A Submicrogram-Scale Protocol for Biomolecule-Based PET Imaging via Rapid $6\pi$-Azaelectrocyclization: First Visualization of Sialic acid-Dependent Circulatory Residence of Glycoproteins

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All commercially available reagents were used without further purification. Dichloromethane were refluxed over and distilled from CaH₂. Anhydrous DMF was purchased from Aldrich, and anhydrous THF was purchased from Kanto Chemicals, Tokyo. Preparative separation was usually performed by column chromatography on silica gel (FUJI silysia LTD, BW-200 and BW-300) and by thin layer chromatography on silica gel (Merek, 20 x 20 cm, Silica gel 60 F254, 1 mm). ¹H NMR and ¹³C NMR spectra were recorded on a JEOL JNM-LA 500 spectrometer and chemical shifts were represented as δ-values relative to the internal standard TMS. IR spectra were recorded on a JASCO FT/IR-8000 Fourier Transform Infrared Spectrometer. ESI-mass spectra were recorded on Applied Biosystems Marinar™ Biospectrometry Workstation. MALDI-TOF-mass spectra were measured on an SHIMADZU AXIMA-CFR mass spectrometer equipped with a nitrogen laser (λ = 337 nm). High resolution mass spectra (HRMS) were measured on a JEOL JMS-T100LC mass spectrometer. Melting points were uncorrected.

**(E)-N-tert-Butoxycarbonyl-2-amino-N-(4-ethenylphenyl)acetamide.** To a solution of 4-ethenylaniline (500 mg, 4.27 mmol) in dry DMF (10 mL) was added N-tert-butoxycarbonyl glycine (747 mg, 4.26 mmol), HBTU (1.94 g, 5.12 mmol) and triethylamine (1.18 mL, 8.53 mmol) at room temperature. After the reaction mixture was stirred under argon atmosphere at room temperature, the mixture was quenched by H₂O and extracted with ethyl acetate. The organic layer was washed with brine, dried over MgSO₄, filtered, and concentrated in vacuo to give the crude product. The residue was purified by column chromatography on silica gel (50 % ethyl acetate in hexane) to give the corresponding acetylene as a yellow powder (1.06 g, 91 %): mp 149-150 °C; IR (neat, cm⁻¹) 3325, 2110, 1688; ¹H NMR (500 MHz, CDCl₃) δ 8.31 (brs, 1H), 7.49 (d, J = 8.7 Hz, 2H), 7.44 (d, J = 8.7 Hz, 2H), 5.26 (brs, 1H), 3.92 (d, J = 4.7 Hz, 2H), 3.04 (s, 1H), 1.48 (s, 9H); ¹³C NMR (125 MHz, acetone-δ₆) δ 169.2, 157.0, 140.3, 133.3, 120.0, 118.0, 84.1, 79.5, 78.2, 45.4, 28.5; ESI HRMS m/z calcd for C₁₃H₁₈N₂O₃Na (M+Na)⁺ 297.1215, found 297.1214.

**(E)-N-tert-Butoxycarbonyl-2-amino-N-{4-[2-(tributylstannyl)vinyl]phenyl}acetamide (1).** To a solution of the acetylene obtained above (582 mg, 2.12 mmol) in dry DMF (3.0 mL) and benzene (3.0 mL) was added AIBN (13 mg, 0.084 mmol) and Bu₃SnH (742 µL, 2.75 mmol) at room temperature. After the reaction mixture was stirred under argon atmosphere at 90 °C for 2h, the mixture was concentrated in vacuo to give the crude product. The residue was purified by column chromatography on silica gel (from 10 % to 25 % ethyl acetate in hexane) to give vinylstannane 1 as a colorless oil (1.0 g, 83 %): mp 79-84 °C; IR (neat, cm⁻¹) 3372, 2956, 2928, 1690, 1669; ¹H NMR (500 MHz, CDCl₃) δ 7.43 (d, J = 8.2 Hz, 2H), 7.23 (d, J = 8.4 Hz, 2H), 6.73 (d, J = 19.4 Hz, 1H), 6.65 (d, J = 19.4 Hz, 1H), 5.83 (brs, 1H), 3.86 (brs, 1H), 2.69 (s, 3H), 1.92 (s, 3H), 1.46 (tt, J = 26.4, 8.7 Hz, 6H), 1.35 (s, 9H), 1.25 (q, J = 7.4 Hz, 6H), 1.14 (t, J = 7.2 Hz, 6H), 0.81 (t, J = 7.3 Hz, 9H); ¹³C NMR (125 MHz, acetone-δ₆) δ 168.8, 154.0, 146.6, 139.4, 131.2, 127.1, 120.1, 113.7, 79.3, 45.3, 38.7, 28.6, 27.9, 14.0, 10.1; ESI HRMS m/z calcd for C₂₇H₄₆N₂O₃SnNa (M+Na)⁺ 589.2433, found 589.2436.

**Ethyl (E,E)-[2-(N-tert-Butoxycarbonyl-2-aminoacetamide)styryl-4-(tetrahydro-2H-pyran-2- yloxy)]but-2-enolate.** To a solution of Pd₂(dba)₃ (17 mg, 0.019 mmol) in DMF (1.0 mL) was added P(2-furyl)₃ (18 mg, 0.078 mmol) at room temperature under argon atmosphere. After the mixture was stirred for 10 min at room temperature giving a green colored solution, a solution of vinylstannane 1 (500 mg,
0.983 mmol) and vinyl bromide 2 (345 mg, 1.18 mmol) in DMF (4.0 mL) and lithium chloride (82 mg, 1.96 mmol) were added at room temperature. After the reaction mixture was stirred for 30 min at 110 °C, 10 % aqueous NH₃ solution was added, and the resulting mixture was extracted with ethyl acetate. The organic layers were combined, washed with brine, dried over MgSO₄, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (from 10 % to 50 % ethyl acetate in hexane) to give the corresponding coupling product (478 mg, 73 %) as a yellow solid: IR (neat, cm⁻¹) 3328, 2939, 1685, 1601; ¹H NMR (500 MHz, CDCl₃) δ 8.51 (brs, 1H), 7.51 (d, J = 8.4 Hz, 2H), 7.39 (d, J = 8.4 Hz, 2H), 6.85-6.71 (m, 3H), 5.44 (brs, 1H), 4.70 (t, J = 3.5 Hz, 1H), 4.60 (dd, J = 14.9, 5.7 Hz, 1H), 4.39 (dd, J = 14.9, 6.5 Hz, 1H), 4.27 (m, 2H), 3.94 (d, J = 6.1 Hz), 3.88 (m, 1H), 3.54 (m, 1H), 1.86 (m, 1H), 1.75 (m, 1H), 1.59 (m, 2H), 1.48 (s, 9H), 1.34 (t, J = 7.1 Hz, 3H); ¹³C NMR (125 MHz, CD₂OD) δ 170.5, 168.3, 158.3, 139.6, 139.3, 135.5, 134.3, 132.8, 128.2, 121.3, 120.9, 100.1, 80.8, 65.2, 63.5, 62.1, 45.1, 31.7, 28.7, 26.5, 20.5, 14.5; ESI HRMS m/z calcd for C₂₀H₄₆N₂O₇Na (M+Na)⁺ 511.2420, found 511.2417.

**Ethyl (E,E)-4-Hydroxy-2-[4-(2-aminoacetamide)styryl]but-2-enoate (3).** To a solution of the coupling product obtained above (208 mg, 0.426 mmol) in MeOH (3.0 mL) was added 6M HCl solution (3.0 mL) dropwise at 0 °C. After the reaction mixture was stirred at room temperature for 2 h, the mixture was extracted with ethyl acetate. The mixture was neutralized by 1M NaOH solution and subjected to size-partitioning gel-filtration through a column filled with HP-20 to give 3 as a colorless oil (130 mg, quant): IR (neat, cm⁻¹) 3335, 3227, 2975, 2935, 1639, 1591; ¹H NMR (500 MHz, CDCl₃) δ 7.56 (2H, d, J = 8.7 Hz), 7.36 (2H, d, J = 8.5 Hz), 6.78 (1H, d, J = 16.3 Hz), 6.70 (1H, d, J = 16.3 Hz), 6.69 (1H, t, J = 6.1 Hz), 4.38 (2H, d, J = 6.1 Hz), 4.17 (2H, q, J = 7.1 Hz), 3.77 (2H, s), 1.23 (3H, t, J = 7.1 Hz); ¹³C NMR (100 MHz, CD₂OD) δ 168.4, 165.3, 142.6, 139.1, 135.3, 134.8, 131.6, 128.3, 121.2, 121.0, 62.1, 60.0, 42.3, 14.5; ESI HRMS m/z calcd for C₁₆H₂₀N₂O₇Na (M+Na)⁺ 327.1321, found 327.1314.

**[(1E,3E)-10-[2-[2-(4-(3-Ethoxycarbonyl-5-oxopenta-1,3,-dienyl)-phenylamino)-2-oxoethylamino]-4,7,10-tetraazacyclodecane]-1,4,7-triacetic acid (4a).** To a solution of 3 (2.4 mg, 7.8 µmol) in DMF (200 µL) was added DOTA-OSu (4.3 mg, 8.6 µmol) and triethylamine (10 µL, 78 µmol) at room temperature. After the mixture was stirred at room temperature for 4 h, the solution was concentrated in vacuo. The residue was directly subjected to size-partitioning gel-filtration through a column filled with HP-20 (CHCl₃ : MeOH : H₂O = 10 : 10 : 1) to give the corresponding DOTA-alcohol (3.1 mg, 58%) as a colorless solid, which was oxidized without further purification: ESI HRMS m/z calcd for C₂₆H₄₆N₂O₁₁Na (M+Na)⁺ 713.3122, found 713.3128.

To a solution of the alcohol obtained above (1.5 mg, 2.1 µmol) in DMF (90 µL) and CH₂Cl₂ (200 µL) was added Dess-Martin periodinane (1.8 mg, 4.3 µmol) at room temperature, and the mixture was stirred at room temperature; the oxidation being estimated to proceed in near quantitative yield after 30 min by ESI-MS analysis of the crude reaction mixtures. The mixture was directly subjected to size-partitioning gel-filtration through a column filled with HP-20 (CHCl₃ : MeOH : H₂O = 10 : 10 : 1) and the yellowish elution fractions were concentrated in vacuo at room temperature to give 4a as a DMF solution, the material being pure enough for subsequent labeling studies. Since the aldehyde partially decomposed during the concentration of DMF or the prolonged exposure of the light at room temperature, a DMF
solution of 4a was immediately reacted with lysines after the identification of 4a by rapid measurement of ESI MS: m/z calcd for C_{32}H_{42}N_{10}O_{11} (M+H)^+ 689.3, found 689.1.

Ethyl (E,E)-4-Oxo-2-{4-[(7-diethylaminocoumarin-3-carboxamide)acetamide]styryl}but-2-enoate (4b). To a solution of the alcohol 3 (200 µg, 650 nmol) in DMF (50 µL) was added coumarin-OSu (230 µg, 650 nmol) at room temperature. After the reaction mixture was stirred at room temperature for 30 min, the mixture was concentrated in vacuo. The crude mixture was purified by preparative thin layer chromatography on silica gel (CHCl₃ : MeOH = 10 : 1) to give the corresponding alcohol (240 µg, 68 %) as a yellow solid, which was oxidized without further purification due to the instability of the coumarin fluorophore: ESI MS m/z calcd for C_{30}H_{34}N_{3}O_{7} (M+H)^+ 548.2, found 548.4.

To a solution of the alcohol obtained above (240 µg, 440 nmol) in DMF (50 µL) and CH₂Cl₂ (50 µL) was added IBX-resin (970 µg, 870 nmol) at room temperature. After the reaction mixture was shaken at room temperature for 30 min, the mixture was filtered, and CH₂Cl₂ was removed in vacuo to give 4b as a red-colored DMF solution, being pure enough and used for subsequent labeling studies after the rapid ESI-MS analysis: m/z calcd for C_{30}H_{32}N_{3}O_{7} (M+H)^+ 546.2, found 546.3.

Ethyl (E,E)-4-Oxo-2-{4-[(6-(tetramethylrhodamine)-5-and-6-carboxamido)hexaneamid]acetamide}styryl]but-2-enoate (4c). To a solution of the alcohol 3 (300 µg, 990 nmol) in DMF (50 µL) was added TAMRA-OSu (630 µg, 990 nmol) at room temperature. After the reaction mixture was stirred at room temperature for 30 min, the mixture was concentrated in vacuo. The residue was purified by preparative thin layer chromatography on silica gel (CHCl₃ : MeOH = 10 : 1) to give the corresponding alcohol (600 µg, 74 %) as a red solid, which was oxidized without further purification due to the instability of the TAMRA fluorophore: ESI MS m/z calcd for C_{47}H_{52}N_{10}O_{9} (M+H)^+ 830.4, found 830.4.

To a solution of the allylic alcohol obtained above (600 µg, 720 nmol) in DMF (50 µL) and CH₂Cl₂ (50 µL) was added IBX-resin (1.6 mg, 1.6 µmol) at room temperature. After the reaction mixture was shaken at room temperature for 30 min, the mixture was filtered, and CH₂Cl₂ was removed in vacuo to give 4c as a red-colored DMF solution, being pure enough and used for subsequent labeling studies after the rapid ESI-MS analysis: m/z calcd for C_{47}H_{50}N_{10}O_{9} (M+H)^+ 828.4, found 828.5.
Gadolinium (1E,3E)-(ß)-10-[2-[4-(3-Ethoxycarbonyl-5-oxopenta-1,3,6-dienyl)-phenyl]-2-oxoethylamino]-2-oxoethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetate. To a solution of the alcohol 3 (500 µg, 720 nmol) in distilled water (100 µL) was added 0.1 M GdCl₃ solution (7.6 µL) at room temperature. After the reaction mixture was allowed to stand at room temperature for 30 min, the mixture was concentrated in vacuo to give Gd-DOTA alcohol, which was oxidized without further purification: ESI-MS m/z calcd for C₃₂H₄₅GdN₆O₁₁ (M+2H)²⁺ 423.6, found 423.5.

To a solution of the crude Gd-DOTA alcohol obtained above in DMF (30 µL) and CH₂Cl₂ (100 µL) was added Dess-Martin periodinane (600 µg, 1.4 µmol) at room temperature. After the reaction mixture was stirred at room temperature for 30 min, the mixture was directly subjected to size-partitioning gel-filtration through a column filled with HP-20 (CHCl₃ : MeOH : H₂O = 10 : 10 : 1). The elution fractions were concentrated in vacuo at room temperature to give the corresponding aldehyde 4c as a yellowish DMF solution, being pure enough and used for subsequent labeling studies after the rapid ESI-MS analysis: m/z calcd for C₃₂H₄₂GdN₆O₁₁ (M+H)⁺ 844.2, found 844.2.
Representative procedures of labeling of biomolecules; labeling of somatostatin by the probe 4a (Table 1, entry 1). To a solution of somatostatin (170 µg, 108 nmol) in distilled water (154 µL) was added a DMF (40 µL) solution of 4a (1.5 mg, 2.2 µmol) at room temperature (reaction concentration: 5.5 x 10^{-4} M for somatostatin, 5.5 x 10^{-2} M for 4a). After the mixture was allowed at room temperature for 30 min, the DOTA-labeled somatostatin was isolated by reverse phase HPLC (conditions shown in Figure S-1), lyophilized, and characterized by MALDI-TOF-MS (Figure S-2).

Figure S-1. HPLC profile of the reaction mixture between somatostatin and 4a.

Figure S-2. MALDI-TOF-MS of labeled somatostatin by 4a (DOTA-somatostatin).

Due to the low ionization property of DOTA conjugated peptides both by positive and negative modes, the determination of the labeled Lys in somatostatin was performed with a similar N-Boc analog 4a’ (Figure S3). To a Tris-HCl buffer solution (pH = 9.0, 10 µL) of somatostatin labeled by 4a’ (39 nmol), being prepared under the same conditions performed with 4a, was added a Tris-HCl buffer solution (pH = 9.0, 10 µL) of lysyl endopeptidase (1.0 µg, 36 pmol, 3.6 x 10^{-5} M, Wako Chem. Co. LTD) at 40 °C. After the mixture was allowed to stand at 40 °C for overnight, the crude peptide fragments were purified by reverse phase HPLC (column: nacalai tesque 5C_{18}-AR300, 4.6 x 250 mm; MeCN in H_2O: 25 % for 0-30 min, 25-30 % for 30-40 min, and 30 % for 40-60 min), lyophilized, and analyzed by mass spectrometry. MALDI-TOF-MS detected a fragment peak (28 min) corresponding to a labeled peptide sequence of Ala-Gly-Cys-Lys(+4a’)-Asn-Phe-Phe-Trp-Lys (m/e (M+H)^+ 1455, found 1455), from which the labeling of lysine at site 1 was concluded (Figure S-3).
**Figure S-3.** Determination of labeled Lys in somatostatin by MALDI-TOF-MS peptide mapping.

**Labeling of orosomucoid by the probe 4a (Table 1, entries 5 and 6).** To a PBS solution of orosomucoid (62 µg, 1.4 nmol, 295 µL, pH = 7.4) was added a DMF solution of 4a (14 nmol, 1.5 µL) at room temperature (reaction concentration: 4.5 x 10⁻⁶ M for orosomucoid, 4.5 x 10⁻⁵ M for 4a). After the mixture was allowed to stand at room temperature for 30 min, the DOTA-labeled protein was purified by ultrafiltration (30,000 cut). To a PBS solution of the labeled protein (6.67 x 10⁻¹⁰ mol, 5.74 mg/mL, 5 µL, pH = 7.4) was added the solutions of ammonium acetate (0.25 M, 20 µL, pH = 7.0) and CoCl₂ (4.0 nmol, 400 µM, 5 µL, containing > 350000 cpm/µL of $^{57}$CoCl₂). After the mixture was incubated at 40°C for 3 h, a solution of DOTA (40 µg, 100 nmol, 10 mM, 10 µL) was added, and incubated at 40°C for 30 min. An aliquot of 1.0 µL of the resulting solution was spotted on a TLC plate, and developed until the solvent (MeOH : H₂O = 1 : 1) reached to 7 cm from the origin. The plate was cut into seven fractions, each of which $^{57}$Co was counted by the gamma counter. The number of the $^{57}$Co chelates per orosomucoid was calculated by the ratio of the $^{57}$Co at the bottom side of TLC (Rf = 0.0-0.14, corresponding to the $^{57}$Co-DOTA-protein) to the rest of the $^{57}$Co chelated to free DOTA (Figure S-4), according to the method of Meares and co-workers.
**Figure S-4.** DOTA introduced to orosomucoid (left) and asialoorosomucoid (right) by $^{57}$Co experiments.

**Fluorescence labeling of human serum albumin by the probe 4c (Table 1, entry 9).** To a PBS solution of HSA (12 µg, 1.8 x 10^{-10} mol, 8 µL) was added a DMF solution of 4c (1.3 x 10^{-9} mol, 0.82 µL) at room temperature (reaction concentration: 2.1 x 10^{-5} M for HSA, 1.5 x 10^{-4} M for 4c). After the mixture was allowed to stand at room temperature for 30 min, the labeled protein was purified by NAP-5 column (GE Healthcare). TAMRA introduced to HAS was estimated by fluorescence spectra in Figure S-5 (excitation at 525 nm).

![Fluorescent spectra of HAS labeled with TAMRA.](image_url)

**Figure S-5.** Fluorescent spectra of HAS labeled with TAMRA.

**Fluorescence labeling of anti-GFP mAb by the probe 4c (Table 1, entry 10).** To a PBS solution of anti-GFP mAb (rat IgG2a, monoclonal, nacalai tesque) (2.0 µg, 1.3 x 10^{-11} mol, 1.6 x 10^{-6} M, 8 µL, pH = 7.4) was added a DMF solution of 4c (1.3 x 10^{-9} mol, 1.6 mM, 0.82 µL) at room temperature (reaction concentration: 1.1 x 10^{-6} M for anti-GFP mAb, 2.2 x 10^{-5} M for 4c). After the mixture was allowed to stand at room temperature for 30 min, the labeled anti-GFP mAb was purified by NAP-5 column (GE Healthcare). The number of TAMRA fluorophore introduced to an antibody molecule was estimated by the fluorescence intensity at 555 nm (excitation at 525 nm). On the other hand, the reaction mixture was treated with a PBS solution of excess GFP (pH = 7.4), and subsequently with excess protein G-Sepharose beads (50 µL, GE Healthcare), and the resulting anti-GFP mAb/GFP complex on beads was purified by centrifuge precipitation. After anti-GFP mAb/GFP complex was eluted from the beads by the treatment...
with a PBS solution including 5 % of SDS (pH = 7.4), the antigen recognition of the labeled anti-GFP mAb was evaluated by fluorescence spectra of GFP in the resulting complex (Figure S-6, excitation at 395 nm).

Figure S-6. Fluorescence spectra of GFP/anti-GFP mAb complex (excitation at 395 nm).
Labeling of DOTA-somatostatin with $^{68}$Ga. $^{68}$GaCl$_3$ was obtained as 1M HCl solution (1.68 mCi, 500 μL) from $^{68}$Ge/$^{68}$Ga radionuclide generator. After the acidic solution was neutralized by 4N NaOH, the resulting $^{68}$GaCl$_3$ solution was added to DOTA-orosomucoid prepared in Table 1, entry 5 (10 μg) at room temperature. After the mixture was incubated at 40 °C for 10 min, a solution of DOTA (1.0 μmol, 10 mM in H$_2$O) was added in order to chelate and excrete the excess $^{68}$Ga from the body during the PET study, and the resulting solution was directly injected to the rabbit without further purification. $[^{68}\text{Ga}]$DOTA-asialoorosomucoid was prepared in the same procedure.

General procedure of $[^{68}\text{Ga}]$PET imaging. The animal studies were performed according to a protocol approved by the Ethics Committee of the Laboratory Animal Center at Osaka City University Graduate School of Medicine. Briefly, the PET study was conducted on female Japanese white rabbits weighing 2.1-2.2 kg at 13 weeks of age (Japan SLC, Inc., Hamamatsu, Japan) under a general anesthesia with ketamine (Ketalar®, Sankyo, Tokyo, Japan) and xylazine (Selactar®, Bayer Yakuhin, Tokyo, Japan), and was used a small animal PET scanner, the microPET P4 system (Siemens Medical Solutions Inc., Knoxville, TN, USA). The system has an animal port 220 mm in diameter, an axial extent of 78 mm, a capacity of 63 parallel slices, a spatial resolution of 1.7 mm in full width at half maximum at the center of the field of view, a detector system composed of 32 crystal rings with lutetium oxyorthosilicate, and a 3-dimensional list-mode method of data acquisition. The rabbits were injected initially with intramuscular a mixture of ketamine (60 mg/kg) and xylazine (6 mg/kg), and were sedated continuously with intravenously administered a mixture of ketamine (60 mg/kg/hr) and xylazine (6 mg/kg/hr) during the experiment. To obtain the abdominal images, the animals were scanned by the single-helical computed tomography (CT) scanner (Pro Speed AI, General Electric Medical Systems, Milwaukee, WI, USA) before the PET scan. $[^{68}\text{Ga}]$DOTA-orosomucoid or asialoorosomucoid conjugates at a dose of 15.8-16.1 MBq in 2.2 mL were injected via an ear vein, and then the emission data was collected for 240 min postinjection as 12 frames (6 x 400 sec, 3 x 1000 sec, 2 x 1600 sec, and 1 x 3400 sec), and was acquired with an energy window of 400-650 keV and a coincidence timing window of 6 nsec. The images were reconstructed from 120 min to 240 min after injection of $[^{68}\text{Ga}]$DOTA-orosomucoid or asialoorosomucoid by an ordered subset expectation maximization (OSEM) algorithm with attenuation correction using CT data or no scatter correction, and were smoothed by using a Gaussian kernel with an FWHM of 3 mm in the all directions. Quantitative analysis was performed using ASIPro VM version 6.3.3.0 software (Siemens Medical Solutions Inc., Knoxville, TN, USA). Regions of interest (ROIs) were placed on the tissue region. As shown in Figure 3, the accumulation of $[^{68}\text{Ga}]$DOTA-orosomucoid or asialoorosomucoid conjugates in the rabbits were visualized by the microPET and CT-fused images, and were accumulated found in the liver and spleen, respectively.