

Synthesis and characterization of glucosamine-bound near-infrared probes for optical imaging

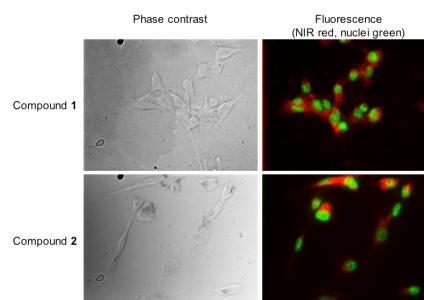
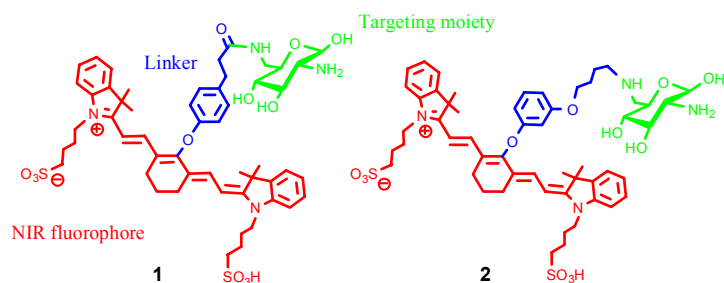
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ABSTRACT



Two novel near-infrared (NIR) fluorescent probes have been synthesized by linking a carbocyanine fluorophore and glucosamine through different linkers. These probes demonstrated a high quantum yield, low cytotoxicity, reversible pH-dependent fluorescence in the physiological pH range, and decreased aggregation tendency in aqueous solutions. *In vitro* NIR optical imaging studies revealed cellular uptake and strong intracellular NIR fluorescence of these two probes in several breast cancer cell lines.

Optical imaging is an emerging non-invasive diagnostic imaging modality, which can be used for visualizing pathological conditions with high sensitivity, low tissue irradiation, and relatively low cost.¹ To this end, it is very important to develop novel optical imaging probes, and in particular probes that fluoresce in the near-infrared (NIR) wavelength range because tissue autofluorescence and light absorption in the NIR range (700–900 nm) are low, and NIR light can penetrate several centimeters into heterogeneous tissues.²

Lysosomes are membranous organelles found in almost all mammalian cells. Recent studies demonstrated that

lysosomes can play a pivotal role in cancer cell invasion and metastasis because several crucial proteolytic enzymes, such as cathepsin D, B, and L, which actively participate in the digestion of extracellular matrix (ECM) proteins, are sequestered in lysosome vesicles.³ Moreover, under conditions of acidic extracellular pH, which are frequently found in the tumor microenvironment, cells with a high degree of malignancy exhibited larger lysosome sizes and increased nucleus-lysosome distances than poorly metastatic cells.⁴ Therefore, assessing lysosomal morphology and trafficking *in vivo* by using NIR optical imaging probes will help elucidate the role of

¹ Shah, N.; Cerussi, A.; Eker, C.; Espinoza, J.; Butler, J.; Fishkin J.; Hornung, R.; Tromberg, B. *Proc. Natl. Acad. Sci. USA* **2001**, 98, 4420–4425.

² Achilefu, S. *Technol. Cancer Res. Treat.* **2004**, 3, 393–409.

³ (a) Roshy, S.; Sloane, B. F.; Moin, K. *Cancer Metastasis Rev.* **2003**, 22, 271–86. (b) Levicar, N.; Strojnik, T.; Kos, J.; Dewey, R. A.; Pilkington, G. J.; Lah, T. T. *J. Neurooncol.* **2002**, 58, 21–32.

⁴ Glunde, K.; Guggino, S. E.; Solaiyappan, M.; Pathak, A. P.; Ichikawa, Y.; Bhujwalla, Z. M. *Neoplasia*, **2003**, 5, 533–545.

lysosomal parameters in cancer invasion and metastasis, and may provide a novel means of diagnosing malignant lesions in their early stages.^{4,5}

We previously demonstrated that lysosomes can be optically imaged by utilizing fluorophores covalently bound to the 6-hydroxyl group of glucosamine.⁶

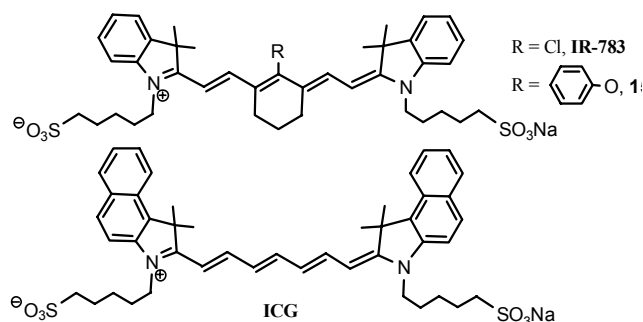
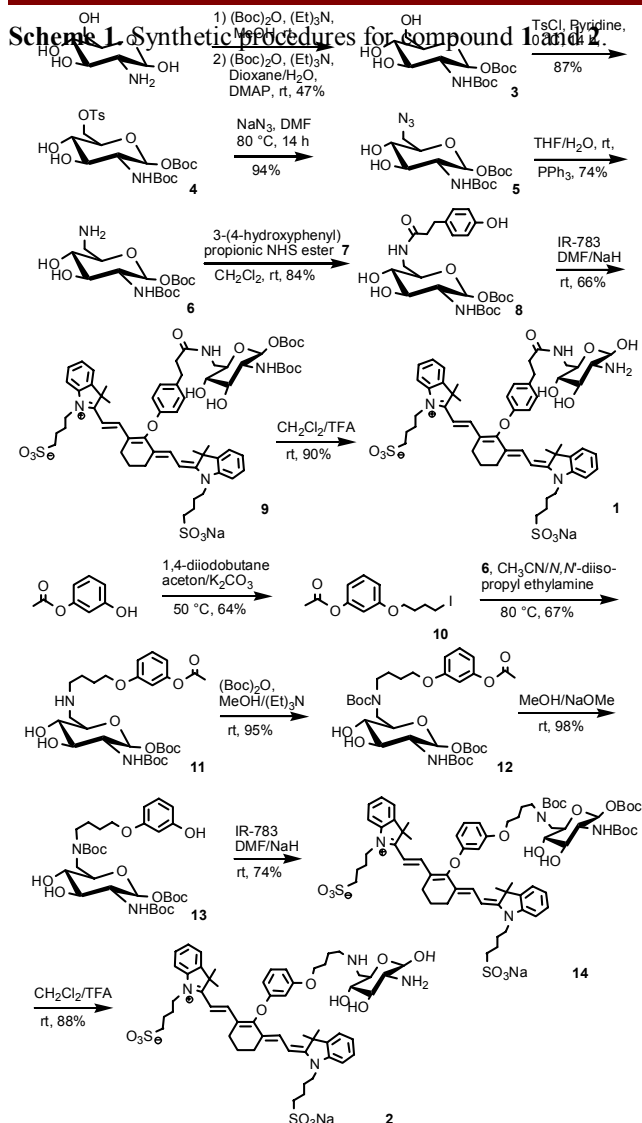


Figure 1. Chemical structures of IR-783, control molecule 15, and indocyanine green (ICG).

Glucosamine is required for the biosynthesis of highly glycosylated lysosomal proteins, some of which are an integral part of lysosomal membranes.⁷ Here, we report two novel compounds, in which an NIR fluorophore was covalently linked to glucosamine. Carbocyanine IR-783 (Figure 1) was chosen as NIR fluorophore because of its high extinction coefficient, optimal emission wavelength, and good photochemical and photophysical stability.⁸ To prevent self-quenching between several IR-783 moieties in the cellular environment⁹ and loss of bioactivity of the glucosamine due to modification with this bulky fluorophore¹⁰, two extended carbon chain linkers were placed between IR-783 and glucosamine.

The synthesis of compound **1** is outlined in scheme 1. Bis-Boc protected glucosamine **3** was prepared as previously described.⁶ Regiospecific reaction of **3** with TsCl in pyridine afforded the product mono-tosylated at the C6 position. Replacement of tosyl with azide was achieved by treatment of **4** with sodium azide in DMF. Reduction of azide to amine was realized in THF/H₂O in the presence of triphenylphosphine. Treatment of 3-(4-hydroxyphenyl) propionic acid with *N*-hydroxysuccinimide in the presence of DCC resulted in NHS-ester **7**,¹¹ which reacted efficiently with **6** to give **8**. Etherification of **8** with IR-783 was accomplished in DMF/NaH.¹² Deprotection of **9** was achieved in TFA/CH₂Cl₂, and the final product **1** was

obtained as deep green powder. Compound **2** was synthesized from **6** in five steps.



The starting material 3-(4-iodobutoxy)-phenyl acetate **10** was prepared by reacting resorcinol monoacetate with 1,4-diiodobutane in acetone. Of several possible reaction conditions to achieve alkylation between **10** and **6**, such as CH₃CN/K₂CO₃, CH₃CN/(Et)₃N, DMF/K₂CO₃, or CH₃CN/*N,N'*-diisopropyl ethylamine, only CH₃CN/*N,N'*-diisopropyl ethylamine provided the desired alkylated product **11** in modest yield. Treatment of **11** with (Boc)₂O produced the fully protected tertiary amine **12**. Selective deprotection of the phenyl acetate in **12** was tested under different hydrolytic reaction conditions. In dioxane/NaOH or EtOH/NaOH, both the phenyl acetate and the *O*-Boc group at position C1 were hydrolyzed. The monohydrolytic product **13** was obtained in CH₃OH by applying a catalytic dose of CH₃ONa, giving a yield above 98%. Similar to **9**, **14** was prepared by treating **13** with IR-783.¹² Deprotection of **14** yielded **2** as deep green solid. Additionally, to clearly identify the function of the glucosamine moiety in **1** and **2**, control molecule **15**

⁵ Fehrenbacher, N.; Jaattela, M. *Cancer Res.* **2005**, *65*, 2993–2995.

⁶ (a) Glunde, K.; Guggino, S. E.; Ichikawa, Y.; Bhujwalla, Z. M. *Mol. Imaging* **2003**, *2*, 24–36. (b) Glunde, K.; Foss, C. A.; Takagi, T.; Wildes, F.; Bhujwalla, Z. M. *Bioconjugate Chem.* **2005**, *16*, 843–851.

⁷ Winchester B. *Glycobiology* **2005**, *15*, 1R–15R.

⁸ (a) Hilderbrand, S. A.; Kelly, K. A.; Weissleder, R.; Tung, C.-H. *Bioconjugate Chem.* **2005**, *16*, 1275–1281. (b) Sasaki, E.; Kojima, H.; Nishimatsu, H.; Urano, Y.; Kikuchi, K.; Hirata, Y.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 3684–3685.

⁹ Weissleder, R.; Tung, C. H.; Mahmood, U.; Bogdanov Jr, A. *Nature Biotechnol.* **1999**, *17*, 375–378.

¹⁰ Zhou, X. T.; Forestier, C.; Goff, R. D.; Li, C.; Teyton, L.; Bendelac, A.; Savage, P. B. *Org. Lett.* **2002**, *4*, 1267–1270.

¹¹ Suijkerbuijk, B. M. J. M.; Slagt, M. Q.; Klein Gebbink, R. J. M.; Lutz, M.; Spek, A. L.; van Koten, G. *Tetrahedron Lett.* **2002**, *43*, 6565–6568.

¹² Narayana, N.; Patonay, G. *J. Org. Chem.* **1995**, *60*, 2391–2395.

(Figure 1), which has an identical fluorophore structure as **1** and **2**, was also prepared.

Photospectroscopic measurements performed in PBS at pH 7.4 revealed that the absorbances of **1** and **2** centered at 766 and 768 nm, with the corresponding emission maxima at 782 and 784 nm, respectively. Even though the molar extinction coefficients of **1** ($\epsilon = 176,000 \text{ M}^{-1}\text{cm}^{-1}$) and **2** ($\epsilon = 157,000 \text{ M}^{-1}\text{cm}^{-1}$) decreased compared to control molecule **15** ($\epsilon = 192,000 \text{ M}^{-1}\text{cm}^{-1}$) due to the “dye dilution” effect¹³, both of these new compounds demonstrated much higher quantum yields than **15** (0.043, Table 1). The fluorescence quantum yields (Φ_f) of **1** and **2** were 0.185 and 0.113, respectively, which correspond to a four- and threefold increase compared to the control molecule. These high quantum yields of **1** and **2** can be explained with the presence of multiple hydrophilic groups in the glucosamine moiety, which may not only improve the hydrophilicity of these conjugates, but also disrupt the π - π interaction between the hydrophobic

Table 1. Photophysical properties of **1**, **2**, and **15**.

	$\lambda_{\text{max, abs}}$	$\epsilon (\text{M}^{-1}\text{cm}^{-1})$	$\lambda_{\text{max, em}}$ ^a	Φ_f ^b
1 ^c	766	176,000	782	0.186
2 ^c	768	157,000	784	0.113
15 ^c	766	192,000	783	0.043
ICG ^c	779	191,000	802	0.041
ICG ^d	794	218,000	816	0.13

^aThe fluorophores were excited at their maximum absorbances. ^bQuantum yields were determined by comparison with indocyanine green (ICG, Figure 1) in DMSO (Q.Y. = 0.13), 298 K. ^cMeasured in PBS buffer, pH 7.4, (< 0.005% methanol as cosolvent). ^dMeasured in DMSO.

Reduced quantum yield of carbocyanine dyes in aqueous environments have previously been reported as a consequence of their self-aggregation.^{14,15} To test this hypothesis, we studied the concentration dependence of the absorbances of **1**, **2** and **15**. An additional broad, blue-shifted band centered at 670 nm increased with increasing concentrations of **15** (Supporting Information, Figure S2), which most likely corresponded to H-aggregates.¹⁴ In contrast, no additional bands were found in the absorption spectra of **1** and **2**, not even at concentrations above 50 μM . These results indicate that **1** and **2** have decreased aggregation tendencies in aqueous solution compared to control molecule **15**. To date, very few pH-dependent NIR dyes have been reported.^{8b,16} pH effects on the fluorescence of **1** and **2** were investigated. Similar to control molecule **15**, the emission intensity of **1** decreased with acidification (Figure 2). Interestingly, compound **2** exhibited a quite unique pH-dependent fluorescence behavior. Between pH 11.0 and 7.2 (slope *a*, Figure 2), its

fluorescence decreased significantly with decreasing pH, reaching a minimum at pH 7.2. Upon further acidification, the fluorescence increased inversely (slope *b*, Figure 2), reaching a maximum at pH 3.0 with a threefold intensity enhancement compared to pH 7.2.

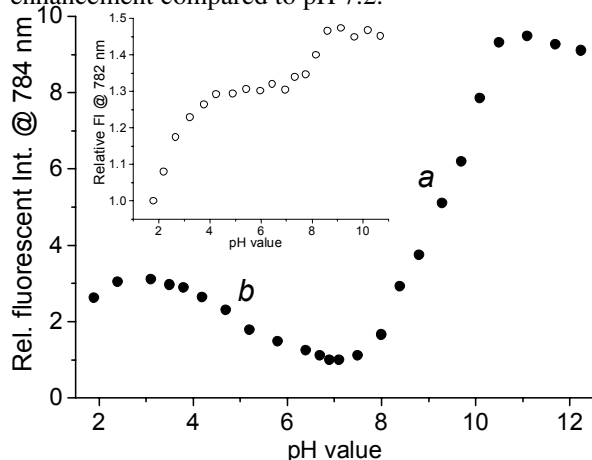


Figure 2. pH-dependent fluorescence intensity profile of **2** and **1** (insert) in 0.1 M sodium phosphate buffer. The fluorescence intensities of **2** and **1** (0.6 μM) were determined at 784 and 782 nm with excitation at 768 and 766 nm, respectively.

This unique pH-dependent fluorescence was reversible in the pH range of 3.0–7.0 for at least three cycles. Compound **2** may prove useful in future applications, where acidic environments, such as the lysosomal lumen maintained at about 5.0^{3,8}, and/or the tumor microenvironment characterized by extracellular pH values of 6.4 to 6.8¹⁷, can enhance its NIR fluorescence. Intermolecular electrostatic repulsions arising from protonation of both secondary and primary amines in glucosamine may alleviate self-quenching between cyanine fluorophores, and hence increase the fluorescence intensity.

Previous studies reported that covalently binding saccharides to initially toxic polymers or small molecules can substantially decrease the toxicity of the resulting conjugates.¹⁸ Here, we tested the cytotoxicity of **1** and **2** relative to **15** in four human mammary epithelial cell (HMEC) lines, MCF-12A, MCF-7, MDA-MB-231, and MDA-MB-435, which represent different stages of malignancy, by MTT assay.¹⁹ In all four HMEC lines, **1** and **2** exhibited significantly lower cytotoxicity than **15**. No cells survived following treatment with **15** at concentrations above 250 μM . Compound **1** demonstrated minimal cytotoxicity, resulting in cell viabilities of 95%–

¹⁷ Stubbs, M.; McSheehy, P. M.; Griffiths, C. L. *Mol. Med. Today* **2000**, 6, 15–19.

¹⁸ (a) Metzke, M.; O'Connor, N.; Maiti, S.; Nelson, E.; Guan, Z. *Angew. Chem. Int. Ed.* **2005**, 44, 6529–6533. (b) Queiroz, E. F.; Roblot, F.; Duret, P.; Figadère, B.; Gouyette, A.; Laprêve, O.; Serani, L.; Hocquemiller, R. *J. Med. Chem.* **2000**, 43, 1604–1610.

¹⁹ The MTT cell proliferation assay determines the ability of viable cells to reduce yellow [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) to blue-colored formazan crystals by mitochondrial enzymes. The concentration of formazan crystals can be determined spectrophotometrically when dissolved in an organic solvent. For a detailed description of this assay see “Cetin, Y.; Bullerman, L. B. *J. Agric. Food Chem.* **2005**, 53, 6558–6563.”

¹³ Ye, Y. P.; Li, W. P.; Anderson, C. J.; Kao, J.; Nikiforovich, G. V.; Achilefu, S. *J. Am. Chem. Soc.* **2003**, 125, 7766–7767.

¹⁴ (a) Khairutdinov, R. F.; Serpone, N. *J. Phys. Chem. B* **1997**, 101, 2602–2610. (b) Egorov, V. V. *J. Chem. Phys.* **2002**, 116, 3090–3103.

¹⁵ Zhang, Z. R.; Achilefu, S. *Org. Lett.* **2004**, 6, 2067–2070.

¹⁶ Zhang, Z. R.; Achilefu, S. *Chem. Commun.* **2005**, 5887–5889.

100% in MCF-12A and MCF-7 cells even at the highest concentration (2.0 mM) (Figure 3). While **1** and **2** exhibited minimal toxicity in MCF-7 and MCF-12A cells within the tested concentration range, both compounds caused significant cytotoxicity in the highly malignant cell lines MDA-MB-231 and MDA-MB-435 (cell viabilities < 5%) when administered highly concentrated (2.0 mM).

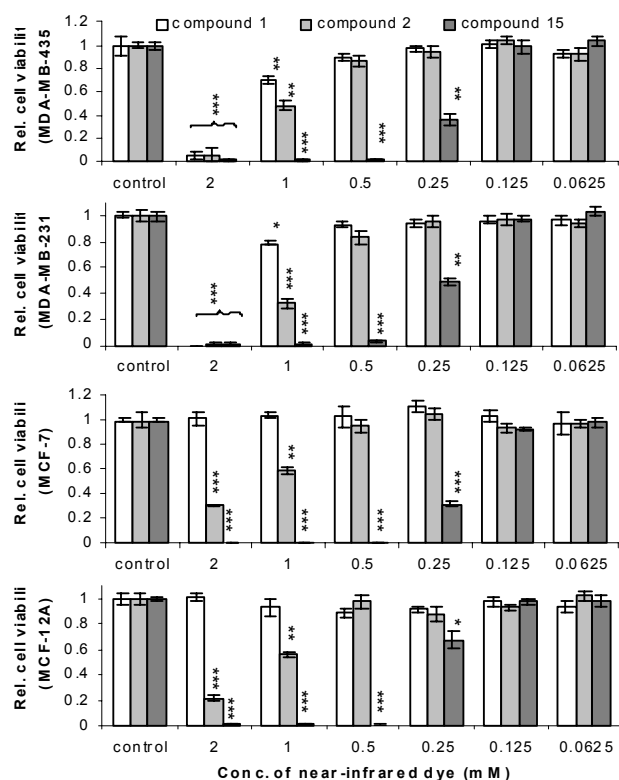
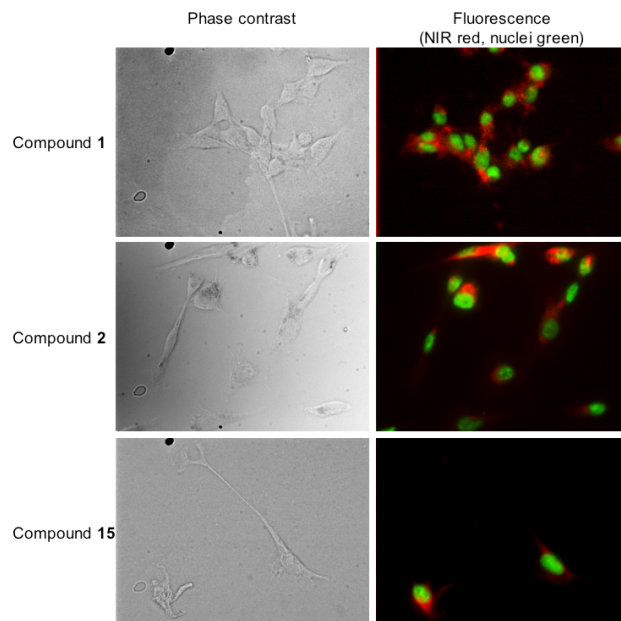


Figure 3. Relative cell viability of human MDA-MB-435 (n = 4), MDA-MB-231 (n = 8), MCF-7 (n = 8), and MCF-12A (n = 8) breast epithelial cells following 24 hours of treatment with compound **1** (white bars), **2** (light grey bars), and control molecule **15** (grey bars). Error bars represent mean \pm S.D., * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$ for relative cell viability after treatment with near-infrared dye versus control

In contrast, **15** exhibited non-specific cytotoxic behavior in all four HMEC lines. The low cytotoxicity of **1** and **2** may be due to multiple hydrophilic groups in glucosamine, which can shield surface charges of carbocyanine, and thereby decrease potentially harmful coulombic interactions of such dyes with cell membranes.^{17a} Compound **1** and **2** show great promise for future *in vivo* optical imaging applications, since fluorescent probes are typically administered in a dose range between 0.1–600 μ M.^{6b,20} NIR fluorescence microscopy demonstrated that **1** and **2** were taken up into the cells, and resulted in strong

intracellular NIR fluorescence in all four HMEC lines, as shown for MDA-MB-231 cells in Figure 4.



Phase contrast images of the same fields of view revealed intracellular localization of **1** and **2** (Figure 4). NIR fluorescence (red) of **1** was localized in small intracellular vesicles, and a partially punctate pattern was detected for **2**, most likely attributable to lysosomal localization. NIR fluorescence from control molecule **15** was detected predominantly in dead cells, and weakly in live cells without giving a punctate pattern (Figure 4).

In summary, we have developed two novel NIR fluorescent probes, which demonstrated high quantum yields, low cytotoxicity, reversible pH-dependent fluorescence, and a low tendency to aggregate in aqueous solution. Incubation of HMEC cultures with **1** or **2** resulted in cellular uptake of these compounds and intense punctate intracellular NIR fluorescence. *In vivo* NIR optical imaging studies with **1** and **2** are currently underway.

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Supporting Information Available: Experimental procedures, full characterization of compounds **1**, **2**, **15**, photospectroscopic analysis, pH-dependent fluorescence, *in vitro* cytotoxic studies, and immunofluorescence imaging. This material is online available free of charge at <http://pubs.acs.org>

