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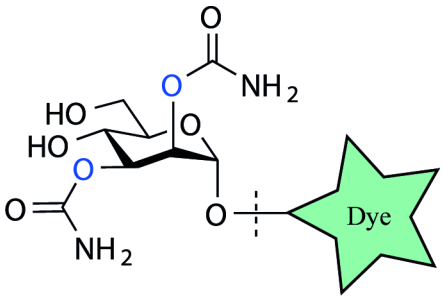


Dicarbamoyl conjugates
Cy5^{GE}, Alexa 647, NBD
Uptake studied by FACS

Synthesis and Cellular Uptake of Carbamoylated Mannose Derivatives

Hidde Elferink[a]‡, Kim Geurts[a]‡, Stijn Jue[a], Somhairle MacCormick[b], Gerrit Veeneman[b], Thomas J. Boltje[a]*

[a] Radboud University, Institute for Molecules and Materials, Heyendaalseweg 135, 6525 AJ, Nijmegen, The Netherlands.

[b] PharmaCytics B.V. Noviotech Campus, Transistorweg 7-N, 6534 AT, Nijmegen, The Netherlands.

* Corresponding author, e-mail: t.boltje@science.ru.nl

‡These authors contributed equally

ABSTRACT: A series of 3-carbamoyl- and 2,3-dicarbamoyl-mannose derivatives were synthesized, conjugated to a fluorescent dye ($Cy5^{GE}$, AF 647 or NBD) and their cellular uptake in A549 and THP-1 cell lines was studied by FACS. In contrast to earlier studies on carbamoyl mannosides, the observed uptake was not related to carbamoyl group on the mannose residue but rather to the cyanine dye attached, a trend previously observed for Cy5-fructose conjugates. The NBD-conjugates however, showed a temperature and concentration dependent uptake in case of mannose conjugates. These results suggest a profound impact of the dye which should be taken into consideration when studying the uptake of small molecules by dye conjugation.

Keywords: Glycoconjugates, Fluorescence Activated Cell Sorting, Cancer Targeting, Bleomycin

1. INTRODUCTION:

The selective uptake of diagnostics and cytostatics in cancerous tissue is one of the main challenges in cancer diagnosis and therapy, respectively. A number of cancers can be targeted by utilizing their aberrant carbohydrate uptake and metabolism e.g. overexpressing of glucose transporter GLUT-1[1] and the enzymes involved in the anaerobe glycolysis related to the Warburg effect.[2-4] For example, positron emission tomography (PET) imaging using 2-deoxy-2-^{18}fluoro-D-glucose (FDG)[5] is frequently used to diagnose cancers that display a heightened glucose metabolism. In addition, it was discovered that the carbohydrate portion of bleomycin, a natural product used to treat various cancers, enables its selective uptake in cancerous cells.[6] The carbohydrate portion of bleomycin is an unusual disaccharide consisting of 3-carbamoyl-mannose (1→3)-α-linked to L-gulose (Figure 1).

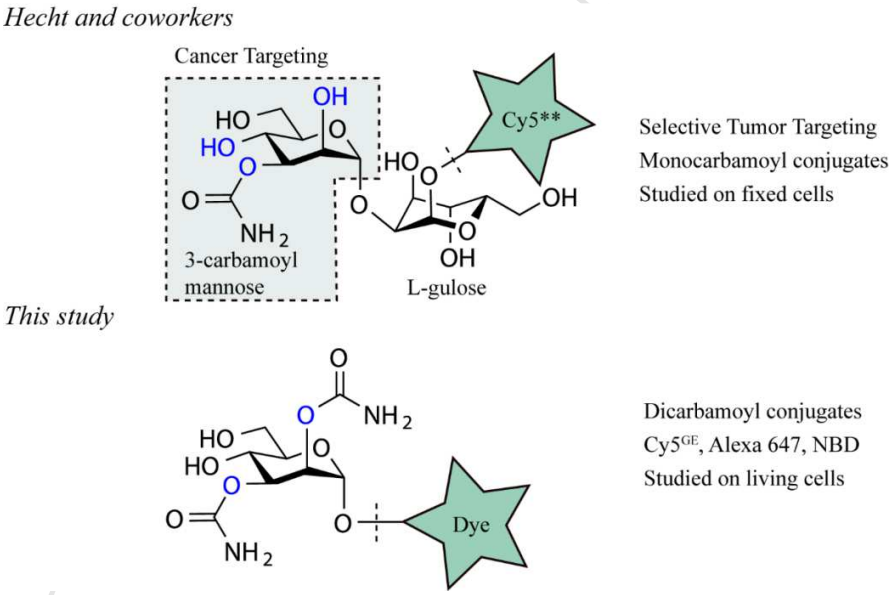


Figure 1: Studies on the selective uptake of 3-carbamoyl mannoside conjugates. Hecht and coworkers (above) showed selective uptake of Cy5^{GE}-coupled monocarbamoyl mono- and disaccharides in cancerous cells using a fluorescent microscopy-based assay. This work explores the synthesis and cellular uptake of dicarbamoyl monosaccharides using fluorescence activated cell sorting (FACS) in live cells.

Hecht and co-workers showed that microbubbles coated with bleomycin where adherent to MCF-7 human breast carcinoma cells whilst microbubbles coated with bleomycin lacking the disaccharide were not.[7] Critically, non-cancerous MCF-10A breast cells were not affected. Subsequent studies showed that the

disaccharide portion itself was sufficient to enable selective uptake using disaccharides conjugated to fluorescent dyes to assay cellular uptake.[8] Cellular uptake of the disaccharide conjugates was temperature dependent and much higher at 37^{o}C compared to 4^{o}C indicating ATP-dependent uptake. Furthermore, the induction of increased glycolysis flux in healthy cells using mitochondrial complex 1 inhibitor rotenone[9] led to enhanced uptake. Conversely, inhibition of glucose transporter GLUT1 with cytochalasin B and phloretin led to a decrease in uptake hinting at the involvement of GLUT transporters. A later study with six different cancerous cell lines showed that the minimal epitope for the targeting and uptake in cancer cells is the 3-carbamoyl mannose residue.[10] When the 3-carbamoyl group was removed, the cellular uptake was significantly lower indicating that the carbamoyl group is crucial for the recognition and internalization.[10] Finally the position and substitution (Figure 1, highlighted in blue) of the carbamoyl functionality in the bleomycin disaccharides was explored.[11] From these studies it became clear that *N*-methyl carbamoyl group at the 2- or 3-position of mannose showed even greater uptake than the parent 3-carbamoyl mannose.

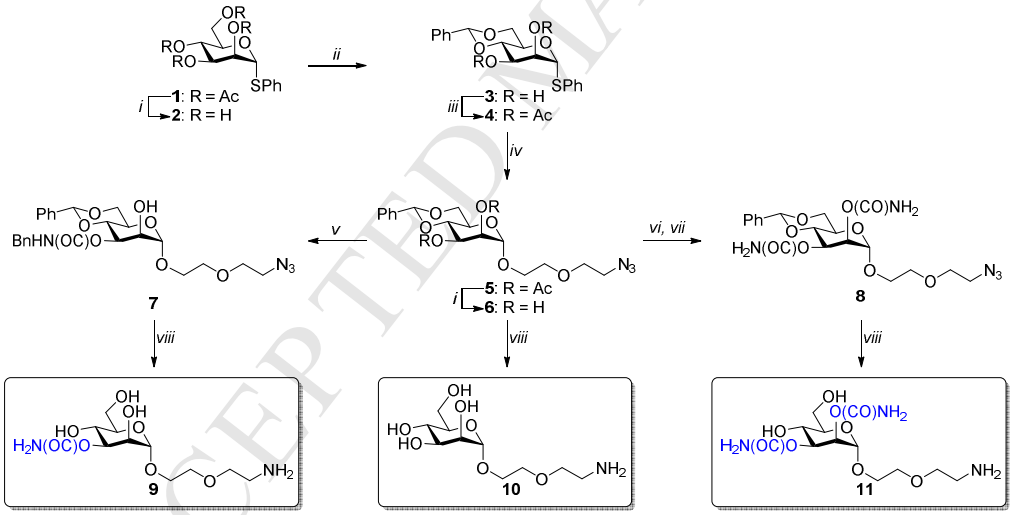
Intrigued by these results we set out to investigate the uptake of a 2,3-dicarbamoyl mannose reasoning that there may be a synergistic effect when installing two carbamoyl groups at once. In addition, as determination of the uptake by confocal microscopy on fixed cells is laborious and does not allow for high throughput screening, we opted for a more convenient method on living cells. Herein we report the synthesis of 3-carbamoyl- and 2,3-dicarbamoyl-mannose derivatives and their conjugation to three different fluorescent dyes. Cellular uptake was assayed by fluorescence activated cell sorting (FACS).

2. RESULTS and DISCUSSION:

2.1 *Chemical Synthesis*

To prepare carbamoylated mannose derivatives **9**-**10** in a divergent manner from known thioglycoside **1** we developed the synthetic route shown in Scheme 1. Deacetylation of **1** followed by installation of a 4,6-

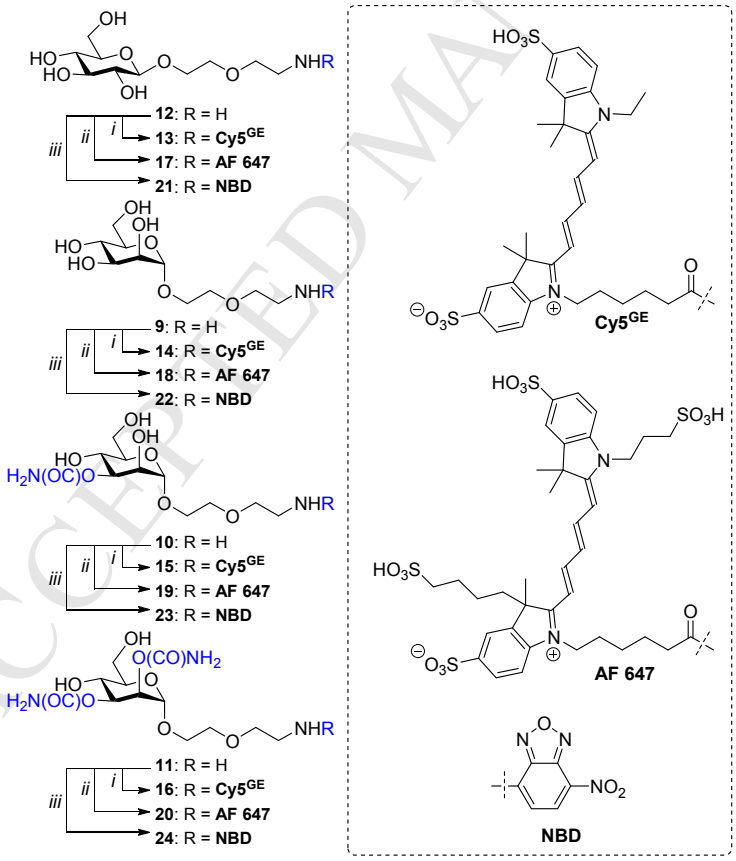
benzylidene afforded **3** in 48% yield after crystallization. Acetylation of the 2,3-diol was carried out to ensure α-selectivity in the ensuing glycosylation. Glycosylation of 2-(2-azidoethoxy)-ethanol using **4**[12] and the NIS/TfOH promoter system afforded mannoside **5** in 64%.[13] Subsequent deacetylation afforded 2,3-diol **6** which was used to prepare carbamoylated derivatives **7**-**8**. An unmodified mannose derivative **10**[14] used for control experiments was prepared first by global deprotection of **6** using Pd/C and H_2. 3-carbamoyl-mannose derivative **7** was prepared by a regioselective reaction with benzylisocyanate. Removal of the protecting groups was achieved using hydrogenolysis (Pd/C, H_2) to afford 3-carbamoyl-mannose derivative **9**[15]. 2,3-dicarbamoylation using benzylisocyanate proved more difficult to achieve. Fortunately, using trichloroacetyl (TCA) isocyanate dicarbomoylation was possible and after removal of the TCA-groups using K_2CO_3 and MeOH **8** was obtained. Hydrogenolysis (Pd/C, H_2) of **8** finally afforded deprotected compound **11**.



Scheme 1: Synthesis of mannoside conjugates **9**-**11**: *i)* MeOH, K_2CO_3; **2**, 99%; **6**, quant.; *ii)* $PhCH(OMe)_2$, $HBF_4.Et_2O$, DMF; **3**, 48%; *iii)* pyridine, Ac_2O; **4**[12], 92%; *iv*) $HOC_2H_4OC_2H_4N_3$, NIS, TfOH, DCM; **5**, 64%; *v*) DMF, benzyl isocyanate; **7**, 23% *vi*) N,N-diisopropylethylamine, trichloroacetyl isocyanate, DCM *vii*) MeOH, K_2CO_3; **8**, 32% over two steps; *viii*) H_2O, tert-butanol, EtOAc, Pd/C, H_2; **9**, 35%; **10**, 39%; **11**, 42%;.

With compounds **9**-**11** in hand we prepared conjugates with a fluorescent label to assay their cellular uptake (see scheme 2). To this end, we selected three fluorescent dyes to investigate the influence of the

dye structure on the uptake. In addition to mannose derivative **10** we also prepared glucose derivative **12**[16] (see supporting information, S8-S9) as a control for the cell experiments. Although most uptake studies involving 3-carbamoyl mannosides published used the Cy5** dye (structure presented supporting information, S10)[17], we switched to more available dyes since Cy5** has a restricted availability. Fluorescent dyes $Cy5^{GE}$ and Alexa Fluor 647 (AF 647), also used in related experiments by Bhattacharya *et al.*[15], were conjugated to amines **9**-**12** using their corresponding *N*-hydroxysuccinimide esters. HPLC purification afforded pure conjugates used for cellular uptake studies. The nitrobenzoxadiazole (NDB)[18] dye is also frequently used to track the cellular uptake[19, 20] and hence we prepared conjugates of this dye as well by reacting 4-chloro-7-nitrobenzofurazan with amines **9**-**12** to afford conjugates **20**-**24**.



Scheme 2: Dye conjugations to carbohydrate conjugates **9**-**12**: *i*) $Cy5^{GE}$-OSu, 0.2M aq. phosphate buffer; **13**, 79%; **14**, 62%; **15**, 32%; **16**, 26%; *ii*) AF 647-OSu, 0.2M aq. phosphate buffer; **17**, 55%; **18**; 69%; **19**, 48%; **20**, 51%; *iii*) NBD-Cl, 0.3M aq. $NaHCO_3$; **21**, 15%; **22**, 25%; **23**, 7%; **24**, 10%.

2.2 Biological Assays

Next, we investigated the cellular uptake of **13**-**24** in two cancer cell lines (figure 1). We selected two cancer cell-lines (A549 and THP-1), derived from lung cancer and acute monocytic leukemia patients, respectively (see supporting information for specific culture conditions). The concentration of conjugates **13**-**24** was set at 2μM after FACS signal optimization. Cells were incubated for 1 hour at 37^{o}C before being rinsed with PBS twice and released from the surface by trypsin containing buffer (A549) or mechanical stress (THP-1). Phosphate buffered albumin (PBA, 1% BSA, 0.02% NaN_3 in PBS) was added followed by propidium iodide (PI) staining (0.1% PI in PBA) to assess cell viability. Cellular uptake was assayed using FACS based on at least 5000 live cells per measurement. To ensure cell viability during the FACS experiments, cells were stained with trypan blue after two hours incubation in buffer. No significant cell death was observed. In addition, the cells were isolated, resolved in medium and showed adherence and growth to normal speed.

The uptake of the conjugates showed a striking difference depending on the fluorescent group (figure 2, **A**-**E**). Conjugates containing $Cy5^{GE}$ (figure 2, **A**-**C**) and AF 647 (figure, **D**-**E**) showed a high uptake regardless of the sugar attached. Internalization of the cyanine-dyes was confirmed by confocal microscopy studies on conjugate **12**-**16** (see supporting information), which showed a non-even distribution throughout the cells, suggesting lysosomal uptake. To assess whether the internalization is largely mediated by an active process or passive diffusion, the uptake was studied at low temperature. The uptake of all conjugates was lowered by incubation at 4^{o}C, most pronounced in the AF 647 conjugates (**17**-**20**), hinting at a specific form of uptake. In contrast, previous studies with the monosaccharide 3-carbamoyl mannose-Cy5** conjugate showed temperature independent uptake[15], suggesting a different mechanism of internalization. Critically, the expected difference in uptake between 3-carbamoyl mannose *vs* mannose itself was not observed and both showed comparable uptake. Additionally, the glucose control as well as the 2,3-dicarbamoyl mannoside showed equal levels of cellular uptake. Uptake of conjugates **13**-**20** therefore seem to stem largely from the fluorescent label and not the sugar attached.

These results are further emphasized by additional studies with linker-conjugates containing only the dye and the linker but lacking a monosaccharide which also give significant signal in both $Cy5^{GE}$ and AF 647 (see supporting information, fig. S1). Gambhir and coworkers observed a similar effect in Cy5.5 labelled fructose for uptake mediated by the human D-fructose transporter (GLUT5). In their case a GLUT5 independent uptake was observed with large fluorophores as Cy5.5.[19] The NBD conjugates **21-24** in comparison show a much lower signal with most conjugates showing the same signal as the blank control. Only mannoside conjugate **22** showed modest increased fluorescence levels, suggesting uptake.

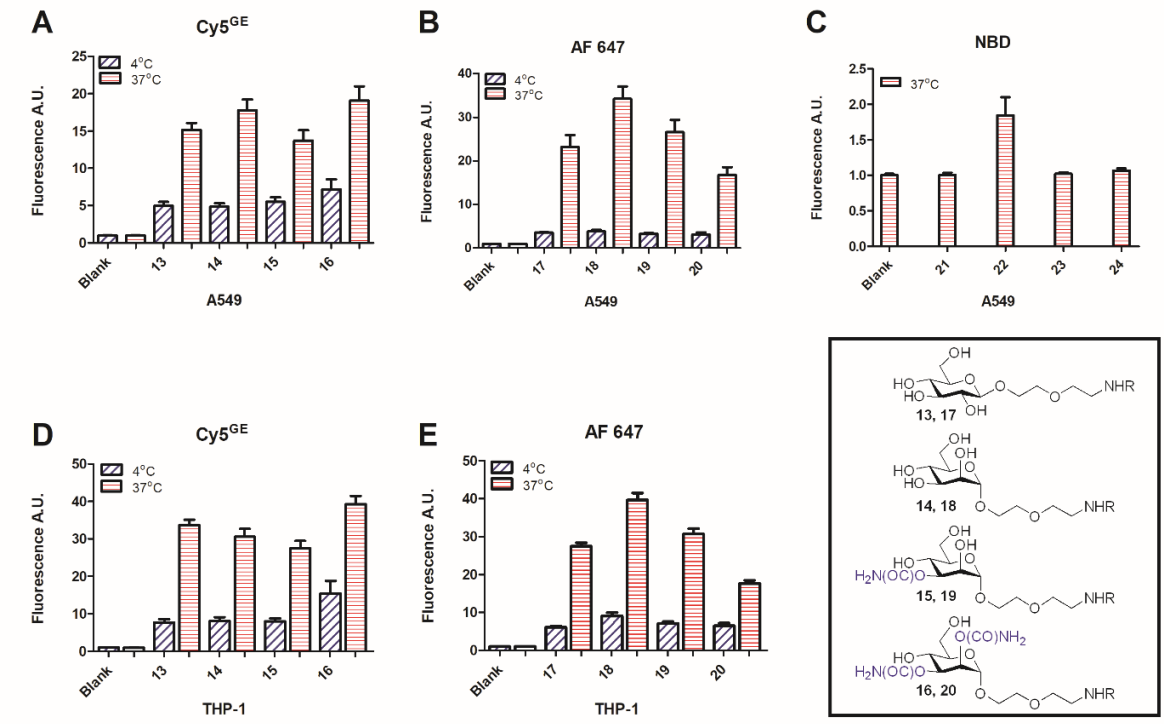


Figure 2: Cellular uptake of fluorescent glycosyl conjugates **14-24** in A549 (**A-C**) and THP-1 (**D-E)** cancer cell lines. Cells were incubated at warm (37°C) or cold (4°C) temperature (final concentration in all temperature experiments was set at 2µM). Fluorescence was normalized according to the blank (H_2O).

In general, the NDB dye (figure 2, **C**) is less bright than dyes $Cy5^{GE}$ and AF 647 and hence provided only a weak signal at 2µM concentration. We therefore performed experiments at increasing concentrations of the NDB conjugates (figure 3, **A**). Whilst the carbamoylated mannosides **23-24** and glucose conjugate **21** showed no increase in uptake upon increasing their concentration, mannose conjugate **22** did show a clear

concentration dependent increase in fluorescence. Uptake of mannose conjugates **22** was temperature dependent in A549 and THP-1 cell-lines hinting at an active uptake process. To evaluate the extent of NDB mannose uptake, we compared the uptake of **22** to NBD-glucosamine (NBDG) a frequently used NBD conjugated probe to measure glucose uptake.[20] In A549 cells, both mannose conjugate **22** and glucosamine conjugate **25** performed equally well showing that the uptake of **22** is significant. In THP-1 cells, mannose conjugates **22** outperformed NBDG and was taken up to a much larger extend, which may be related to the increased expression of the human passive glucose transporter GLUT3 in monocytes.[21, 22] Overall these results indicate that large fluorophores such as $Cy5^{GE}$ and AF 647 may influence the properties of the small molecules attached, whereas small fluorophores such as NBD are more likely to show a substrate dependent uptake.

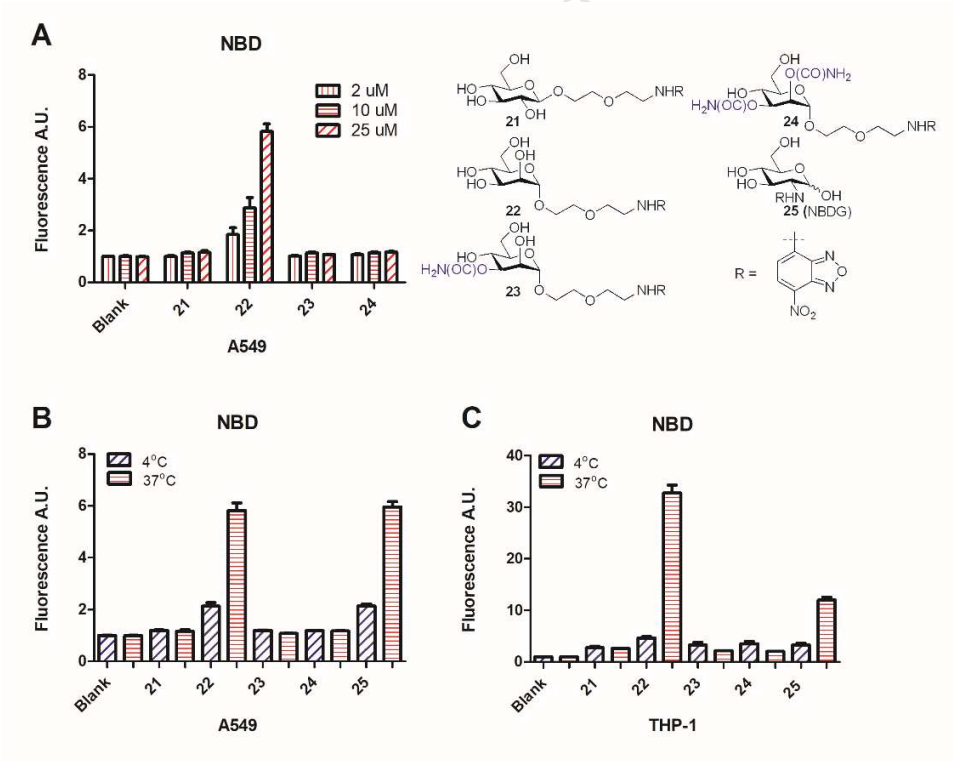


Figure 3: Cellular uptake of fluorescent NBD-glycosides **21-25** in A549 (**A-B**) and THP-1 (**C)** cancer cell lines. Fluorescence was normalized according to the blank (H_2O). Final substrate concentrations used in B and C were set at 25 µM.

3. Conclusion:

In conclusion, we developed a robust synthetic route towards carbamoyl functionalized mannosides for conjugate synthesis. The mannosides were conjugated to $Cy5^{GE}$, AF 647 and NBD and their temperature dependent uptake was studied by FACS in two cancerous cell lines (A549 and THP-1). In contrast to earlier studies, the uptake was not related to carbamoylation of the conjugates but rather to the cyanine dye attached. The NBD-conjugates however, gave evidence of a temperature and concentration dependent uptake in case of mannose conjugates. These results suggest a profound impact of the dye which should be taken into consideration when studying the uptake of small molecules by dye conjugation.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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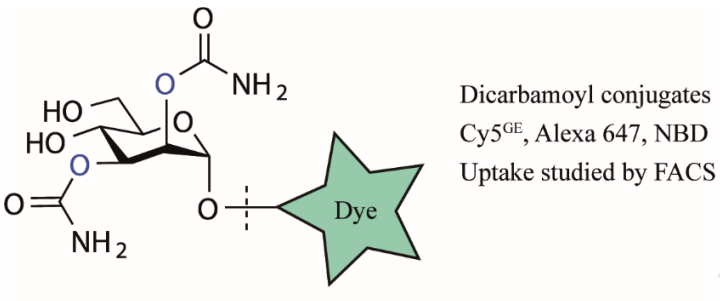
Graphical Abstract



- (di)carbamoylated mannosides were synthesized.
- The (di)carbamoylated mannose derivatives were conjugated to fluorescent dyes Cy5^{GE}, AF 647 or NBD.
- Cellular uptake was assed using FACS and confocal microscopy.
- Cellular uptake was dependent on the fluorescent dye used.
- Carbamoylation did not result in increased or selective cellular uptake.