, 30 μmol) in DCM (300 μL) was added a 0.6 M propargyl alcohol solution (150 μL, 90 μmol) in DCM and a 0.3 M $BF_3{\cdot}Et_2O$ solution (150 μL, 45 μmol) in DCM. The mixture was heated to 50 °C for 6 h after which the reaction mixture was cooled to RT, diluted with EtOAc, and quenched with sat. $NaHCO_3$ (aq.). EtOAc was added until the organic phase was transferred to the top phase. The organic phase was washed with sat. $NaHCO_3$ (2×), H_2O (3×), and brine (2×), dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography yielded per-*O*-acetylated propargyl trimer **18** as a white milky oil (17.6 mg, 18.3 μmol, 61% yield). $[\alpha]_d^{22} = +80.4°$ ($c = 1.0$, DCM). FTIR (neat): $\tilde{\nu} = 978.00$, 1038.64, 1136.67, 1214.43, 1368.90, 1433.77, 1741.73, 2926.85 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.35$–5.17 (m, 7 H), 5.04–4.98 (m, 3 H), 4.82 (d, $J = 1.8$ Hz, 1 H), 4.29–4.25 (m, 3 H), 4.24 (s, 1 H), 4.21 (dd, $J =$ 9.9, 3.5 Hz, 1 H), 4.16 (t, $J = 2.6$ Hz, 1 H), 4.13 (d, $J = 2.4$ Hz, 1 H), 4.11 (d, $J = 1.9$ Hz, 1 H), 4.10–4.06 (m, 1 H), 3.88 (ddd, $J = 9.7$, 6.6, 2.5 Hz, 1 H), 3.78 (dd, $J = 10.8$, 6.6 Hz, 1 H), 3.53 (dd, $J = 10.8$, 2.5 Hz, 1 H), 2.50 (t, $J = 2.4$ Hz, 1 H), 2.23 (s, 3 H), 2.16 (s, 3 H), 2.14–2.13 (m, 9 H), 2.12 (s, 3 H), 2.06 (s, 3 H), 2.05 (d, $J = 1.1$ Hz, 3 H), 1.99 (s, 3 H), 1.98 ppm (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.9$, 170.8, 170.6, 170.3, 170.2, 170.2, 170.1, 169.9, 169.74, 99.0, 97.4, 96.0, 78.1, 75.7, 74.1, 70.8, 70.2, 70.1, 69.6, 69.5, 69.2, 68.8, 68.4, 68.4, 67.0, 66.1, 66.1, 62.6, 62.5, 54.8, 21.0, 21.0, 20.9, 20.9, 20.9, 20.8, 20.8 ppm. HRMS: $[M+H]^+$ calcd for $C_{41}H_{55}O_{26}$ 963.29761, found 963.29723.

Propargyl 3-*O*-(α-D-mannopyranosyl)-6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (19): To a solution of per-*O*-acetylated propargyl trimer **18** (18.3 mg, 17.6 μmol) in MeOH (370 μL) was added a 5 mM NaOMe solution (370 μL, m1.83 μmol) in MeOH. After complete conversion the reaction was quenched with Amberlite IR-120 H^+ (pH ≤ 7). The solids were filtered and the filtrate was concentrated in vacuo. Purification by column chromatography followed by lyophilization yielded propargyl trimer **19** as a white powder (6.2 mg, 11.3 μmol, 61% yield). $[\alpha]_d^{22} = +114.0°$ ($c = 1.0$, MeOH). FTIR (neat): $\tilde{\nu} = 981.81$, 1042.80, 1131.53, 1363.00, 2490.41, 2929.08, 3285.13 cm^{-1}. ^{1}H NMR (400 MHz, MeOD): $\delta = 5.06$ (s, 1 H), 4.91 (d, $J = 1.8$ Hz, 1 H), 4.84 (d, $J = 1.8$ Hz, 1 H), 4.26 (t, $J = 2.3$ Hz, 2 H), 4.04 (dd, $J = 3.1$, 1.8 Hz, 1 H), 3.98 (dd, $J = 3.3$, 1.7 Hz, 1 H), 3.94 (dd, $J = 11.1$, 5.2 Hz, 1 H), 3.89–3.84 (m, 3 H), 3.84–3.81 (m, 2 H), 3.81–3.78 (m, 2 H), 3.78–3.65 (m, 5 H), 3.65–3.58 (m, 3 H), 2.88 ppm (t, $J = 2.5$ Hz, 1 H). ^{13}C NMR (100 MHz, MeOD): $\delta = 104.0$, 101.5, 100.2, 80.7, 79.9, 76.2, 74.9, 74.4, 73.8, 72.6, 72.4, 72.1, 72.0, 71.2, 68.7, 68.5, 67.3, 67.1, 62.8, 55.0 ppm. HRMS: $[M+H]^+$ calcd for $C_{21}H_{35}O_{16}$ 543.19196, found 543.19224.

Acetyl 2,4-*O*-benzyl-α-D-mannopyranoside (20): To a 0 °C cooled solution of NIS (0.247 g, 1.10 mmol) in DCM/AcOH (1:1) (20 mL) was added dropwise a 0.1 M solution of thiomannose **13** (10 mL, 1.0 mmol) in DCM. The reaction mixture was stirred at 0 °C for 1 h and allowed to warm to RT. After complete consumption of the starting material the reaction was quenched with 10% $Na_2S_2O_3$ (aq.) and the product was extracted with EtOAc (4×). The combined organic phases were washed with H_2O (2×), sat. $NaHCO_3$ (aq.) (3×), H_2O (3×), and brine (2×), dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography yielded α-*O*-acetyl mannose **20** as a colorless amorphous solid (0.148 g, 0.37 mmol, 40% yield). $[\alpha]_d^{22} = +14.6°$ ($c = 1.0$, DCM). FTIR (neat): $\tilde{\nu} = 1026.60$, 1071.89, 1239.98, 1366.52, 1454.28, 1496.96, 1720.48, 2930.75, 3031.24, 3420.07 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.40$–7.25 (m, 10 H), 6.20 (d, $J = 1.8$ Hz, 1 H), 4.90 (d, $J = 11.1$ Hz, 1 H), 4.76 (d, $J = 11.7$ Hz, 1 H), 4.66 (d, $J = 11.0$ Hz, 1 H), 4.59 (d, $J =$ 11.7 Hz, 1 H), 3.99 (d, $J = 7.0$ Hz, 1 H), 3.83 (dd, $J = 12.1$, 2.6 Hz, 1 H), 3.80–3.73 (m, 2 H), 3.72–3.65 (m, 2 H), 2.52 (s, 1 H), 2.32 (s, 1 H), 2.05 ppm (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 169.3$, 138.2, 137.25, 128.7, 128.7, 128.3, 128.1, 128.1, 128.0, 91.0, 77.0, 75.6, 75.2, 74.0, 73.1, 71.4, 61.8, 21.0 ppm. HRMS: $[M+H]^+$ calcd for $C_{22}H_{27}O_7$ 403.17518, found 403.17527.

Acetyl 2,4-*O*-dibenzyl-3-*O*-(2,4-*O*-dibenzyl-3-*O*-(2-*O*-acetyl-3,4,6-*O*-tribenzyl-α-D-mannopyranosyl)-6-*O*-(2-*O*-acetyl-3,4,6-*O*-tribenzyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-6-*O*-(2,4-*O*-dibenzyl-3-*O*-(2-*O*-acetyl-3,4,6-*O*-tribenzyl-α-D-mannopyranosyl)-6-*O*-(2-*O*-acetyl-3,4,6-*O*-tribenzyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-α-D-mannopyranoside (21): Trimer donor **14** (4.20 g, 3.0 mmol) and acceptor **20a** (0.402 g, 1.0 mmol) were dissolved in DCM (20 mL) and the solution was stirred over activated molecular sieves (3 Å) at RT for 30 min. NIS (0.74 g, 3.3 mmol) was added and the solution was stirred at RT. After 15 min the reaction mixture was cooled to −40 °C and TfOH (0.3 mmol, 27 μL) was added to the mixture. The reaction mixture was gradually warmed to RT and quenched with Et_3N. The mixture was filtered over celite and diluted with DCM. The organic phase was washed with 10% $Na_2S_2O_3$ (aq.) and the aqueous phase was extracted with DCM (5×). The combined organic layers were washed with H_2O (1×) and brine (1×), dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by size exclusion chromatography (DCM/MeOH, 1:1) yielded benzylated heptamer **21** as a colorless oil (1.84 g, 0.62 mmol, 62% yield). $[\alpha]_D^{22} = +43.6$ ($c = 1.0$, DCM). FTIR (neat): $\tilde{\nu} = 977.68$, 1026.77, 1051.75, 1232.44, 1368.31, 1453.93, 1496.62, 1742.78, 2868.56, 3032.00 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta =$ 7.36–7.07 (m, 90 H), 6.17 (d, $J = 1.8$ Hz, 1 H), 5.54–5.46 (m, 4 H), 5.21 (s, 3 H), 4.97 (d, $J = 1.8$ Hz, 1 H), 4.93 (s, 1 H), 4.90 (s, 1 H), 4.84 (dt, $J = 10.5$, 5.2 Hz, 5 H), 4.75–4.69 (m, 3 H), 4.64 (d, $J = 3.8$ Hz, 1 H), 4.63–4.46 (m, 12 H), 4.46–4.35 (m, 14 H), 4.34 (d, $J = 2.2$ Hz, 2 H), 4.24 (d, $J = 12.1$ Hz, 1 H), 4.15 (ddd, $J = 18.1$, 9.2, 3.0 Hz, 2 H), 4.05 (dd, $J = 9.2$, 3.2 Hz, 2 H), 3.95 (d, $J = 8.9$ Hz, 2 H), 3.93–3.82 (m, 10 H), 3.83–3.71 (m, 3 H), 3.71–3.46 (m, 15 H), 3.39 (dd, $J = 10.8$, 5.9 Hz, 2 H), 2.12 (s, 6 H), 2.06 (s, 3 H), 2.04 (s, 3 H), 2.02 ppm (s, 3 H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.3, 170.2, 170.1, 170.1, 169.0, 138.8, 138.7, 138.6, 138.5, 138.3, 138.3, 138.2, 138.2, 138.2, 138.0, 138.0, 137.9, 137.9, 137.9, 137.8, 137.2, 128.7, 128.6, 128.6, 128.5, 128.4, 128.4, 128.4, 128.3, 128.3, 128.3, 128.2, 128.1, 128.1, 128.0, 128.0, 127.9, 127.9, 127.8, 127.8, 127.8, 127.8, 127.7, 127.7, 127.6, 127.6, 127.6, 127.5, 127.5, 127.4, 127.4, 127.2, 127.1, 99.7, 98.5, 98.3, 97.2, 90.7, 78.3, 78.2, 78.0, 77.5, 77.2, 76.8, 76.4, 75.2, 75.1, 74.9, 74.9, 74.6, 74.5, 74.2, 74.1, 74.1, 73.8, 73.45, 73.4, 73.4, 72.3, 72.1, 72.0, 72.0, 71.9, 71.8, 71.8, 71.6, 71.6, 71.3, 71.0, 68.9, 68.8, 68.8, 68.6, 68.3, 68.2, 66.3, 21.2, 21.2, 21.1, 21.1, 21.1 ppm. MALDI: $[M+H]^+$ calcd for $C_{178}H_{191}O_{41}$ 2985.28889, found 2985.28898

Acetyl 2,4-*O*-diacetyl-3-*O*-(2,4-*O*-diacetyl-3-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-6-*O*-(2,4-*O*-diacetyl-3-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-α-D-mannopyranoside (23): Benzylated heptamer **21** (896 mg, 0.30 mmol) was dissolved in EtOAc/MeOH/H_2O (5:4:1) (6 mL) and the solution was purged with argon. To the solution was added cat. Pd/C (10%) and the mixture was stirred overnight at RT under H_2 (g) atmosphere. Pd/C was removed by filtration through a pad of celite, which was rinsed with methanol, and the filtrate was concentrated in vacuo. The ^{1}H NMR spectrum of the crude showed the presence of aromatic signals. The crude was taken up in MeOH/H_2O (1:1) (6 mL) and purged with argon. To the solution was added cat. Pd/C (10%) and the mixture was stirred at RT under H_2 (g) atmosphere overnight. Pd/C was removed by filtration through a pad of celite, which was rinsed with methanol, and the filtrate was concentrated in vacuo. The debenzylated intermediate was co-evaporated with pyridine (3×) and dissolved in pyridine (10 mL), and the solution was cooled to 0 °C. To the cooled solution was added Ac_2O (1 mL) dropwise and the reaction mixture was allowed to warm to RT. After complete conversion of the starting material the mixture was cooled to 0 °C and the reaction was quenched with MeOH. The reaction mixture was concentrated in vacuo and the crude was dissolved in EtOAc. The organic phase was washed with 1 M HCl (aq.) (1×), sat. $NaHCO_3$ (aq.) (2×), H_2O (3×) and brine (3×), dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography yielded peracetylated heptamer **23** as a colorless amorphous solid (635 mg, quantitative yield). FTIR (neat): $\tilde{\nu}$ = 976.88, 1038.56, 1084.01, 1138.17, 1213.21, 1369.06, 1432.60, 1742.41, 2935.07 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.98 (d, J = 1.9 Hz, 1H), 5.37–5.17 (m, 13H), 5.15 (s, 1H), 5.10–5.04 (m, 2H), 5.01 (s, 2H), 4.99 (s, 2H), 4.93 (s, 1H), 4.88–4.78 (m, 2H), 4.28 (dtd, J = 18.4, 10.1, 8.2, 4.0 Hz, 4H), 4.20–3.86 (m, 13H), 3.85–3.65 (m, 4H), 3.62–3.52 (m, 2H), 3.50 (d, J = 2.5 Hz, 1H), 2.27–1.92 ppm (m, 69H). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 170.7, 170.6, 170.6, 170.6, 170.5, 170.5, 170.4, 170.4, 170.3, 170.2, 170.0, 170.0, 169.9, 169.9, 169.8, 169.8, 169.8, 169.7, 169.7, 169.6, 169.6, 169.6, 169.5, 169.5, 168.4, 168.0, 99.6, 99.2, 99.0, 98.7, 97.4, 97.3, 90.5, 90.5, 77.5, 76.0, 75.6, 75.3, 75.2, 75.0, 74.7, 73.4, 71.4, 70.9, 70.8, 70.7, 70.6, 70.1, 70.0, 69.8, 69.8, 69.6, 69.5, 69.4, 69.4, 69.3, 69.1, 69.0, 69.0, 68.8, 68.7, 68.5, 68.5, 68.4, 67.7, 67.5, 67.5, 67.2, 66.9, 66.3, 66.0, 65.9, 65.8, 65.7, 65.6, 62.3, 62.2, 62.1, 62.0, 20.8, 20.8, 20.8, 20.8, 20.7, 20.7, 20.6, 20.6, 20.6, 20.5 ppm. MALDI: $[M+H]^+$ calcd for $C_{88}H_{119}O_{59}$ 2119.63059, found 2119.63084.

2,4-*O*-Diacetyl-3-*O*-(2,4-*O*-diacetyl-3-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-6-*O*-(2,4-*O*-diacetyl-3-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-α/β-D-mannopyranoside (24): To a 0 °C cooled solution of peracetylated heptamer **23** (0.498 g, 0.235 mmol) in DMF (2.8 mL) was added hydrazine acetate (23.3 mg, 0.259 mmol). The reaction mixture was stirred at 0 °C for 1 h and 30 min at RT. After TLC showed complete conversion the reaction was quenched with acetone and the reaction mixture was concentrated in vacuo. The crude was dissolved in Et_2O and the organic phase was washed with brine (3×), dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by column chromatography yielded 1-OH-acetylated heptamer **24** as a white amorphous solid (0.384 g, 0.185 mmol, 79% yield). FTIR (neat): $\tilde{\nu}$ = 978.58, 1038.62, 1081.66, 1137.06, 1215.09, 1369.26, 1433.38, 1741.35, 2926.28 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.37 (dd, J = 3.4, 1.6 Hz, 1H), 5.33–5.24 (m, 8H), 5.21 (dd, J = 10.4, 3.2 Hz, 2H), 5.17 (d, J = 3.0 Hz, 2H), 5.11 (t, J = 10.1 Hz, 1H), 5.07–5.02 (m, 3H), 4.99 (dd, J = 4.8, 1.8 Hz, 2H), 4.95 (s, 1H), 4.86–4.82 (m, 3H), 4.76 (d, J = 5.0 Hz, 1H), 4.37–4.22 (m, 4H), 4.18 (dd, J = 9.8, 3.4 Hz, 1H), 4.15–3.96 (m, 12H), 3.94 (ddd, J = 9.7, 5.0, 2.2 Hz, 1H), 3.86 (ddd, J = 10.2, 6.0, 2.5 Hz, 1H), 3.76 (tt, J = 7.6, 2.7 Hz, 3H), 3.55 (dd, J = 11.4, 3.2 Hz, 1H), 3.51 (dd, J = 11.4, 3.2 Hz, 1H), 3.47 (d, J = 9.5 Hz, 1H), 2.21 (s, 3H), 2.18 (s, 3H), 2.17–2.13 (m, 24H), 2.13–2.11 (m, 12H), 2.06 (s, 3H), 2.05–2.03 (m, 9H), 2.00–1.97 ppm (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.1, 170.9, 170.8, 170.8, 170.7, 170.5, 170.5, 170.5, 170.4, 170.3, 170.2, 170.2, 170.1, 170.1, 167.0, 169.9, 169.9, 169.8, 169.7, 169.7, 99.5, 99.2, 99.0, 97.9, 97.6, 97.4, 91.4, 77.5, 77.2, 76.8, 75.3, 75.0, 74.5, 72.7, 70.9, 70.8, 70.1, 70.0, 69.9, 69.7, 69.6, 69.6, 69.5, 69.4, 69.3, 69.2, 68.6, 68.5, 68.5, 68.4, 68.2, 68.1, 67.7, 67.3, 66.8, 66.7, 66.3, 66.0, 65.7, 62.4, 62.3, 62.2, 62.1, 21.2, 21.1, 21.0, 21.0, 20.9, 20.8, 20.8, 20.7 ppm. HRMS: $[M+H]^+$ calcd for $C_{86}H_{117}O_{58}$ 2077.62003, found 2077.62039.

2,4-*O*-Diacetyl-3-*O*-(2,4-*O*-diacetyl-3-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-6-*O*-(2,4-*O*-diacetyl-3-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-1-*O*-(*N*-phenyltrifluoroacetimidoyl)-α/β-D-mannopyranoside (25): To a solution of 1-OH-acetylated heptamer **24** (56.9 mg, 27.4 μmol) in acetone (274 μL) was added a 0.15 M *N*-phenyl trifluoroacetimidoyl chloride solution (274 μL, 41.1 μmol) in acetone. To the reaction mixture was added Cs_2O_3 (15.5 mg, 41.1 μmol) and the mixture was stirred at RT until TLC showed complete conversion of the starting material. The reaction mixture was concentrated in vacuo and directly purified without further workup. Purification by column chromatography yielded heptamer imidate donor **25** as a slightly yellow oil (61.6 mg, 27.3 μmol, quantitative yield). FTIR (neat): $\tilde{\nu}$ = 1040.72, 1084.61, 1138.28, 1215.66, 1370.23, 1435.52, 1674.33, 1743.57, 2854.33, 2924.27 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.28 (t, J = 7.5 Hz, 2H), 7.09 (t, J = 7.5 Hz, 1H), 6.85 (d, J = 7.7 Hz, 2H), 5.40–5.16 (m, 16H), 5.10–5.05 (m, 2H), 5.04–4.95 (m, 4H), 4.91 (s, 1H), 4.84 (d, J = 1.7 Hz, 1H), 4.81 (s, 1H), 4.36–4.22 (m, 4H), 4.22–3.88 (m, 13H), 3.87–3.79 (m, 1H), 3.79–3.70 (m, 3H), 3.60–3.46 (m, 3H), 2.25–2.01 (m, 54H), 2.01–1.95 (m, 9H), 1.92 ppm (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.8, 170.7, 170.7, 170.7, 170.5, 170.2, 170.1, 170.1, 170.1, 167, 167.0, 169.9, 169.8, 169.8, 169.7, 169.7, 169.5, 143.2, 128.9, 124.7, 119.5, 99.6, 99.4, 99.1, 97.6, 97.6, 75.5, 75.2, 71.9, 70.8, 70.8, 70.0, 69.9, 69.9, 69.8, 69.6, 69.5, 69.5, 69.4, 69.1, 68.7, 68.7, 68.6, 68.6, 67.8, 67.4, 66.8, 66.4, 66.1, 66.0, 65.9, 65.7, 62.5, 62.2, 62.1, 29.8, 21.0, 20.9, 20.9, 20.8, 20.8, 20.7, 20.7, 20.6 ppm.

Propargyl 2,4-*O*-diacetyl-3-*O*-(2,4-*O*-diacetyl-3-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl)-6-*O*-(2,4-*O*-diacetyl-3-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4,6-*O*-tetraacetyl-α-D-mannopyranosyl)-α-d-mannopyranosyl)-α-D-manno-

pyranoside (26): Heptamer imidate donor **25** (43.9 mg, 19.5 μmol) was dissolved in a 0.274 M propargyl alcohol solution (356 μL, 97.5 μmol) in DCM and the mixture was stirred over activated molecular sieves (3 Å) for 30 min at RT. Then the mixture was cooled to −40 °C and to the cooled mixture was added a 0.11 M TfOH solution (36 μL, 3.9 μmol) in DCM and the reaction mixture was gradually warmed to 0 °C. The reaction was quenched with a 0.1 M Et_3N solution (0.1 mL) in DCM and the solution was filtered over celite and concentrated in vacuo. Purification by column chromatography yielded peracetylated propargyl mannose heptamer **25** as a white milky oil (16.5 mg, 7.8 μmol, 40% yield). FTIR (neat): $\tilde{\nu}$ = 1037.06, 1136.93, 1214.09, 1369.36, 1433.95, 1740.68, 2926.41 cm^{-1}. ^1H NMR (600 MHz, $CDCl_3$): δ = 5.35 (dd, J = 9.9, 3.5 Hz, 1H), 5.33–5.32 (m, 1H), 5.29–5.22 (m, 7H), 5.21 (dd, J = 10.0, 3.4 Hz, 2H), 5.19–5.16 (m, 1H), 5.07 (dd, J = 3.0, 1.9 Hz, 1H), 5.05 (dd, J = 3.4, 1.8 Hz, 1H), 5.00–4.97 (m, 3H), 4.97–4.94 (m, 3H), 4.92–4.89 (m, 1H), 4.85 (d, J = 1.8 Hz, 1H), 4.84 (d, J = 1.9 Hz, 1H), 4.32–4.23 (m, 6H), 4.17 (td, J = 10.0, 3.4 Hz, 2H), 4.14–4.09 (m, 2H), 4.08 (s, 1H), 4.07–3.99 (m, 4H), 3.97 (dd, J = 9.9, 3.4 Hz, 2H), 3.97–3.88 (m, 3H), 3.87 (ddd, J = 10.3, 5.2, 2.7 Hz, 1H), 3.75 (dq, J = 10.8, 5.5 Hz, 3H), 3.71 (d, J = 5.6 Hz, 1H), 3.56 (dd, J = 11.4, 2.2 Hz, 1H), 3.53 (d, J = 2.6 Hz, 1H), 3.52 (d, J = 2.6 Hz, 1H), 2.52 (t, J = 2.4 Hz, 1H), 2.21 (s, 3H), 2.19 (s, 3H), 2.17 (s, 3H), 2.16–2.13 (m, 21H), 2.12–2.11 (m, 12H), 2.06 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.04 (s, 3H), 1.98 (s, 3H), 1.98 (s, 3H), 1.97 ppm (d, J = 1.3 Hz, 6H). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 171.1, 171.0, 170.9, 170.8, 170.8, 170.8, 170.8, 170.8, 170.6, 170.6, 170.6, 170.5, 170.4, 170.3, 170.3, 170.2, 170.2, 170.2, 170.2, 170.1, 170.1, 170.1, 170.0, 170.0, 170.0, 170.0, 169.9, 169.9, 169.8, 169.8, 169.8, 169.7, 99.4, 99.2, 97.8, 97.7, 97.3, 96.6, 78.8, 75.6, 75.5, 75.3, 75.0, 71.2, 71.0, 70.9, 70.3, 70.0, 70.0, 69.8, 69.6, 69.6, 69.5, 69.5, 69.5, 69.3, 69.2, 68.8, 68.7, 68.6, 68.6, 68.0, 67.8, 67.6, 66.8, 66.8, 66.4, 66.1, 66.1, 66.0, 66.0, 65.8, 62.5, 62.3, 62.2, 55.0, 21.0, 21.0, 21.0, 21.0, 20.9, 20.9, 20.9, 20.9, 20.8, 20.8, 20.8, 20.8 ppm. HRMS: $[M+H]^+$ calcd for $C_{89}H_{119}O_{58}$ 2115.63568, found 2115.63581.

Propargyl 3-*O*-(3-*O*-(α-D-mannopyranosyl)-6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranosyl)-6-*O*-(3-*O*-(α-D-mannopyranosyl)-6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranosyl)-α-D-mannopyranoside (27): To a solution of per-*O*-acetylated propargyl mannose heptamer **26** (16.5 mg, 7.8 μmol) in MeOH (156 μL) was added a 125 mM NaOMe (156 μL, 3.9 μmol) solution in MeOH and the reaction was stirred overnight at RT. TLC-MS analysis showed incomplete deacetylation of the starting material and the reaction was quenched with Amberlite IR-120 H^+ (pH ≤ 7). The crude was taken up in H_2O (0.5 mL) and a 0.2 M NaOH (aq.) (0.5 mL) solution was added to the solution. The reaction was followed on TLC-MS and after completion the reaction was quenched with Amberlite IR-120 H^+ (pH ≤ 7). Solids were filtered and the filtrate was concentrated in vacuo. The product was lyophilized from H_2O without further purification yielding propargyl mannose heptamer **27** as a white powder (9.3 mg, 7.8 μmol, quantitative yield). ^1H NMR (600 MHz, D_2O): δ = 5.17 (d, J = 1.8 Hz, 1H), 5.14 (d, J = 1.8 Hz, 1H), 5.07 (d, J = 1.8 Hz, 1H), 5.02 (d, J = 1.8 Hz, 1H), 4.92 (d, J = 1.8 Hz, 1H), 4.91 (d, J = 1.8 Hz, 1H), 4.88 (d, J = 1.9 Hz, 1H), δ 4.36 (s, 2H), 4.26 (dd, J = 3.3, 1.8 Hz, 1H), 4.16 (dd, J = 3.3, 1.8 Hz, 1H), 4.11 (dd, J = 3.2, 1.7 Hz, 1H), 4.08 (dt, J = 3.6, 1.9 Hz, 2H), 4.03 (dd, J = 9.6, 3.5 Hz, 1H), 4.01–3.97 (m, 5H), 3.96–3.92 (m, 2H), 3.92–3.87 (m, 11H), 3.87–3.83 (m, 2H), 3.80–3.72 (m, 8H), 3.70 (ddd, J = 12.6, 7.3, 2.1 Hz, 4H), 3.67 (s, 2H), 3.65 ppm (s, 1H). ^{13}C NMR (150 MHz, D_2O): δ = 103.8, 103.7, 103.5, 100.8, 100.7, 100.6, 100.3, 80.1, 79.7, 79.7, 74.70, 74.6, 74.0, 73.9, 73.1, 72.7, 72.2, 71.9, 71.9, 71.7, 71.4, 71.3, 71.3, 71.2, 70.9, 70.9, 70.8, 68.1, 68.1, 68.0, 67.1, 67.1, 66.8, 66.7, 66.6, 66.4, 62.4, 62.2, 56.2 ppm. HRMS: $[M+H]^+$ calcd for $C_{45}H_{75}O_{36}$ 1191.40325, found 1191.40308.

Man_1-BODIPY-DCG-04 (2): To a solution of propargyl mannose **7** (1.75 mg, 8 μmol) and BODIPY-DCG-04 **(5)** (8.6 mg, 7.6 μmol) in DMF/H_2O (1:1) (3 mL) was added 0.1 M sodium ascorbate (aq.) (160 μL, 16 μmol) and 0.1 M $CuSO_4$ (aq.) (16 μL, 1.6 μmol). The resulting mixture was stirred for 2 h at room temperature, before being concentrated and co-evaporated with toluene. Purification by HPLC-MS (A: 25 mM NH_4OAc, B: linear gradient 20→35% acetonitrile (ACN) in 12 min) followed by lyophilization from H_2O yielded Man_1-BODIPY-DCG-04 (**2**) (4.3 mg, 3.2 μmol, 42% yield). ^1H NMR (600 MHz, $CDCl_3$/MeOD): δ = 8.02 (s, 1H), 7.92 (t, J = 5.6 Hz, 1H), 7.86 (d, J = 7.5 Hz, 2H), 7.83 (d, J = 7.6 Hz, 1H), 7.76 (t, J = 5.6 Hz, 1H), 7.40 (s, 1H), 7.06 (d, J = 4.1 Hz, 1H), 7.01 (d, J = 8.5 Hz, 2H), 6.95 (d, J = 8.9 Hz, 2H,), 6.69 (d, J = 8.5 Hz, 2H, 2), 6.59 (d, J = 4.1 Hz, 1H), 4.80 (d, J = 12.4 Hz, 1H), 4.67–4.60 (m, 3H, CH_2), 4.45 (t, J = 7.5 Hz, 1H), 4.40–4.36 (m, 1H), 4.30–4.19 (m, 3H), 4.08 (t, J = 5.8 Hz, 2H), 3.85 (dd, J = 11.8, 2.1 Hz, 1H), 3.82–3.77 (m, 1H), 3.74–3.65 (m, 3H), 3.64–3.55 (m, 3H), 3.15–3.11 (m, 3H), 3.06–3.01 (m, 1H), 2.99–2.93 (m, 1H), 2.86–2.80 (m, 1H), 2.75 (t, J = 7.4 Hz, 2H), 2.50 (s, 3H), 2.42 (p, J = 6.5 Hz, 2H), 2.33 (t, J = 7.3 Hz, 2H), 2.25 (s, 3H), 2.21–2.16 (m, 2H), 1.78–1.69 (m, 1H), 1.62–1.48 (m, 6H), 1.46–1.41 (m, 2H), 1.41–1.35 (m, 2H), 1.31–1.29 (m, 5H), 1.22–1.15 (m, 2H), 0.92 (d, J = 6.4 Hz, 3H), 0.88 ppm (d, J = 6.4 Hz, 3H,). ^{13}C NMR (150 MHz, $CDCl_3$/MeOD): δ = 177.02, 176.02, 174.68, 173.56, 172.96, 168.66, 168.31, 160.68, 160.59, 157.16, 156.37, 145.22, 141.69, 136.49, 135.67, 131.84, 131.81, 131.78, 131.68, 131.30, 129.27, 128.81, 127.14, 125.60, 124.63, 119.12, 116.19, 115.14, 100.70, 74.84, 72.42, 71.93, 68.54, 65.67, 63.19, 62.90, 60.64, 56.36, 54.34, 54.11, 53.33, 53.14, 48.49, 41.56, 40.15, 40.12, 38.14, 36.86, 36.58, 32.64, 30.93, 29.87, 29.74, 27.32, 26.39, 25.80, 24.17, 23.29, 22.02, 21.29, 14.35, 13.28, 9.62 ppm. HRMS: $[M+H]^+$ calcd for $C_{65}H_{89}BF_2N_{11}O_{17}$ 1344.64935, found 1344.65139.

Man_3-BODIPY-DCG-04 (3): To a solution of propargyl mannoside **19** (2.4 mg, 4.5 μmol) and BODIPY-DCG-04 **(5)** (5.1 mg, 4.5 μmol) in DMF/H_2O (1:1) (2 mL) was added 0.1 M sodium ascorbate (aq.) (90 μL, 9 μmol) and 0.1 M $CuSO_4$ (aq.) (2.2 μL, 0.22 μmol). The resulting mixture was stirred for 1 h at room temperature, before being concentrated and co-evaporated with toluene. Purification by HPLC-MS (A: 25 mM NH_4OAc, B: linear gradient 20→35% ACN in 12 min) followed by lyophilization from H_2O yielded Man_3-BODIPY-DCG-04 (**3**) (1.8 mg, 1.1 μmol, 24%) ^1H NMR (600 MHz, MeOD): δ = 8.08 (s, 1H), 7.88 (d, J = 8.9 Hz, 2H,), 7.43 (s, 1H), 7.07 (d, J = 4.0 Hz, 1H), 7.01 (d, J = 8.5 Hz, 2H), 6.97 (d, J = 8.9 Hz, 2H), 6.69 (d, J = 8.5 Hz, 2H), 6.62 (d, J = 4.1 Hz, 1H), 5.05 (s, 1H), 4.85–4.77 (m, 3H), 4.69–4.61 (m, 3H), 4.45 (t, J = 7.6 Hz, 1H), 4.38 (dd, J = 9.3, 5.7 Hz, 1H), 4.29–4.23 (m, 3H), 4.09 (t, J = 5.9 Hz, 2H), 4.05 (d, J = 2.5 Hz, 1H), 3.98–3.90 (m, 2H), 3.88–3.54 (m, 17H), 3.16–3.09 (m, 3H), 3.07–2.99 (m, 1H), 2.99–2.93 (m, 1H), 2.86–2.80 (m, 1H), 2.76 (t, J = 7.3 Hz, 2H), 2.51 (s, 3H), 2.42 (p, J = 6.5 Hz, 2H), 2.33 (t, J = 7.3 Hz, 2H), 2.26 (s, 3H), 2.20–2.14 (m, 2H), 1.77–1.70 (m, 1H), 1.64–1.47 (m, 6H), 1.47–1.41 (m, 2H), 1.41–1.35 (m, 2H), 1.32–1.29 (m, 5H), 1.20–1.15 (m, 2H), 0.92 (d, J = 6.4 Hz, 3H), 0.88 ppm (d, J = 6.4 Hz, 3H). ^{13}C NMR (150 MHz, MeOD): δ = 177.14, 176.13, 174.79, 173.69, 173.08, 168.74, 168.43, 165.61, 163.01, 160.81, 160.67, 157.29, 156.48, 150.29, 145.18, 142.19, 136.59, 135.75, 131.90, 131.77, 131.36, 129.35, 128.91, 127.23, 125.71, 124.73, 119.16, 116.24, 115.22, 103.97, 101.33, 100.79, 80.67, 74.96, 74.39, 73.70, 72.66, 72.46, 72.10, 71.27, 68.80, 68.64, 67.48, 67.14, 65.75, 63.23, 62.91, 60.71, 56.47, 54.38, 54.21, 53.42, 53.19, 41.62, 40.20, 38.19, 36.91, 36.63, 32.71, 31.05, 29.95, 29.82, 27.40, 26.48, 25.88, 24.25, 23.30,

22.02, 21.34, 14.35, 9.59. LC/MS analysis (linear gradient 10% → 90% ACN) t_R: 6.53 min, ESI-MS (*m/z*): $[M+H]+$: 1668.40.

Man_7-BDP-DCG-04 (4): To a solution of propargyl mannoside **27** (4 mg, 3.4 μmol) and BODIPY-DCG-04 **(5)** (3.8 mg, 3.4 μmol) in DMF/H_2O (1:1) (2 mL) was added 0.1 M sodium ascorbate (aq.) (68 μL, 6.8 μmol) and 0.1 M $CuSO_4$ (aq.) (6.8 μL, 0.68 μmol). The resulting mixture was stirred for 8 h at room temperature, before being concentrated and co-evaporated with toluene. Purification by HPLC-MS (A: 25 mM NH_4OAc, B: linear gradient 20→35% ACN in 12 min) followed by lyophilization from H_2O yielded Man_7-BDP-DCG-04 (**4**) (2.5 mg, 1.1 μmol, 32%). ^1H NMR (600 MHz, MeOD): δ = 8.28 (s, 1H), 8.09 (d, J = 8.1 Hz, 1H), 7.95 (s, 1H), 7.90–7.85 (m, 2H), 7.79 (t, J = 5.6 Hz, 1H), 7.42 (s, 1H), 7.07 (d, J = 4.1 Hz, 1H), 7.01 (d, J = 8.5 Hz, 2H), 6.98 (d, J = 8.9 Hz, 2H), 6.69 (d, J = 8.5 Hz, 2H), 6.62 (d, J = 4.1 Hz, 1H), 5.11 (s, 1H), 5.07 (s, 1H), 4.99 (s, 1H), 4.81–4.64 (m, 8H), 4.50–4.43 (m, 1H), 4.41–4.36 (m, 1H), 4.31–4.23 (m, 3H), 4.19 (d, J = 2.8 Hz, 1H), 4.12–4.07 (m, 4H), 4.03–3.53 (m, 41H), 3.16–3.10 (m, 3H), 3.07–3.02 (m, 1H), 3.00–2.94 (m, 1H), 2.88–2.80 (m, 1H), 2.76 (t, J = 7.3 Hz, 2H), 2.51 (s, 3H), 2.44 (q, J = 6.4 Hz, 2H), 2.34 (t, J = 7.2 Hz, 2H), 2.26 (s, 3H), 2.21–2.17 (m, 2H), 1.78–1.71 (m, 1H), 1.66–1.47 (m, 6H), 1.47–1.41 (m, 2H), 1.41–1.36 (m, 2H), 1.33–1.28 (m, 5H), 1.23–1.17 (m, 2H), 0.92 (d, J = 6.4 Hz, 3H), 0.88 ppm (d, J = 6.4 Hz, 3H). HRMS: $[M+H]^+$ calcd. for $C_{101}H_{149}BF_2N_{11}O_{47}$ 2317.96965, found 2317.97256.

Cell culture of primary cells: Immature dendritic cells were obtained from the bone marrow of C75BL/6 mice and were a gift from the Biopharmaceutical Department (Leiden University). The use of animals was approved by the ethics committee of Leiden University. Mice were sedated; bone marrow of tibiae and femurs was flushed out and washed with PBS. Cells were grown in dendritic cell selection medium (IMDM containing granulocyte-macrophage colony stimulating factor (GM-CSF) (2:1 v/v) containing 8% FCS, penicillin/streptomycin (100 units/mL), glutamax (2 mM), and β-mercaptoethanol (20 μM). Cells were selected for 10 days (37 °C; 5% CO_2) and subcultured every 2–3 days before use in the assays.

Labeling of cathepsins in mouse liver and immature dendritic cell lysate: Lysates (8–10 μg total protein, determined on a Qubit 2.0 fluorometer, Life Technologies-Invitrogen) in 50 mM sodium citrate pH 5.5 or pH 7 (as indicated), 5 mM DTT, 0.2% CHAPS, and 0.1% Triton X-100 were incubated with the indicated concentration of probe (total volume: 10 μL) for 1 h at 37 °C. For competition experiments, lysates were first incubated with N_3-DCG-04 (1 or 10 μM), AS44 (10 μM) or BODIPY(FL)-DCG-04 (1 or 10 μM) for 1 h, 37 °C, before addition of the probe and incubation was continued for 1 h. After treatment, 5x Laemli's sample buffer (including β-mercaptoethanol) was added and the samples were boiled (100 °C, 5 min) and resolved on 12.5% SDS-PAGE. Gels were scanned on a Typhoon 2000 imager (GE Healthcare) using the Cy2 (λ_{ex} = 532 nm; λ_{em} = 526 nm) and Cy3 (λ_{ex} = 532 nm; λ_{em} = 580 nm) settings. Total protein loading was determined by staining with Coomassie brilliant blue and subsequent scanning on a BioRad GS800 calibrated densitometer. Image processing was done with ImageJ, representative gels from at least three independent experiments are shown.

Labeling of cathepsins in live immature mouse dendritic cells: Cells were seeded onto tissue-culture coated 24-well plates (200.000 cells per well, 250 μL medium) and allowed to attach for 2 h (37 °C; 5% CO_2), before addition of inhibitor or probe to the medium. Pre-incubations with N_3-DCG-04 (20 μM) or mannan (3 mg mL^{-1}) were conducted for 1 h, followed by addition of compound **2**, **3**, or **4** (1 μM) and incubation was continued for 2 h. For direct labeling experiments, cells were cultured for 2 h (37 °C; 5% CO_2) in the presence of probes **2**, **3**, or **4** (0.1, 0.25, 0.5, 0.75 or 1 μM). After incubation, cells were washed with PBS (2×), lysed (35 μL Invitrogen complete cell extraction buffer), and proteins resolved on 12.5% SDS-PAGE, followed by fluorescence scanning (Cy3 settings) and CBB staining. Image processing was done with ImageJ, representative gels from at least three independent experiments are shown.

Confocal fluorescence microscopy: Experiments were conducted on a Leica TCS SPE confocal microscope, using dsRed filter settings for BODIPY (λ_{ex} = 532 nm) and Cy5 settings for Draq5 (λ_{ex} = 635 nm). Cells (30–75×10^4 cells per well) were seeded onto sterile Labtek II 4- or 8-chamber borosilicate cover glass systems (Fisher Emergo). Dendritic cells were allowed to attach for 2 h before pre-incubation with mannan (3 mg mL^{-1}) (1 h, 37 °C, 5% CO_2) and subsequent probe incubation (1 μM, 2 h). Cells were then thoroughly washed (PBS), fixed (4% formaldehyde in PBS), washed again with PBS, nuclei stained with Draq5 (Thermo Scientific), and imaged. All experiments were performed at least in duplicate.

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