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A Mechanism-Based Affinity-Labeling Agent for Possible Use in Isolating *N*-Acetylglucosaminidase

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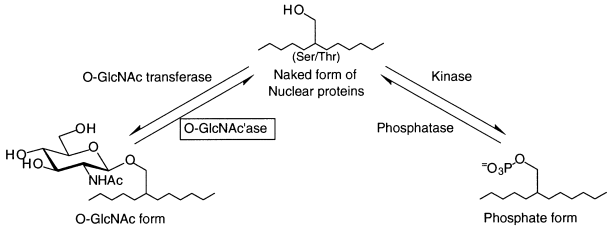
Abstract—We have prepared several mechanism-based affinity-labeling agents for possible use in isolating *N*-acetylglucosaminidase, in which an *N*-acetylglucosamine is linked to an *o*-monofluoro- or difluoro-methyl phenoxy glycoside with or without a cleavable disulfide group in the tether to biotin.

Glycosidases hydrolyze glycoconjugates, including glycoproteins, glycolipids, DNA, RNA, glycosaminoglycans, and peptidoglycans, into sugars and aglycons and consequently play critical roles in carbohydrate metabolism,[1,2] DNA repair mechanisms,[3] and cell physiology.[4] Therefore, it is important to identify and characterize the specific enzymes involved in order to understand their direct roles in such biological events. Although several affinity-labeling agents have been developed (sugars with an epoxy-[5] or α-haloketo-containing aglycon[6] or fluorinated sugars)[7] for active-site mapping, only a few iminosugar-based inhibitors with a photoaffinity-labeling group have been used in attempts to identify specific glycosidases on SDS-PAGE.[8,9]

O-Linked *N*-acetylglucosaminidase (*O*-GlcNAc'ase) cleaves the *O*-glycosidic linkage between GlcNAc and Ser/Thr residues of proteins to liberate the free OH group of the Ser/Thr residue for subsequent phosphorylation by a kinase.[10] This on–off glycosylation of Ser/Thr residues, and therefore the activity of the enzyme, is thought to be responsible for regulating the biological function of many nuclear and cytosolic proteins.[11] However, this important enzyme has not yet been completely isolated or characterized because of the lack of a suitable affinity-labeling agent.[12] We had previously prepared several benzophenone-based GlcNAc thioglycosides and examined the photolabeling of *O*-GlcNAc'ase partially purified from rat spleen.[13] After introducing a radioactive marker on the photoaffinity-labeled proteins, we observed ~5 positive bands on an SDS-PAGE gel together with several minor bands (probably because of its hydrophobic nature the acetophenone-based compound tended to exhibit nonspecific interaction with other co-existing proteins that may bind to the GlcNAc moiety), and subsequently excised and sequenced the radioactive bands from SDS-PAGE gel (G. Parker and Y. Ichikawa, unpublished).[13] Disappointingly we were unable to identify the enzyme sequence. We reasoned that, because there were a large number of other proteins comigrating with the radioactive *O*-GlcNAc'ase on the SDS-PAGE gel, we might have sequenced one of them instead of the target enzyme. We therefore decided to develop a procedure that allows us to effectively isolate/purify a specific glycosidase.



Janda and co-workers have reported the use of a biotin-conjugated mechanism-based tagging agent in the isolation of an antibody with a β-galactosidase-like activity from a phage library.[14] Because no standard protocol has yet been established for isolating a specific glycosidase, we decided to evaluate and further develop such a mechanism-based tagging agent as a tool for isolating glycosidases, including *O*-GlcNAc'ase.

A possible mechanism for such tagging (affinity labeling) or inactivation of an enzyme, mediated by a 2-halobenzyl-phenol group, has been well documented.[15,16] In

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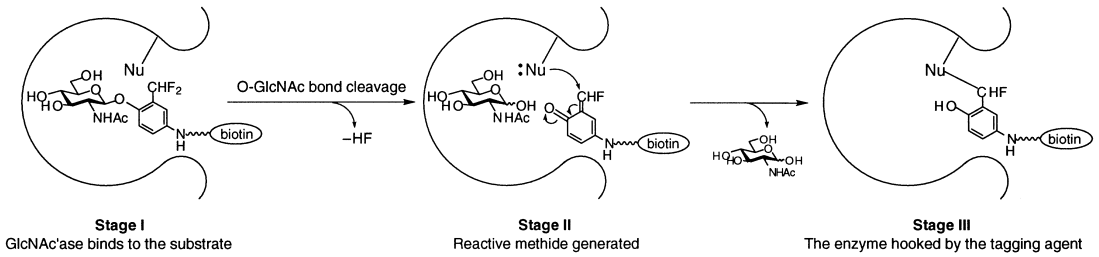


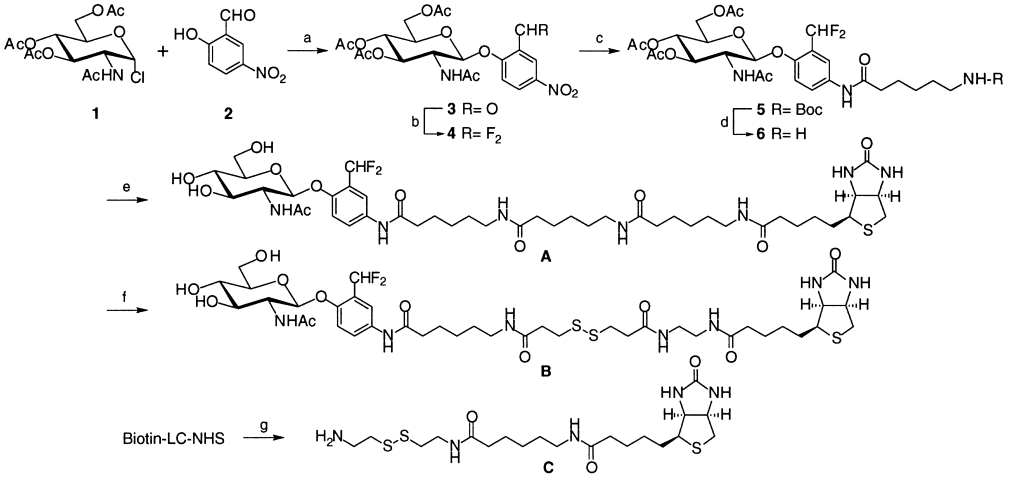
our case, when an enzyme cleaves a β-GlcNAc linkage (stage I→II), a reactive methide is generated with a loss of HF. The methide, at stage II, serves as a Michael acceptor to react with a potential nucleophile (i.e., the carboxylate of Asp or Gln) inside or near the active site to form a covalent linkage between the enzyme and the released aryl moiety (stage III), which could be easily isolated by streptavidin-based affinity column chromatography.

Synthesis of Difluoromethyl Aryl β-*N*-Acetyl-D-glucosaminide (Scheme 1)

The basic strategy for synthesis of the tagging agents was similar to that by Janda[14] and Danzin.[16] *N*-Acetylglucosaminyl chloride **1**[17] was treated with 2-hydroxy-5-nitro-benzaldehyde **2** in the presence of tetrabutylammonium bromide in a mixture of 5% NaOH and CH_2Cl_2[16,18] to give the β-glycoside **3**[19] in 20–30% yield. Treatment of **3** with diethylaminosulfurtrifluoride (DAST) gave a difluoromethyl aryl derivative **4**[19] in 60% yield. The nitro group of **4** was reduced to an amino group, which was coupled with *N*-Boc-6-aminohexanoic acid in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDAC) to give **5**[19] in 74% overall yield. Acidic treatment of **5** generated the free amino group that was used to prepare the biotin conjugates. We have prepared two agents with different tethers for comparison: agent **A**[19] possesses a stable alkyl chain-based tether, and agent **B**[19] carries, on the other hand, a cleavable disulfide group in the tether. These were added by reacting **6** with commercially available biotin LCLC NHS[20] and biotin disulfide NHS,[20] respectively. In addition, we also prepared another disulfide-containing biotin conjugate **C**, which lacks *N*-acetylglucosaminyl moiety, by treating a commercially available Biotin-LC NHS[20] with cystamine in order to see a stability of the disulfide group.

Affinity-Labeling Experiments

A 50% aq DMSO solution of each agent **A**–**D** was added, to a final concentration of 2 mM (4% DMSO final concentration), to a sodium cacodylate buffer (100 μL, 50 mM, pH 6.5) containing a partially purified *O*-GlcNAc'ase,[10] EDTA (10 mM), Triton X-100 (1%), and NaCl (200 mM), and the solution was incubated for 2 h at 37 °C. An aliquot (10 μL) was taken from each incubation mixture and solubilized in SDS sample buffer (under nonreducing conditions). The remaining mixture was treated with pretreated streptavidin-agarose beads (40 μL, Pierce) overnight at 4 °C. The beads were washed three times with a 100 mM Tris pH 7.0 solution containing 2.5 M KCl and 1% Triton X-100 (the suspension was centrifuged and the supernatant was removed by pipeting), and boiled in SDS sample



Scheme 1. Synthesis of difluoromethyl aryl-containing tagging agents. Reagents and conditions: (a) Bu_4NBr/5% NaOH–CH_2Cl_2 (24%); (b) DAST/CH_2Cl_2 (60%); (c) (i) H_2/Pd–C/EtOAc; (ii) 6-*N*-Boc-aminohexanoic acid/EDAC/THF (74% overall); (d) CF_3CO_2H–CH_2Cl_2 (1:4)/0 °C; (e) (i) biotin LCLC NHS/Et_3N/DMF; (ii) EtOH–H_2O–Et_3N (4:1:1)/rt; (f) (i) biotin disulfide *N*-hydroxysuccinimide ester/Et_3N/DMF; (ii) EtOH–H_2O–Et_3N (4:1:1)/rt; (g) cystamine/Et_3N/DMF.

buffer (under non-reducing conditions). The eluted proteins were separated by SDS-PAGE (10% gel) and electrophoretically transferred to nitrocellulose (Fig. 1). The protein bands were visualized with alkaline phosphatase-streptavidin and BCIP/NBT. In separate experiments, when the enzyme preparation was treated with agents **A**, **B**, and **C** and *O*-deacetylated forms of **4**, **5**, **8**, and **9**, we did not observe the apparent time-dependent inactivation of the enzymes (*O*-GlcNAc'ase activity), which may indicate the labeling takes place outside the enzyme active site.[14] When the affinity-labeling experiments were conducted in the presence of a thio-glycoside derivative **11** (see ref 13), which had been identified as a potent inhibitor of *O*-GlcNAc'ase ($K_i = 50 \pm 5\,\mu M$), the tagging agents **A**, **B**, and **D** were not hydrolyzed (spectrophotometric and TLC analysis), and consequently, no protein bands were observed in SDS-PAGE by the Western blotting.

An affinity tagging experiment in which a partially purified *O*-GlcNAc'ase from rat spleen was treated with the difluoromethyl aryl alkyl-tethered tagging agent **A** showed that several protein bands were tagged by the biotin (lane 1); however, these bands stained very weakly and were not detected after the attempted affinity purification probably because only a small fraction of the proteins were conjugated with the biotin (lane 2). A possible explanation for this low tagging efficiency might involve the difluoromethyl group. After the covalent linkage was formed between the tagging agent **A** and the enzyme, these conjugates were still present in the incubation mixture for 2 h in the presence of 4% DMSO which helped solubilize the tagging agent. Under such conditions,[21] the phenol group of the conjugate might have readily become ionized to facilitate the formation of an active methide if a fluorine atom still remains (**I**→**II**→**III**, Fig. 2), which would react with water to form a hemiacetal (**III**→**IV**). The hemiacetal would easily undergo formation of the stable aldehyde, with a concomitant loss of the once-hooked enzyme (**IV**→**V**).

In the case of agent **B** with a disulfide group in the tether, we observed a pattern of protein bands before the affinity purification that was similar to that obtained with agent **A** (lane 3, Fig. 1) and one major band retained after the affinity purification (lane 4). To our surprise, however, these protein bands in lane 3 were also seen when we used agent **C**, a disulfide-containing biotin conjugate without a GlcNAc aryl moiety (lane 5). We therefore speculated that a sulfide-exchange reaction might have taken place during the incubation (Fig. 3). The enzyme preparation had other proteins present, including GlcNAc-binding and free cysteine-containing proteins such as **VI** in Fig. 3. In this situation, when the tagging agent binds to a GlcNAc-binding protein, a free cysteine near the binding site reacts with the disulfide group to cause a sulfide-exchange reaction (**VII**).[22] In path a (Fig. 3), a cysteine reacts with a sulfur atom close to the biotin to form a new disulfide bond, with a biotin with a shorter tether length, which is visible after Western blotting; however, this biotin-conjugate cannot bind to streptavidin-agarose beads probably because only a small portion of agent C undergoes the disulfide-exchange reaction to form biotin-conjugates or the tether may be too short to be retained with streptavidin beads.[23] On the other hand, if the cysteine reacts with a sulfur atom away from the biotin,

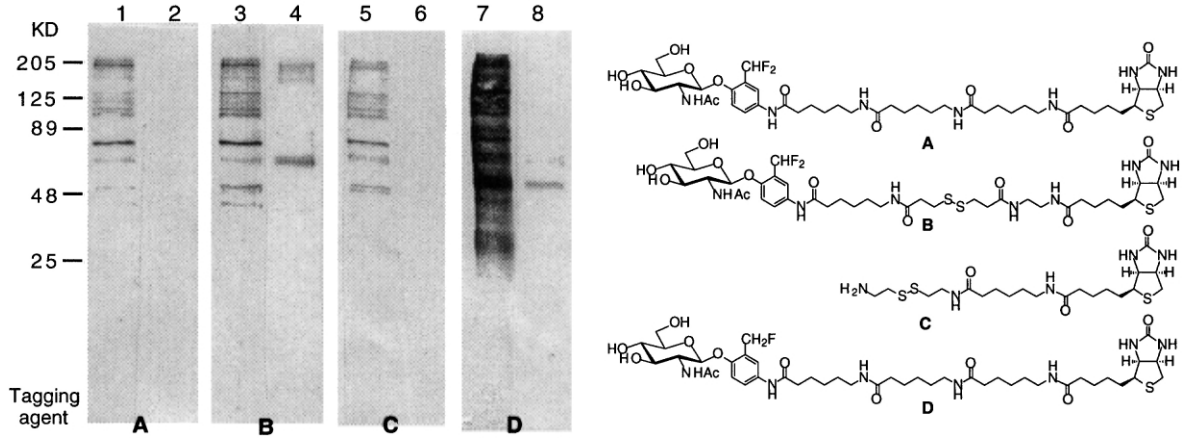


Figure 1. SDS-PAGE of the affinity-tagging experiments. After incubation with each biotin-conjugated tagging agent (2 mM of **A**, **B**, **C**, or **D**), the samples were separated by SDS-PAGE before (lanes 1, 3, 5, and 7) and after affinity purification with streptavidin-agarose beads (lanes 2, 4, 6, and 8). The biotinylated protein bands were visualized with streptavidin-alkaline phosphatase and BCIP/NBT.

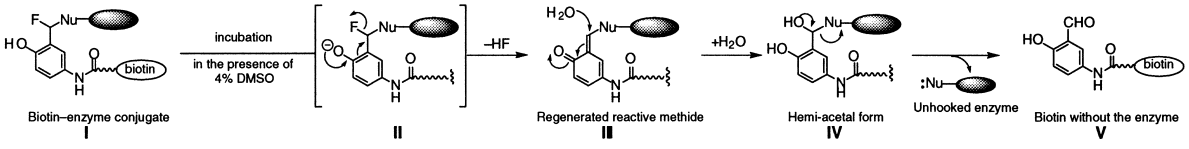


Figure 2. A possible explanation for disappearance of the protein bands after affinity purification.

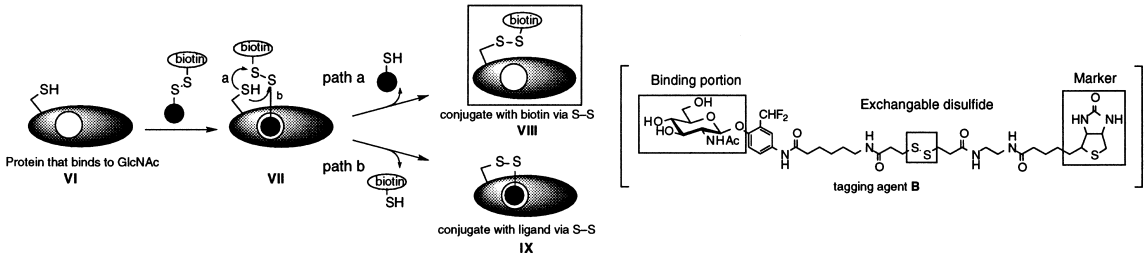
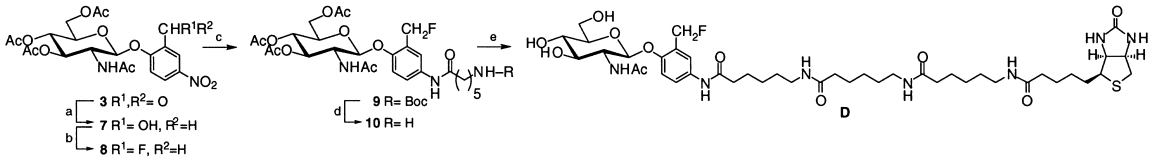


Figure 3. A possible explanation for protein tagging via a disulfide group.



Scheme 2. Synthesis of a monofluoromethyl aryl-containing tagging agent. Reagents and conditions: (a) $NaBH_3CN$/THF/aq HCl; (b) DAST/CH_2Cl_2 (60%); (c) (i) H_2/Pd–C/EtOAc; (ii) 6-*N*-Boc-aminohexanoic acid/EDAC/THF (74% overall); (d) CF_3CO_2H–CH_2Cl_2 (1:4)/0 °C; (e) (i) biotin LCLC NHS/Et_3N/DMF; (ii) EtOH–H_2O–Et_3N (4:1:1)/rt.

as in path b, the GlcNAc-aryl group becomes attached to the protein which is not visible after Western blotting.

Synthesis of Another Tagging Agent with a Monofluoromethyl Aryl Group

Because we hypothesized that a monofluoromethyl group would prevent such 'release' of the already tagged enzyme as depicted in Fig. 2, we decided to prepare another type of tagging agent with a monofluoromethyl aryl group (Scheme 2). The aldehyde **2** was subjected to reduction with $NaBH_3CN$[16] to convert it to the corresponding alcohol **7**. The rest of the synthetic strategy was almost identical to that for the synthesis of a difluoromethyl derivative, agent **A**.

We then performed affinity tagging experiments with the monofluoromethyl aryl agent **D**. When the enzyme was treated with tagging agent **D** and analyzed by SDS-PAGE, one major protein band was visible (lane 7, Fig. 1) which was retained after affinity purification (lane 8). Its molecular weight was about 51,000 which is consistent with the reported value of *O*-GlcNAc'ase from rat spleen.[10] We are now in the process of isolating this protein band and microsequencing it.

Acknowledgements

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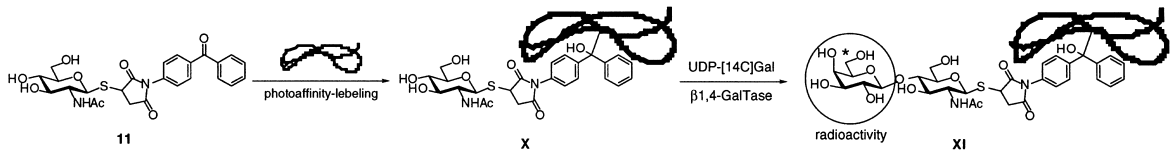
13. A photoaffinity-labeling agent **11** was prepared by reacting a 1-thio-GlcNAc (Horton, D.; Wolfrom, M. L. *J. Org. Chem.* **1962**, *27*, 1794. Ohnishi, Y.; Ichikawa, M.; Ichikawa, Y. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1289) and 4-(*N*-maleimido)benzophenone (Sigma) followed by *O*-deacetylation. The enzyme preparation was pretreated with UDP-Gal and β1,4-galactosyltransferase, which was then photoaffinity-labeled with the agent **11** by 365 nm (**X**), and treated with UDP-[14C]Gal and β1,4-galactosyltransferase in order to introduce radioactivity onto the photoaffinity-labeled protein (Roquemore, E. P.; Chou, T.-Y.; Hart, G. W. *Methods Enzymol.* **1994**, *230*, 433) (**XI**). The radioactive protein bands were excised from the SDS-PAGE gel and microsequenced.



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19. Compound **3**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.77, 1.97, 2.008, 2.010 (3H each, s, 3×OAc, NHAc), 4.14–4.35 (4H, m), 4.98 (1H, t, J = 9.60 Hz, H-4), 5.27 (1H, J = 10.2 Hz, H-3), 5.58 (1H, d, J = 8.40 Hz, H-1), 7.60 (1H, d, J = 9.30 Hz, aromatic), 8.10 (1H, d, J = 9.00 Hz, NH), 8.39 (1H, d, J = 2.70 Hz, aromatic), 8.52 (1H, dd, J = 2.70, 9.30 Hz, aromatic), and 10.17 (1H, s, aldehydro).

Compound **4**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.94, 2.06, 2.08, 2.10 (3H each, s, 3×OAc, NHAc), 3.98 (1H, ddd, J = 2.70, 5.40, 9.90 Hz), 4.18–4.32 (3H, m), 5.16 (1H, t, J = 9.30 Hz), 5.34–5.43 (2H, m), 5.91 (1H, d, J = 8.70 Hz, NH), 6.88 (1H, t, J = 54.6 Hz, CF_2H), 7.22 (1H, br s), 8.29 (1H, dd, J = 1.80, 9.30 Hz, aromatic), and 8.45 (1H, dd, J = 1.80, 2.10 Hz, aromatic).

Compound **5**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.25–1.49 (4H, m), 1.43 (9H, s, NH*t*Boc), 1.94 (3H, s), 2.05 (6H, br s), 2.08 (3H, s), 2.31–2.37 (2H, m), 3.05–3.12 (2H, –CH_2–NHBoc), 3.83 (1H, ddd, J = 2.10, 5.10, 9.90 Hz, H-5), 4.16 (1H, dd, J = 2.40, 12.3 Hz, H-6a), 4.24–4.34 (2H, m), 4.66 (1H, br s), 5.03 (1H, d, J = 8.40 Hz, H-1), 5.12 (1H, J = 9.30 Hz, H-4), 5.32 (1H, dd, J = 9.30, 10.50 Hz, H-3), 6.20 (1H, d, J = 9.00 Hz, NH), 6.79 (1H, t, J = 54.9 Hz, –CF_2H), 7.02 (1H, d, J = 9.00 Hz), 7.47 (1H, br s), 7.71 (1H, d, J = 9.60 Hz), and 8.05 (1H, br s).

Agent **A**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.14–1.64 (m), 1.81 (3H, s, NHAc), 3.71–3.76 (2H, m), 4.10–4.15 (1H, m), 4.28–4.33 (1H, m), 4.80 (1H, d, J = 9.00 Hz), 6.37 (1H, br s), 6.43 (1H, br s), 6.92 (1H, t, J = 55.5 Hz, –CF_2H), 7.17 (1H, d, J = 9.00 Hz), 7.61–7.86 (4H, m), and 9.98 (0.8H, s). Agent **B**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.15–1.65 (15H, m), 1.81 (3H, s, NHAc), 3.69–3.80 (2H, m), 4.10–4.15 (1H, m), 4.27–4.34 (1H, m), 4.63 (1H, br t, J = 6.30 Hz), 4.81 (1H, d, J = 8.70 Hz, H-1), 5.11 (1H, d, J = 5.40 Hz), 5.16 (1H, d, J = 5.10 Hz), 6.35 (1H, br s), 6.41 (1H, br s), 6.92 (1H, t, J = 55.5 Hz, –CF_2H), 7.19 (1H, d, J = 9.00 Hz), 7.63 (1H, br d, J = 9.60 Hz), 7.70–8.02 (5H, m), and 9.96 (0.6H, s). Agent **D**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.81 (3H, s, NHAc), 3.70–3.77 (2H, m), 4.10–4.14 (1H, m), 4.29–4.32 (1H, m), 4.77 (1H, d, J = 8.00 Hz), 5.31 (2H, dd, J = 1.50, 47.50 Hz, –CFH_2), 6.37 (1H, s), 7.10 (1H, d, J = 9.00 Hz), 7.49–7.82 (4H, m), and 9.86 (0.8H, s).

20. These biotin conjugates are: biotin LCLC NHS, (6-biotinamidocaproylamido)caproic acid *N*-hydroxysuccinimide ester from Sigma B3295; biotin disulfide NHS, biotin disulfide *N*-hydroxysuccinimide ester from Sigma B4531; biotin-LC NHS, biotinamidocaproate *N*-hydroxysuccinimide ester from Sigma B 2643.
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