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Efficient Solid-Phase Synthesis of Trifunctional Probes and Their Application to the Detection of Carbohydrate-Binding Proteins

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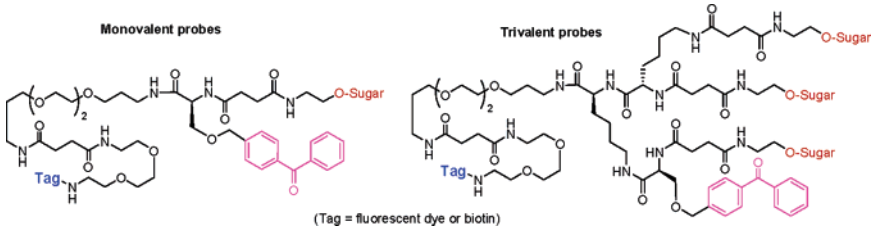
ABSTRACT



An efficient solid-phase synthesis of trifunctional probes containing a photoreactive group, a reporter tag, and a carbohydrate ligand was developed. Labeling studies with these probes demonstrate that specific lectins can be labeled with high sensitivity and selectivity. This technique serves as a powerful tool for the rapid detection and profiling of lectins.

In the post-genomic era, numerous studies of the cellular functions of proteins have been performed in order to elucidate the nature of biological processes and to discover novel therapeutic agents. Among the myriad of proteins found in nature, those that bind carbohydrates (lectins) are extremely important since their specific interactions with glycans play key roles in a wide variety of physiological and pathogenic processes.[1] Analysis of the human genomic sequence suggests that about 100 gene products are lectins.[2] However, the binding properties of only about half of these proteins have been relatively well characterized; the functions of the remaining proteins in this family have not yet been evaluated. To fully understand the biological implications of carbohydrate−protein interactions in living organisms and to help develop novel carbohydrate-based drugs, it is imperative to identify and characterize these unknown lectins and determine their binding specificities. Moreover, a quantitative assessment of the variations in lectins expressed in different cell states and cell types is essential for elucidating their biological roles and identifying disease-related markers.

A new carbohydrate microarray-based technology has emerged recently for the rapid analysis of carbohydrate−protein recognition events.[3,4] This technology has the potential for broad research fields, such as functional glycomics, diagnosis of diseases, and drug discovery. On the other hand, several chemical strategies have been exploited to detect carbohydrate-related proteins in cells, including carbohydrate-

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binding proteins, carbohydrate transporters and carbohydrate-processing enzymes. For example, biotinylated photoaffinity labels have been developed to detect lectins and a fructose or glucose transporter.[5,6] In addition, biotinylated mechanism-based inhibitors have also been designed and utilized to identify glycosidases.[7,8] However, difficulties are still encountered when attempts are made to apply this methodology to the identification of unknown cellular lectins. Thus, although these chemical approaches have been used successfully to identify carbohydrate-related proteins, more efficient methods that are suitable for a systematic study of carbohydrate-binding proteins in cells are required. Toward this end, we have designed an approach for readily detecting and profiling cellular lectins, which relies on trifunctional probes that contain a photoreactive group, a reporter tag and a carbohydrate ligand. These probes, prepared by a highly efficient solid-phase methodology, play a key role in a new technique to detect lectins in cells.

The general structure and strategy for preparation of trifunctional probes developed in this effort are shown in Figure 1. In the procedure, a photoreactive group is

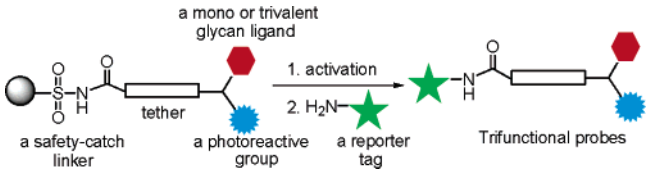
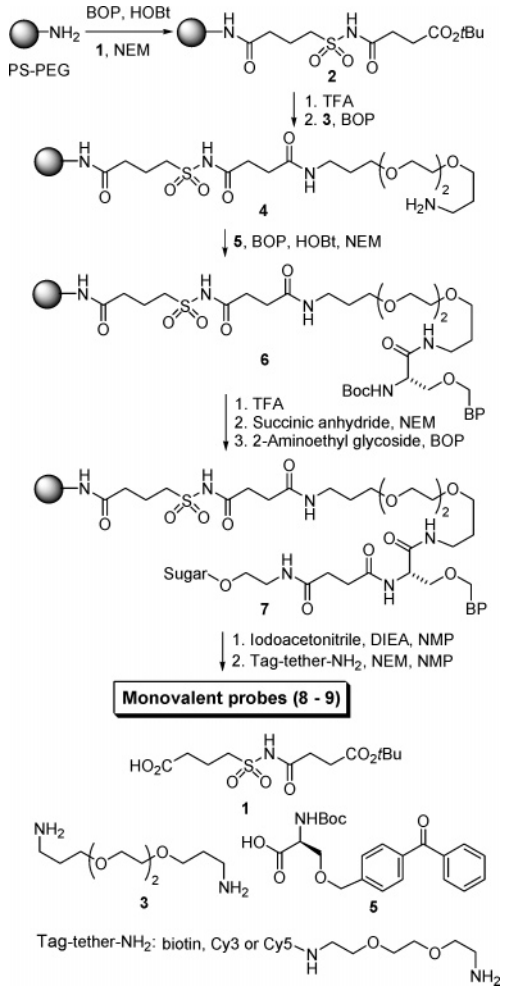


Figure 1. Strategy for preparation of trifunctional probes.

incorporated into the probe for irreversible, covalent labeling of weakly bound lectins, in particular, cell-surface lectins and a reporter (biotin or fluorophore) is inserted to detect and quantify the labeled protein. The general synthetic strategy involves an initial coupling step to covalently link the carbohydrate ligand and photoreactive group to a solid support, and a final step in which the desired probe is released from the resin by reaction with an amine-linked reporter tag after activation of a safety-catch linker.[9]

The initial step in the general route used to synthesize monovalent carbohydrate probes involves attachment of the safety-catch linker-coupled acid **1** to a PS-PEG amine resin to produce **2** (Scheme 1). Since coupling of succinic acid or

Scheme 1. Reaction Sequence for Preparation of Monovalent Carbohydrate Probes[a]



[a] Abbreviations: Boc = *tert*-butyloxycarbonyl; BOP = 1-benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate; Fmoc = 9-fluorenylmethoxycarbonyl; HOBt = 1-hydroxybenzotriazole; NEM = *N*-ethylmorpholine; NMP = *N*-methylpyrrolidinone; PS-PEG = polystyrene-polyethyleneglycol; TFA = trifluoroacetic acid.

succinic anhydride to the safety-catch linker-connected resin was inefficient, a preformed handle strategy was employed.[10] After removal of the *t*-Bu group in **2**, coupling to 4,7,10-trioxa-1,13-tridecanediamine (**3**) was performed to generate the amine terminated resin **4**. Reaction of **4** with the benzophenone (BP)-conjugated serine derivative **5** gave **6**. Sequential Boc deprotection, reaction with succinic anhy-

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dride, and coupling with 2-aminoethyl α-L-fucopyranoside or 2-aminoethyl α-D-mannopyranoside yielded **7**. Finally, after activation of the safety-catch linker in **7** with iodoacetonitrile, reaction with Cy3-tether-NH_2 afforded the monovalent carbohydrate probes **8** and **9** (Figure 2).

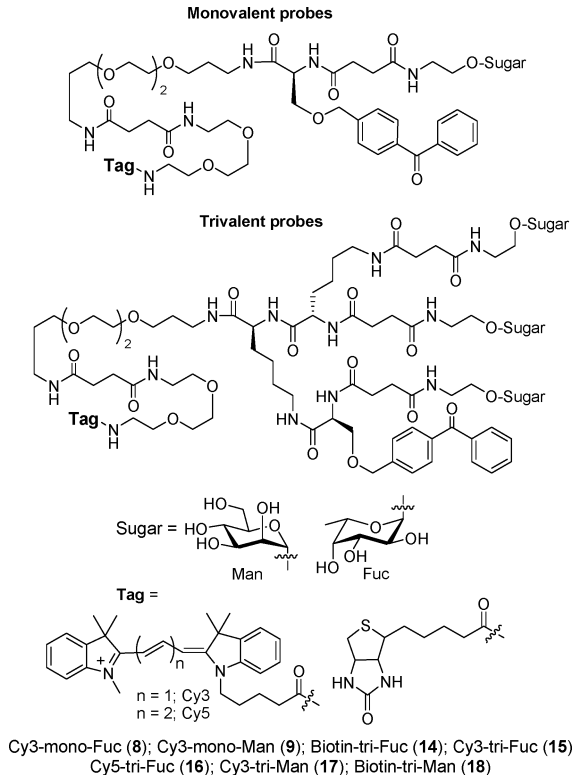
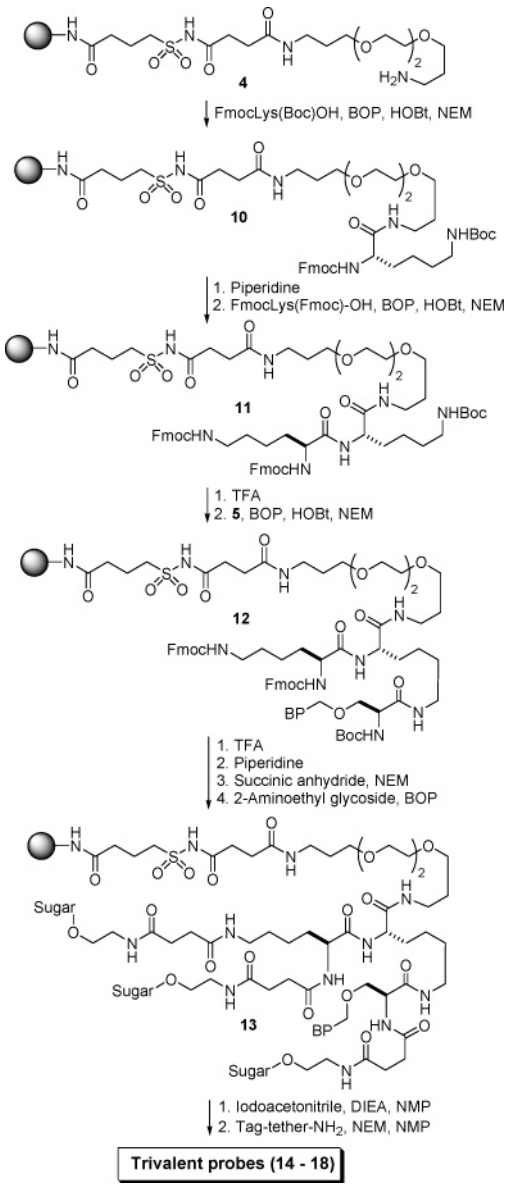


Figure 2. Structures of synthesized trifunctional probes.

In general, interactions between monovalent carbohydrates and proteins are weak. Consequently, multivalent carbohydrate probes are required in order to facilitate labeling of the lectins in cells.[11] In the sequence we have developed for preparation of trivalent carbohydrate probes, the tether-conjugated amine resin **4** was reacted with FmocLys(Boc)OH to give **10** (Scheme 2). Fmoc deprotection in **10** and subsequent coupling with FmocLys(Fmoc)OH produced the selectively protected trisamine resin **11**. The amine formed by removal of the Boc group in **11** was then coupled with **5** to produce **12**. Subjection of **12** to the chemical processes used to transform **6** to monovalent carbohydrate probes resulted in formation of trivalent probes **14**−**18** (Figure 2).

HPLC analyses of the monovalent and trivalent carbohydrate probes showed that the solid-phase synthetic routes were highly efficient. It is worth mentioning that in contrast to monovalent probes, which were easily separated from excess Tag-tether-NH_2 by HPLC, most of the trivalent probes were difficult to separate from excess Tag-tether-NH_2. In these cases, the probes were purified after they were first reacted with Boc_2O (Figure 3).

Scheme 2. Reaction Sequence for Preparation of Trivalent Carbohydrate Probes



The efficiency of protein labeling by the monovalent and trivalent carbohydrate probes were evaluated. The fucose (**8**, **15**) and mannose (**9**, **17**) probes containing Cy3 were preincubated with *A. aurantia* (AA) and ConA, respectively, for 20 min, and the resulting mixtures were irradiated for 5−30 min at 4 °C. Analysis of fluorescence intensities of the proteins after gel separation showed that the amounts of protein labeling by the trivalent probes **15** and **17** were higher than those induced by the monovalent counterparts **8** and **9**.[12] These observations demonstrate that the efficiencies for protein labeling by the trivalent probes are greater than those by the monovalent probes.

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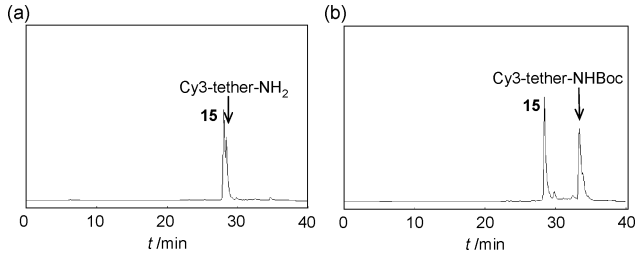

Figure 3. HPLC chromatograms (a) after release of **15** by Cy3-tether-NH_2 and (b) after reaction of the released mixture with Boc_2O.

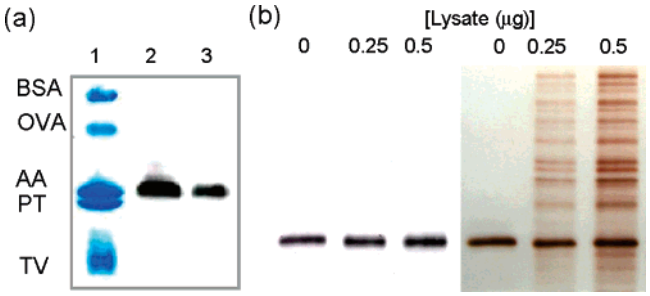

Figure 4. (a) Quantitation of AA labeling under two different conditions (lane 1, Coomassie blue staining; lane 2, Cy3 fluorescence detection; lane 3, Cy5 fluorescence detection): BSA, bovine serum albumin; OVA, ovalbumin; PT, *P. tetrangonolobus*; TV, *T. vulgaris*. (b) Selective labeling of AA by **15** in cell lysates (left, fluorescence image; right, silver staining).

Next, the detection sensitivity of the fluorescence assay for protein labeling was compared with that obtained by using the Western blotting, silver, and Coomassie blue staining techniques. Comparison of the results of labeling experiments, performed by using AA with Biotin-tri-Fuc (**14**) and Cy3-tri-Fuc (**15**), showed that the detection limit (0.4 ng of AA) for the fluorescence assay was superior to that for the Western blotting (3.7 ng of AA), silver (11 ng of AA), and Coomassie blue staining (100 ng of AA) techniques.[12]

Investigations have been carried out to test application of the trifunctional probes for rapid determination of the amount of specific lectins present in two different cell states. To evaluate quantitative aspects of the technique, the mixture of five proteins (BSA, OVA, AA, PT, and TV) containing 800 ng of AA or 200 ng of AA was separately incubated with Cy3-tri-Fuc (**15**) or Cy5-tri-Fuc (**16**), respectively, and then irradiated. The mixtures were combined and then subjected to protein gel separation. The fucose probes selectively labeled AA, and the fluorescence intensities of Cy3 and Cy5 were found to have a ratio of 4:1 (Figure 4a).[13] This result shows that the trifunctional probes can be used to accurately and rapidly quantify lectins. In addition, this methodology was employed in the detection of an exogenous lectin in *E. coli* lysates to demonstrate its potential applications in large-scale proteomic studies. Accordingly, cell lysates (0, 0.25, and 0.5 μg of proteins) containing AA (60 ng) were incubated with **15** and then irradiated. As shown in Figure 4b, AA was selectively labeled by the probe.

In conclusion, an efficient method for synthesizing doubly labeled carbohydrate probes to detect cellular lectins has been developed. This synthetic pathway is applicable to the preparation of various chemical probes, including peptides and small molecules.[14] The results show that probes prepared using this methodology label proteins in a highly sensitive and selective manner. Therefore, they may be useful in the study of poorly characterized lectins predicted from genomic studies. In addition, the probes can be also used for profiling carbohydrate-binding proteins in different cell types or cell states.

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Supporting Information Available: Synthesis of carbohydrate probes and labeling experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) A 1:1 mixture of Cy3 and Cy5 exhibits a fluorescence intensity ratio of 1:1. For additional quantitative protein labeling experiments, see the Supporting Information.

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