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Syntheses and Biological Evaluations of α-D-Mannosyl [60]Fullerenols

Haruhito Kato,[a] Arihiro Yashiro,[b] Akiko Mizuno,[b] Yoshihiro Nishida,[b] Kazukiyo Kobayashi[b] and Hisanori Shinohara[a,*]

[a]*Department of Chemistry, Nagoya University, Nagoya 464-8602, Japan*
[b]*Department of Molecular Design and Engineering, Graduate School of Engineering, Nagoya University, Nagoya 464-8603, Japan*

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Abstract—[60]Fullerenols carrying mono- and bis-α-D-mannosyl linkages on the surface were prepared via a [3+2]-cycloaddition reaction between 2-azidoethyl α-D-mannoside and C_{60} followed by polyhydroxylation with aqueous NaOH. Their biological activity was evaluated in terms of binding affinity to lectins by hemagglutination assay and surface plasmon resonance. [60]Fullerenols without the mannosyl linkage caused aggregation of erythrocytes and binding to a β-D-galactopyranoside specific lectin (RCA_{120}). In contrast, mono- and bis-mannosyl fullerenols were found to decrease the activity for both aggregating erythrocytes and binding to RCA_{120}, and mono-mannosyl fullerenols turned to binding to α-D-mannose specific lectin (Con A).

Introduction

Polyhydroxylated fullerenes (fullerenols) have gained many interests in their medical applications owing to their excellent water solubility and unique biological activities.[1–4] [60]Fullerenols have been reported to function as radical scavengers,[1,2] anticancer reagents,[3] and HIV-1 inhibitors.[4] Chiang et al.[1] reported in their chemiluminescence study that some of these activities were attributable to their strong ability to scavenge superoxide radicals (O_2^-). Systematic removal of these excessive oxygen radicals by the fullerenols is useful to a preventive therapy against many diseases. Another notable utility can be ascribed to the capability of higher fullerenols (fullerenols based on higher fullerenes) to trap metal atoms inside of the cage. Recently, we reported the synthesis of metallo-fullerenols, $Gd@C_{82}(OH)_n$, which were designed to act as highly sensitive magnetic resonance imaging (MRI) contrast agents.[5,6] Current in vivo studies of these metallo-fullerenols and other water soluble fullerenes will provide much information on the scope and limitation of [60]fullerenes as a novel class of biologically active compounds.

In the present study, we report the synthesis and characterization of glycosyl [60]fullerenols, because cytoselectivity may be embedded to the biological functions of fullerenols. Previously,[7] we reported the general synthesis of [60]fullerene glycoconjugates. Here, we have further developed and extended the idea of glycosylation to the polyhydroxylated fullerenes.

Synthesis

Molecular design of mannosyl [60]fullerenols M-1 and M-2

Cell surface glycosyl chains are biologically active components of glycoproteins and glycolipids and become the ligand of carbohydrate receptor proteins. Each of the carbohydrate residues at the non-reducing terminal is expected to construct a key interactive module[8] in the carbohydrate-protein binding interactions. For example, α-D-mannoside linkage in *N*-glycoside glycoproteins is recognized by macrophages and certain types of pathogenic bacteria. A simple D-mannose or a 3,6-branched mannotriose can, therefore, be applied to create bioactive compounds of these species.[9,10] In this study, we designed α-D-mannosyl [60]fullerenols (**M-1**, Scheme 1) to examine the binding property to carbohydrate specific proteins (lectins).

*Corresponding author. Fax: +81-0-52-789-2962; e-mail: nori@nano.chem.nagoya-u.ac.jp

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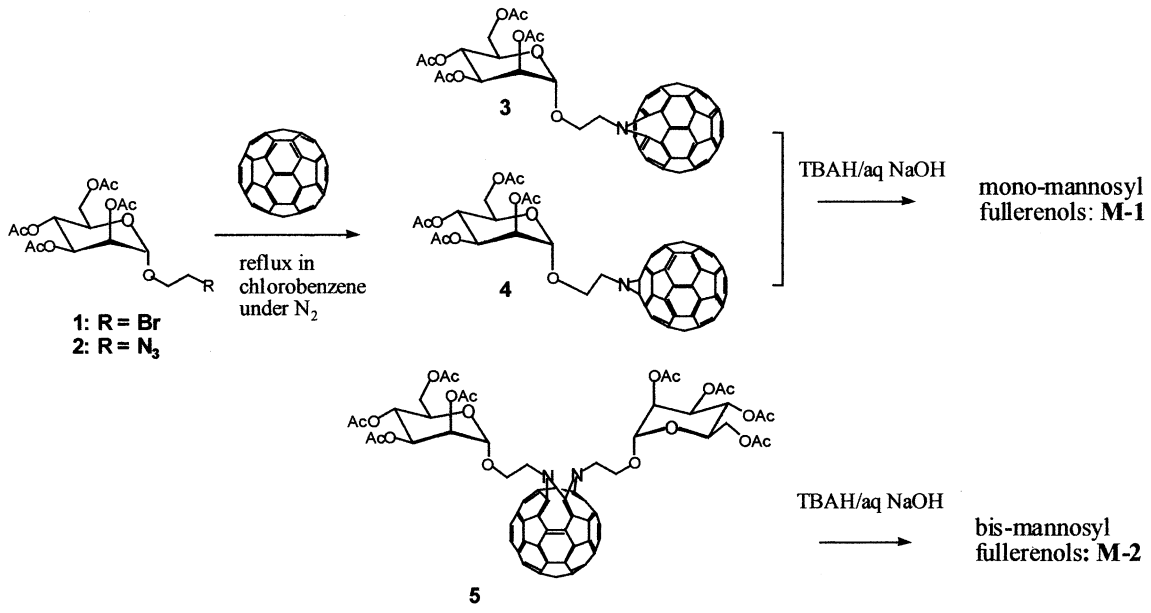

Scheme 1. Syntheses of α-D-mannosyl [60]fullerenes and fullerenols.

Application of our previous method[7] to α-mannosyl [60]fullerene was hampered by the difficulty in deriving per-*O*-acetyl α-D-mannosyl azide from α-mannosyl bromide. The S_N2 displacement reaction with sodium azide was also depressed by the axial vicinal 2-OAc group. We, therefore, undertook an alternative synthetic route which starts from 2-bromoethyl α-D-mannoside **1**[11] towards **M-1** via mannosyl [60]fullerenes (Scheme 1). The 2-bromoethyl sugar **1** was treated with sodium azide to give 2-azido derivative **2**. The thermal coupling reaction between **2** and an equimolar amount of C_{60} in chlorobenzene gave a mixture of mono- and bis-mannosyl adducts (silica gel TLC, $R_f = 0.66$ and $R_f = 0.48$, toluene/ethyl acetate = 10:1).[12] The products were separated on a silica gel column into a mixture of mono-mannosyl adducts **3** and **4** [LD-TOF mass: $m/z = 1109$ (M^-), base peak at m/z 720 (C_{60}^-)] and a bis-adduct **5** [$m/z = 1498$ (M^-)].

The two mono-adducts were separated in 5:2 ratio on an HPLC column (silica gel, toluene/ethyl acetate = 9:1) and were identified as α-D-mannosyl [5,6]-azafulleroid **3** and α-D-mannosyl [6,6]-aziridino fullerene **4** by ^{13}C NMR spectroscopy in reported manners.[13–16] In the spectrum of **3**, 48 lines of sp^2 carbons of the fullerene cage were observed between 133 and 150 ppm. No sp^3 carbon was detected between 70 and 100 ppm.[17] In contrast, isomer **4** gave a sp^3 carbon at 94 ppm together with 15 lines of sp^2 carbon between 140 and 150 ppm.[18] From the ^1H NMR spectra, both isomers **3** and **4** have formed to take a $^4C_1$ ring conformation at the mannoside moiety.

The bis-mannosyl adduct **5** gave broad ^1H NMR signals for the sugar ring protons, suggesting the rotational restriction at the glycoside moiety, presumably due to steric hindrance between the two geminal mannoside linkages. The ^1H NMR spectrum showed that the two glycosides having a $^4C_1$ ring conformation are magnetically non-equivalent. Any HPLC conditions studied here, however, did not give two peaks for the bis-adduct. These data as well as the reaction mechanism reported previously[13–16] support the idea that the bis-adduct was a 1,1-bis-mannosyl [5,6]azafulleroid **5**.[19]

Simultaneous deacetylation and polyhydroxylation were conducted in a reported way.[20] At present, a mixture of **3** and **4** was converted to mono-mannosyl fullerenols **M-1**.[21] C_{60} without the mannosyl linkage and its bis-adduct were also converted to fullerenols **M-0** and to bis-mannosyl fullerenols **M-2**,[22] respectively. The average degree of hydroxylation under the present conditions was estimated to be 29×OH per C_{60} on the basis of the elementary analysis.[6] The ^1H NMR spectra of **M-1** and **M-2** gave broad signals between 3.5 and 4.4 ppm for sugar C–H protons. The broadening suggests that the polyhydroxylation occurred heterogeneously at the position which gives rise to a complex mixture of fullerenols.

Biological Evaluations

Lectin induced hemagglutination inhibition assay

We can evaluate the biological activity of **M-1** and **M-2** in terms of blocking activity against carbohydrate-protein interactions. A lectin-induced hemagglutination assay was performed using an α-D-mannoside-specific protein (Con A) for a series of non-, mono-, and bis-mannosyl fullerenols in a reported manner.[23] Fullerenols **M-0** without the mannoside linkage and 2-azidoethyl α-D-mannopyranoside without the fullerenols moiety were used as the reference compounds. The minimum inhibition concentration of **M-2** (MIC = 1.0×10^{-4} M) resulted in a moderate blocking activity comparable to the activity of 2-azidoethyl α-D-mannopyranoside (1.3×10^{-4} M).

The MIC values, on the other hand, could not be determined for **M-0** and **M-1** because these fullerenols

themselves caused hemagglutination even in the absence of the lectin. Some kinds of polyhydroxyl compounds such as dextrans and arabinoxylans are known to cause a similar activity,[24,25] though the precise mechanism has not been clarified yet. Under the present assay conditions, dextrans ($M_n = 1.6\times10^5$) induced the aggregation at the minimum concentration of 1.3×10^{-4} M. The mono-mannosyl fullerenols **M-1** and the monnose-free fullerenols **M-0** showed aggregation activity at lower concentrations of 7×10^{-5} and 7×10^{-6} M, respectively, whereas the bis-mannoside **M-2** did not show any aggregation even at the concentrations of $>1\times10^{-2}$ M (Table 1). These results indicate that the strong biological activity of fullerenols to aggregate erythrocytes can be reduced by mono-mannosylation and diminished by bis-mannosylation on the surface.

The erythrocyte aggregation seems to be a serious problem for in vivo applications of fullerenols. This biological property as well as the effect of mannosylation as described above have fundamental significance to study the modification of fullerenols for biological applications.

SPR measurements of the binding property of mannosyl fullerenols to lectins

Surface plasmon resonance (SPR) was employed as an alternative approach to determine the lectin binding activity of the mannosyl fullerenols. A biotin-labeled Con A was immobilized on avidine-modified gold surfaces in a reported way.[26–29] β-D-Galactose specific lectin RCA_{120} and bovine serum albumin (BSA) were used as reference proteins to evaluate the specificity in the binding interactions.

Figure 1a plots the angle changes ($\Delta\theta$) at the equibrium binding to protein monolayers against the concentrations of **M-1**. The Langmuir-type binding isotherms having the maximum angle changes ($\Delta\theta_{max}$) suggested that these C_{60} fullerenols were adsorbed as monolayers. Apparent affinity constants (K_a, Table 1) were calculated from the slopes and intercepts according to eq 1, where [S] stands for the concentration of fullerenols.

$$[S]/\Delta\theta = 1/(K_a\Delta\theta_{max}) + [S]/(K_a\Delta\theta_{max}) \quad (1)$$

Table 1. Erythrocyte aggregating activity and lectin binding activity of fullerenols and mannosyl [60]fullerenols[a]

Sample	Erythrocytes aggregating activity (M)	Binding constant ($K_a\times10^{-4}$ M)[b]		
		Con A	RCA_{120}	BSA
M-0	7×10^{-6}	5.1	200	8.4
M-1	7×10^{-5}	24	15	3.6
M-2	$>1\times10^{-2}$	5.1	30	3.1

[a]Arbitrarily assuming general formula of $C_{60}(OH)_{29}(NCH_2CH_2O\text{-}C_6H_6O_5)_n\cdot12H_2O$ ($n = 0, 1, 2$).
[b]Which gave molecular weights **M-0** ($M_n = 1443.84$), **M-1** ($M_n = 1650.73$), and **M-2** ($M_n = 1867.82$). The hydration degree may vary. Determined by SPR measurements.

The mannose-free fullerenols **M-0** showed strong binding to RCA_{120} ($K_a = 2.0\times10^6$ M). Judging from the activity of **M-0** to the other proteins, the binding activity seems to be selective to the β-galactoside specific protein, whereas mannosyl fullerenols **M-1** and **M-2** did not induce any notable binding to RCA_{120}. Instead, the mono-mannosyl fullerenols **M-1** showed moderate affinity for Con A ($K_a = 2.4\times10^5$ M) rather than RCA_{120} and BSA. This means that the mannosyl linkage may change the binding property of fullerenols from the β-galactoside specific protein into the α-D-mannoside specific protein. The bis-mannosyl adduct **M-2** showed a similar tendency, though the binding to Con A was less prominent than that of **M-1**. The geminal mannosides may give rise to steric hindrance upon the binding to

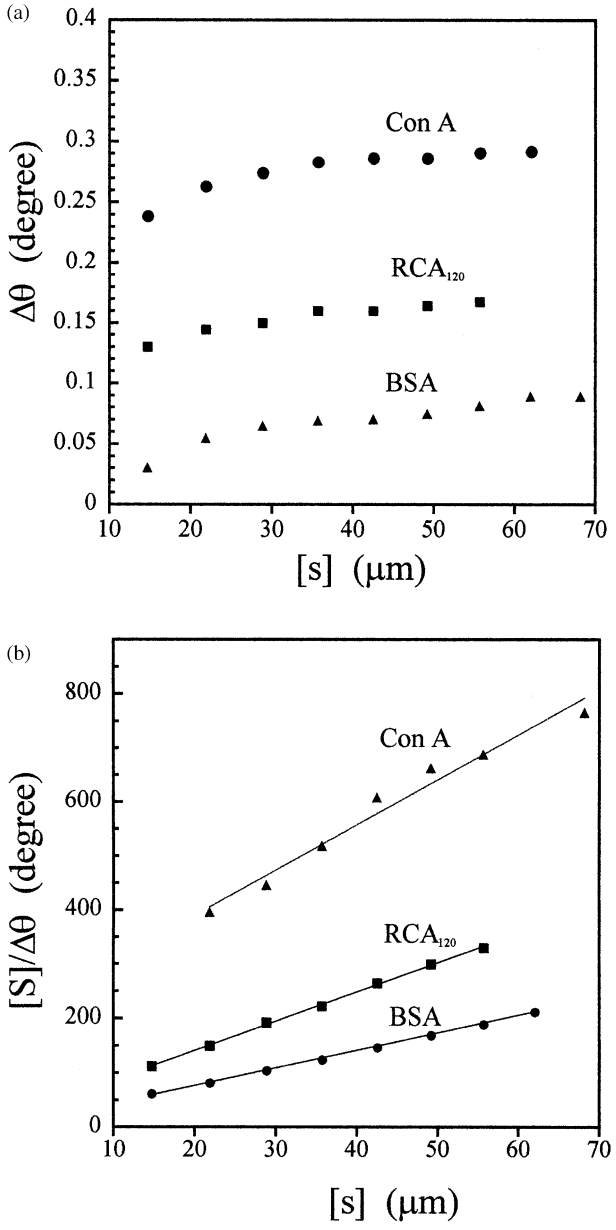

Figure 1. SPR analysis of interaction between mannosyl fullerenols **M-1** and biotin-labelled lectins: (a) typical time courses of the angle change ($\Delta\theta$) of SPR response by the addition of aqueous solution of **M-1**; (b) reciprocal plots to determine binding constants (K_a).

Con A in a manner similar to a negative carbohydrate-clustering effect.[30]

These data indicate that fullerenols **M-0** can become the artificial ligand of β-galactoside specific proteins. This property may be rationalized by the unique molecular topology carrying poly *cis*-diols on the surface. The mannosyl fullerenols **M-1** and **M-2** showed reduced activity for the binding to RCA_{120}. This result might be correlated with the effect of mannoside linkage to minimize the cell–cell aggregation as observed in the preceding biological assay.

Conclusion

We have reported the first synthesis and some biological characterization of α-D-mannosyl [60]fullerenols. We found that fullerenols without the glycoside linkage per se possess notable biological activities of erythrocyte aggregation and binding to a β-galactoside specific protein. Mannosyl linkage on the fullerenols was found to significantly reduce these activities. Although the mannosyl fullerenols could not show strong binding to the mannoside specific protein (Con A) compared to the activity of mannosyl neoglycopolymers[9] ($K_a = 1.0 \times 10^7$ M), these results can definitely provide significant bases for the future molecular design of cytoselective [60]fullerenols and higher metallo-fullerenols.

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12. A solution of C_{60} (72 mg, 0.10 mmol) and **2** (42 mg, 0.10 mmol) in chlorobenzene (50 mL) was heated under reflux for 10 h and then the solvent was removed in vacuo. The crude product was purified by silica gel column chromatography (eluent toluene/ethyl acetate = 10:0–10:2), affording 21 mg (19%) of a mixture of **3** and **4**, 15 mg (10%) of **5**, and 41 mg (58%) of recovered C_{60}.
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17. **2-(Aza-1,6-homo C_{60} fulleryl) ethyl tetra-*O*-acetyl-α-D-mannopyranoside (3).** ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.39 (dd, ^3J (H, H) = 3.0, 10.0 Hz, 1H), 5.36 (dd, ^3J (H, H) = 2.0, 3.0 Hz, 1H), 5.35 (t, ^3J (H, H) = 10.0 Hz, 1H), 5.06 (d, ^3J (H, H) = 1.5 Hz, 1H), 4.29 (dd, ^3J (H, H) = 5.5, 12.0 Hz, 1H), 4.13 (dd, ^3J (H, H) = 2.5, 12.0 Hz, 1H), 4.39–4.31 (m, 1H), 4.36 (ddd, ^3J (H, H) = 2.5, 5.0, 10.0 Hz, 1H), 4.25–4.17 (m, 1H), 4.05 (ddd, ^3J (H, H) = 3.5, 5.0, 13.0 Hz, 1H), 4.02 (ddd, ^3J (H, H) = 3.5, 5.0, 13.0 Hz, 1H), 2.19 (s, 3H), 2.13 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.70, 20.79, 20.84, 20.92, 51.57, 62.53, 66.16, 67.36, 68.87, 69.00, 69.49, 97.63, 133.92, 135.97, 136.12, 136.44, 136.52, 136.59, 137.82, 137.94, 138.02, 138.36, 138.40, 138.56, 139.41, 139.42, 140.43, 140.87, 140.91, 141.51, 141.55, 142.75, 142.76, 142.77, 142.79, 142.93, 142.94, 143.14, 143.25, 143.42, 143.43, 143.61, 143.63, 143.67, 143.85, 143.87, 143.88, 143.94, 144.15, 144.17, 144.29, 144.30, 144.33, 144.42, 144.57, 144.60, 144.69, 144.72, 144.90, 144.95, 145.78, 145.86, 147.73, 147.83, 169.71 (2C), 170.07, 170.65. MS (LD-TOF, dithranol matrix) *m*/*z* 1109 (M^-), 720. IR (KBr, ν cm^{-1}): 2924, 1751, 1558, 1427, 1367, 1225, 1138, 1084, 1049, 526.
18. **2-(*N*,*N*-Aziridino C_{60} fulleryl)-ethyl tetra-*O*-acetyl-α-D-mannopyranoside (4).** ^{1}H NMR(500 MHz, $CDCl_3$) δ 5.54 (dd, ^3J (H, H) = 3.4, 10.3 Hz, 1H), 5.45 (dd, ^3J (H, H) = 1.5, 3.4 Hz, 1H), 5.34 (t, ^3J (H, H) = 9.8 Hz, 1H), 5.15 (d, ^3J (H, H) = 1.5 Hz, 1H), 4.60 (ddd, ^3J (H, H) = 3.6, 7.6, 11.3 Hz, 1H), 4.49 (ddd, ^3J (H, H) = 2.4, 4.5, 10.3 Hz, 1H), 4.41 (dd, ^3J (H, H) = 4.9, 12.3 Hz, 1H), 4.28 (dd, ^3J (H, H) = 2.5, 12.2 Hz, 1H), 4.27 (ddd, ^3J (H, H) = 3.9, 5.4, 11.3 Hz, 1H), 4.06 (ddd, ^3J (H, H) = 3.6, 7.8, 13.3 Hz, 1H), 3.99 (ddd, ^3J (H, H) = 3.9, 4.9, 12.7 Hz, 1H), 2.18 (s, 3H), 2.15 (s, 3H), 1.97 (s, 3H), 1.94 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.61, 20.75, 20.85, 20.91, 49.91, 62.46, 66.18, 67.46, 68.84, 69.02, 69.48, 94.74, 97.86, 140.97, 142.12, 142.27, 142.87, 143.08, 143.79, 143.87, 144.55, 144.57, 144.62, 144.73, 144.82, 145.17, 145.23, 169.50, 169.69, 170.04, 170.68. MS (LD-TOF, dithranol matrix) *m*/*z* 1109 (M^-), 720. IR (KBr, ν cm^{-1}) 2922, 1751, 1429, 1367, 1225, 1049, 526.
19. **Bis(octa-*O*-acetyl-α-D-mannopyranosyl) C_{60} fullerene (5).** ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.54 (dd, ^3J (H, H) = 3.5, 10.0 Hz, 1H), 5.47 (dd, ^3J (H, H) = 1.5, 3.5 Hz, 1H), 5.42 (d, ^3J (H, H) = 3.5, 10.0 Hz, 1H), 5.37 (dd, ^3J (H, H) = 1.5, 3.5 Hz, 1H), 5.36 (t, ^3J (H, H) = 10.0 Hz, 1H), 5.35 (t, ^3J (H, H) = 10.0 Hz, 1H), 5.10 (d, ^3J (H, H) = 1.5, 1H), 5.07 (d, ^3J (H, H) = 1.5 Hz, 1H), 4.35–4.07 (m, 10H), 2.19 (s, 3H), 2.18 (s, 3H), 2.10 (s, 3H), 2.09 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.94 (s, 3H). ^{13}C NMR (125 Mz, $CDCl_3$) δ 20.02 (br), 49.56 (br), 62.40 (br), 66.02 (br), 69.39 (br), 97.48 (br), 130.22, 131.03, 132.37, 132.75, 132.85, 133.78, 135.02, 135.51, 135.87, 136.09,

136.86, 137.90, 138.63, 139.37, 139.39, 139.43, 139.70, 139.82, 140.96, 141.44, 141.66, 141.73, 141.77, 141.78, 142.57, 142.60, 142.79, 143.28, 143.41, 143.47, 143.80, 143.82, 143.90, 143.95, 144.03, 141.10, 144.47, 144.48, 144.54, 144.55, 144.59, 144.60, 144.70, 144.80, 144.87, 145.00, 145.08, 145.20, 145.49, 145.61, 146.63, 146.94, 147.20, 147.52, 161.92, 169.64 (2C), 169.97, 170.00 (2C), 170.21, 170.50, 170.63. MS (LD-TOF, dithranol matrix) m/z 1498 (M^-) 1109, 720. IR (KBr, ν cm^{-1}) 2924, 2854, 1749, 1433, 1369, 1225, 1085, 1049, 526.

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21. **Mono-α-D-mannopyranosyl C_{60} fullerenols (M-1).** To a solution of a mixture (15.0 mg, 0.016 mmol) of **3** and **4** in toluene (5 mL) were added tetrabutyl ammonium hydroxide (TBAH, 5 drops) and 50 wt% of sodium hydroxide (2.0 g), and then the mixture was stirred at ambient temperature for 30 min. After toluene was removed in vacuo, the residue was diluted with water (50 mL) and then stirred at ambient temperature for 12 h. The solution was poured into an excess amount of methanol to yield a brown precipitate. The precipitate was dissolved in water, dialyzed in a methyl cellulose tube (MW cut off 500) against water for 3 days, and freeze-dried to give 10.0 mg of 6 (37%) as a brown solid. ^{1}H NMR (500 MHz, D_2O) δ 3.5–4.6 brs. IR (KBr, ν cm^{-1}) 3398, 2924, 1714, 1622, 1392, 1088, 579.

22. **Bis-α-D-mannopyranosyl C_{60} fullerenols (M-2). M-1** was prepared from 5 in the same way as described for **M-1** (24 mg, 48%). ^{1}H NMR (500 MHz, D_2O) δ 3.5-4.6 brs. IR (KBr, ν cm^{-1}) 3404, 1622, 1604, 1383, 1061, 579.

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29. **Preparation of an avidine-modified gold-deposited glass plate**. A freshly-prepared gold-deposited glass plate was soaked in a 1.0 mM ethanol solution of dithiododecanoic acid overnight, washed with ethanol solution, and treated with a 90% 1,4-dioxane aqueous solution (10 mL) of 1-ethyl-3-(3-dimethylpropyl)-carbodiimide (25 mg) and *N*-hydroxysuccimide (15 mg) for 15 min. Then the plate was washed with ethanol several times, treated with avidine PBS solution (10 mL) for 3 h, immersed into 1.0 mM 2-aminoethanol PBS solution for 2 h, and rinsed with distilled water. **SPR measurement**. The avidine-modified gold plate was attached to the prism (n = 1.518) on a surface plasmon resonance apparatus (SPR 670, Nippon Laser & Electronics Lab., Nagoya, Japan). Aliquots (10 μL) of a 18.3 μM PBS solution of biotin-labeled lectin was injected into PBS buffer (100 μL per a batch) cell until the reflectron angle became constant, followed by washing excess lectin from the cell. Aliquots (2.0 mL) of a stock solution of fullerenols–glycoconjugates were injected into PBS buffer (200 μL) in the cell. The change of the incident angle (Δθ) was recorded as a function of time at 298 K.

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