Self-assembling small-molecule adjuvants as antigen nano-carriers

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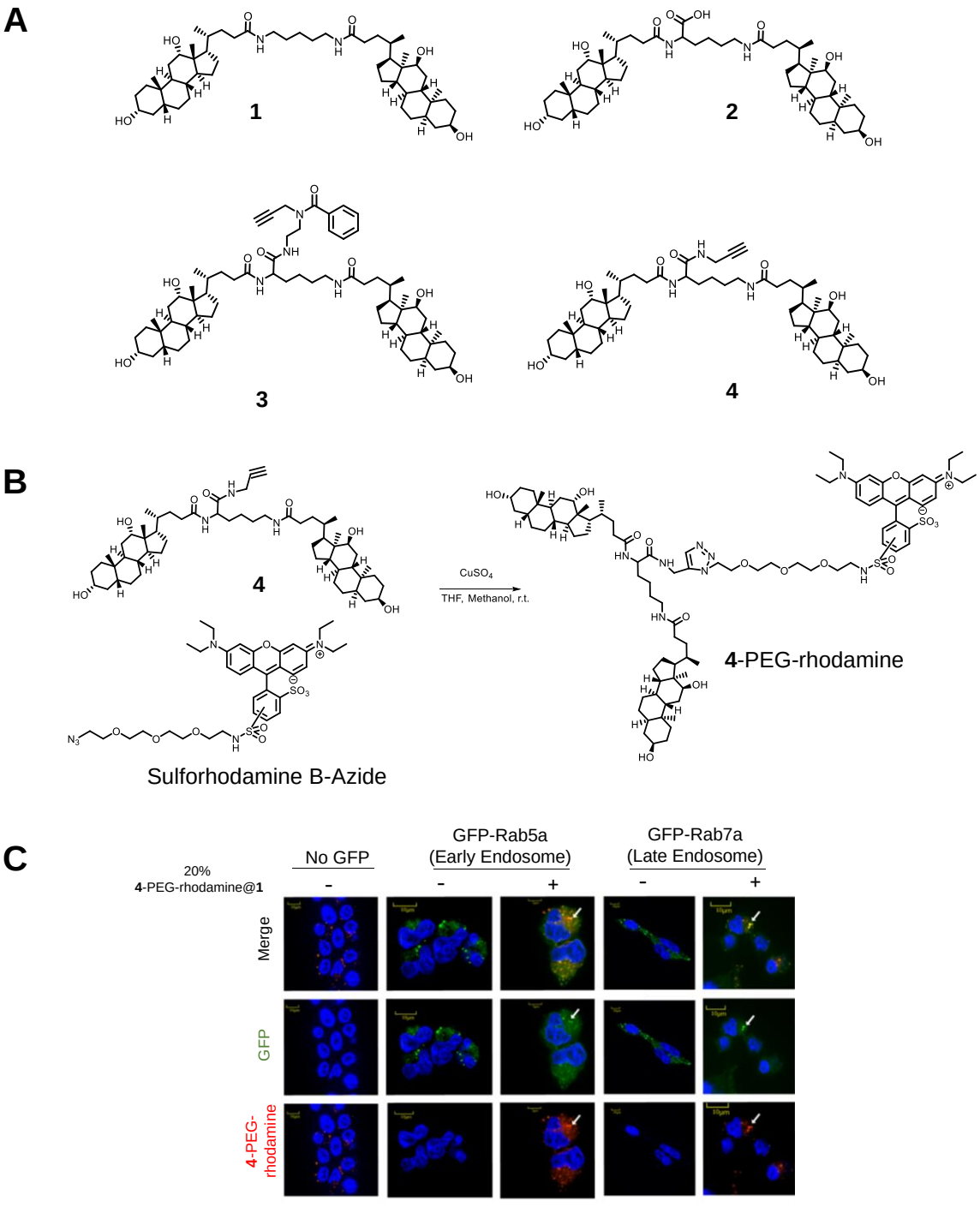


Fig. S1. Structures of cholicamide analogs. (A) Chemical structures of molecule **1**, **2**, **3** and **4**. (B) Synthesis of **4**-PEG-rhodamine. (C) Co-localization of early and late endosome with 20% **4**-PEG-rhodamine@**1**. HEK293 cells was transfected with CellLight™ Early Endosomes-GFP or CellLight™ Late Endosomes-GFP to express GFP fusions of Rab5a and Rab7a, early and late endosome markers, respectively. After incubating at 37°C for 24 h, the cells were incubated with 20% **4**-PEG-rhodamine@**1** (50 μM) at 37°C. After 4 h, the cells were washed with PBS 3 times and stained with Hoechst 33342 (1:1000). Scale bar: 10 μm.

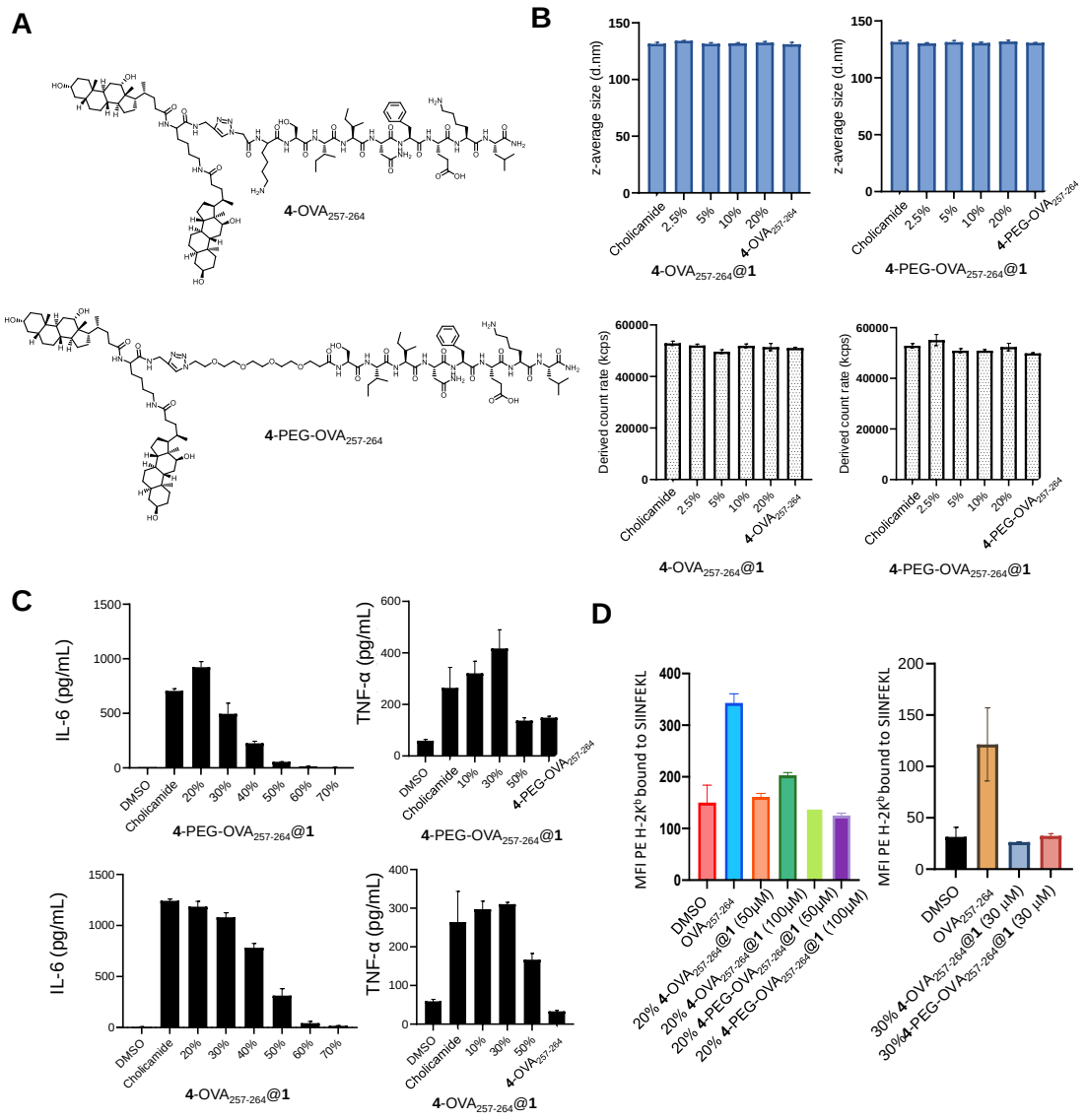
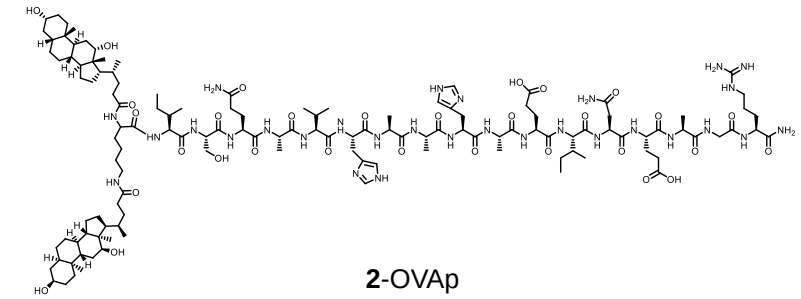
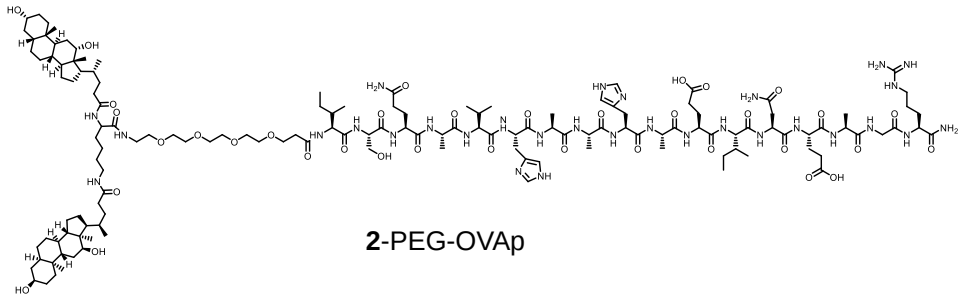


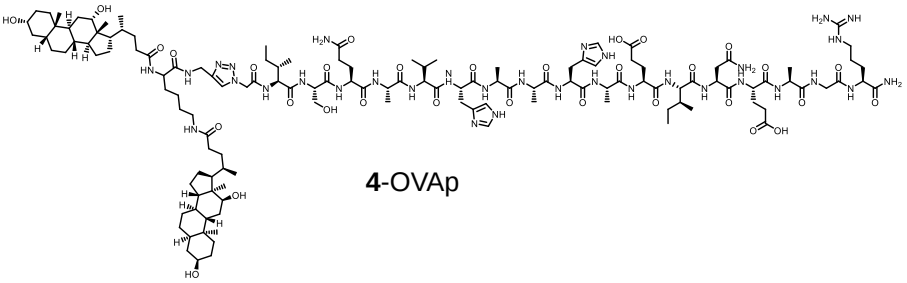
Fig. S2. Characterization and evaluation of conjugates derived from $OVA_{257-264}$. (A) Chemical structures of **4**-$OVA_{257-264}$ and **4**-PEG-$OVA_{257-264}$. (B) The Z-average sizes and derived count rates of **4**-$OVA_{257-264}$**@1** and **4**-PEG-$OVA_{257-264}$**@1** at 30 μM in culture media as measured by DLS. (C) TNF-α and IL-6 production upon treatment of RAW264.7 cells with **4**-$OVA_{257-264}$**@1** (30 μM) and **4**-PEG-$OVA_{257-264}$**@1** (30 μM). (D) Flow cytometric analysis of antigen presentation in BMDCs. BMDCs were treated with 2 μg $OVA_{257-264}$, **4**-$OVA_{257-264}$**@1**, or **4**-PEG-$OVA_{257-264}$**@1** for 24 h and collected for antigen presenting efficiency study. The presented antigens were detected with PE H-$2K^b$ bound SIINFEKL antibody (0.5 μg in 100 μL FACS buffer). Mean fluorescence intensity (MFI) was quantified by FlowJo V10.



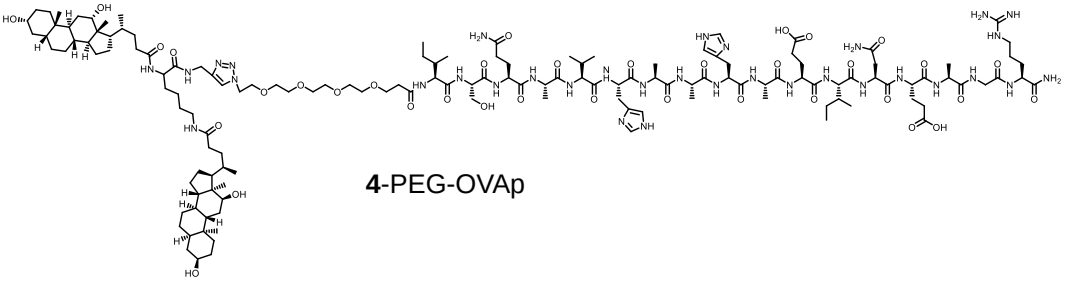
2-OVAp



2-PEG-OVAp



4-OVAp



4-PEG-OVAp

Fig. S3. Chemical structures of **2**-OVAp, **2**-PEG-OVAp, **4**-OVAp, and **4**-PEG-OVAp.

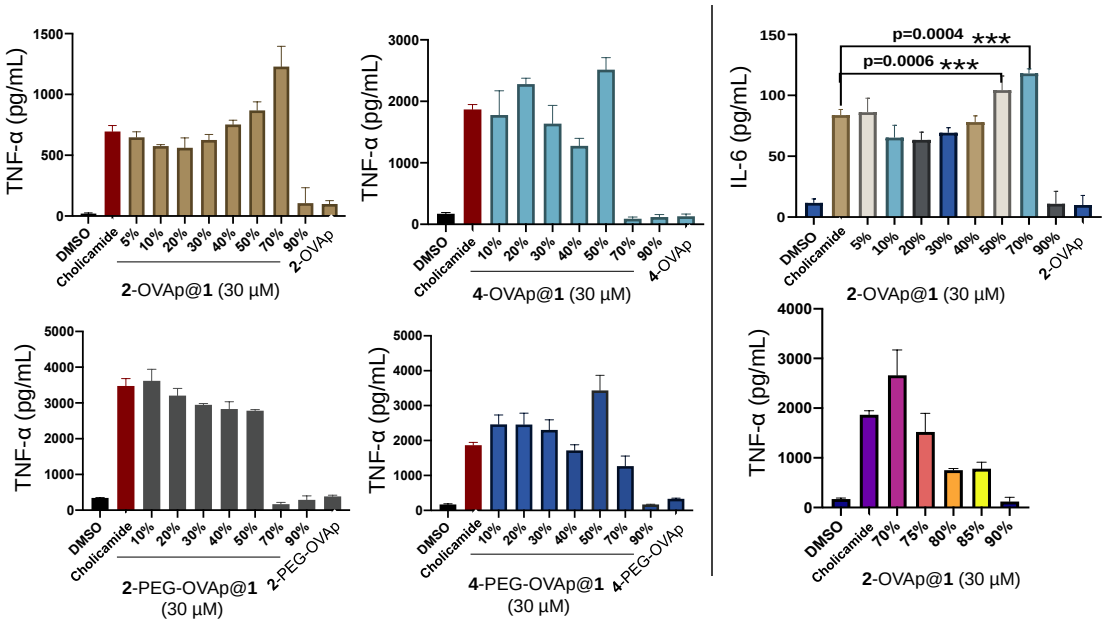


Fig. S4. TNF-α and IL-6 production upon treatment with co-assemblies. RAW264.7 macrophage cells were incubated with conjugate@**1** (30 μM) at various percentages for 24 h. The cytokines (IL-6 and TNF-α) in the supernatants were quantified by ELISA. *Left panel*: Comparison of the ability of the four conjugates to induce TNF-α. *Right panel*: In-depth analysis of **2**-OVA@**1**. Its ability to induce TNF-α at 70-90% and IL-6 at 10-90% is shown. All assays were performed in triplicates.

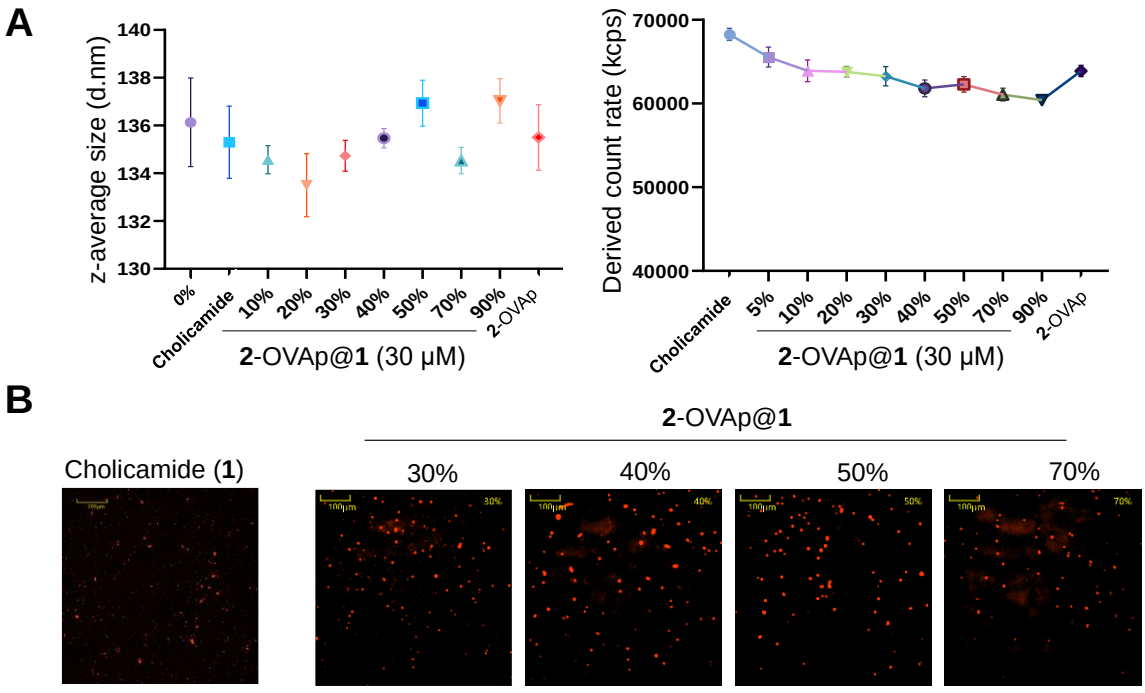


Fig. S5. Co-assembly of **2**-OVAp@**1**. (A) The z-average sizes and derived count rates of **2**-OVAp@**1** in a DMEM culture medium as measured by DLS. (B) Confocal microscopic images of 50 μM **2**-OVAp@**1** at 30%, 40%, 50%, and 70% in a DMEM culture medium with Nile Red (15 μM).

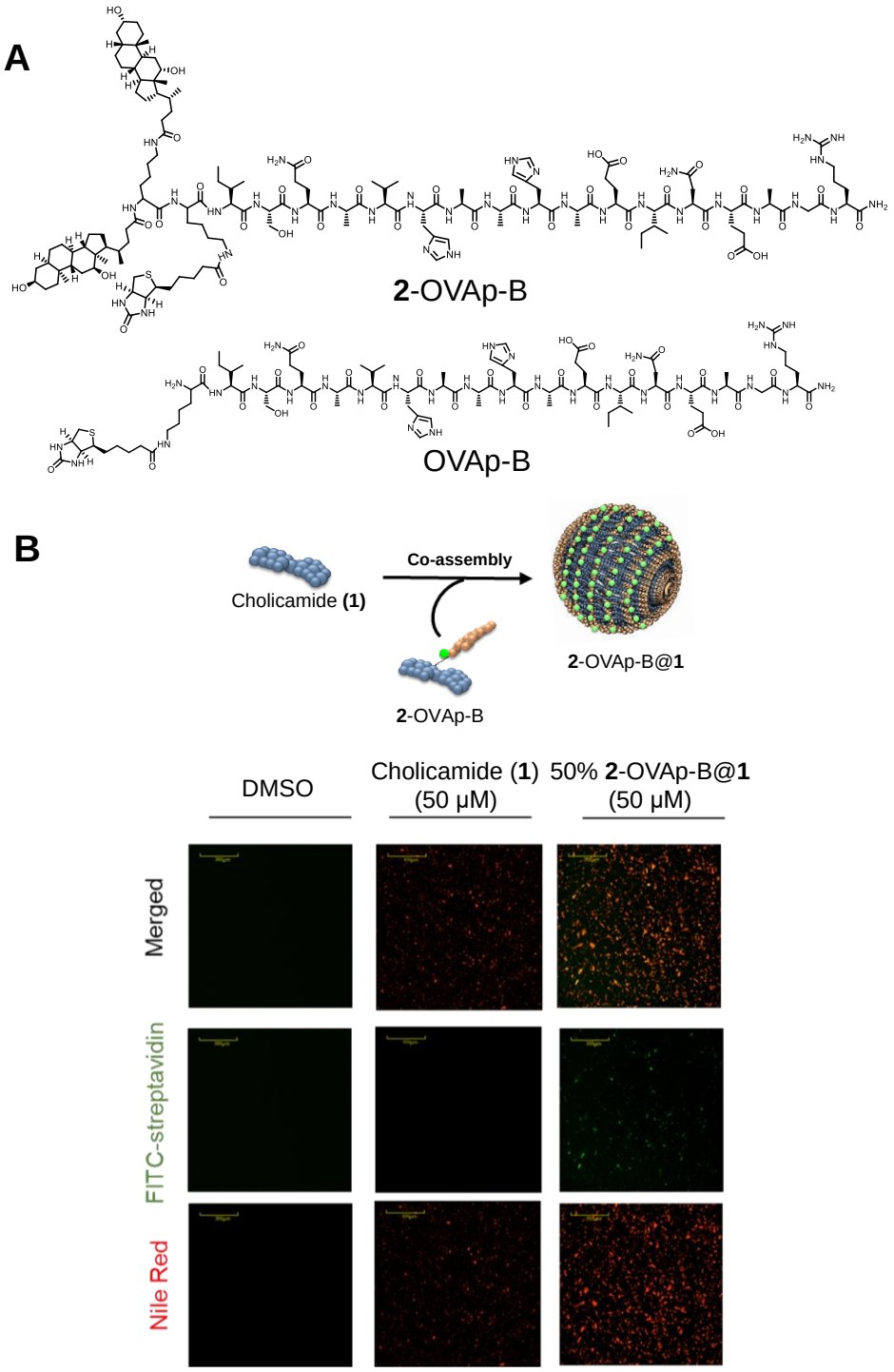


Fig. S6. Design of **2**-OVAp-B and OVA-B. (A) Chemical structures of **2**-OVAp-B and OVAp-B. (B) Confocal images of 50% **2**-OVAp-B@**1** and cholicamide (**1**) at 50 μM stained with Nile Red (15 μM) and FITC-streptavidin. 50% **2**-OVAp-B@**1** or cholicamide (**1**) was added to DMEM containing 1.25% FBS. After 30 min, 1 μL FITC-streptavidin (0.5 mg/mL) was added to each sample. Images were captured after incubation for overnight at 37 °C.

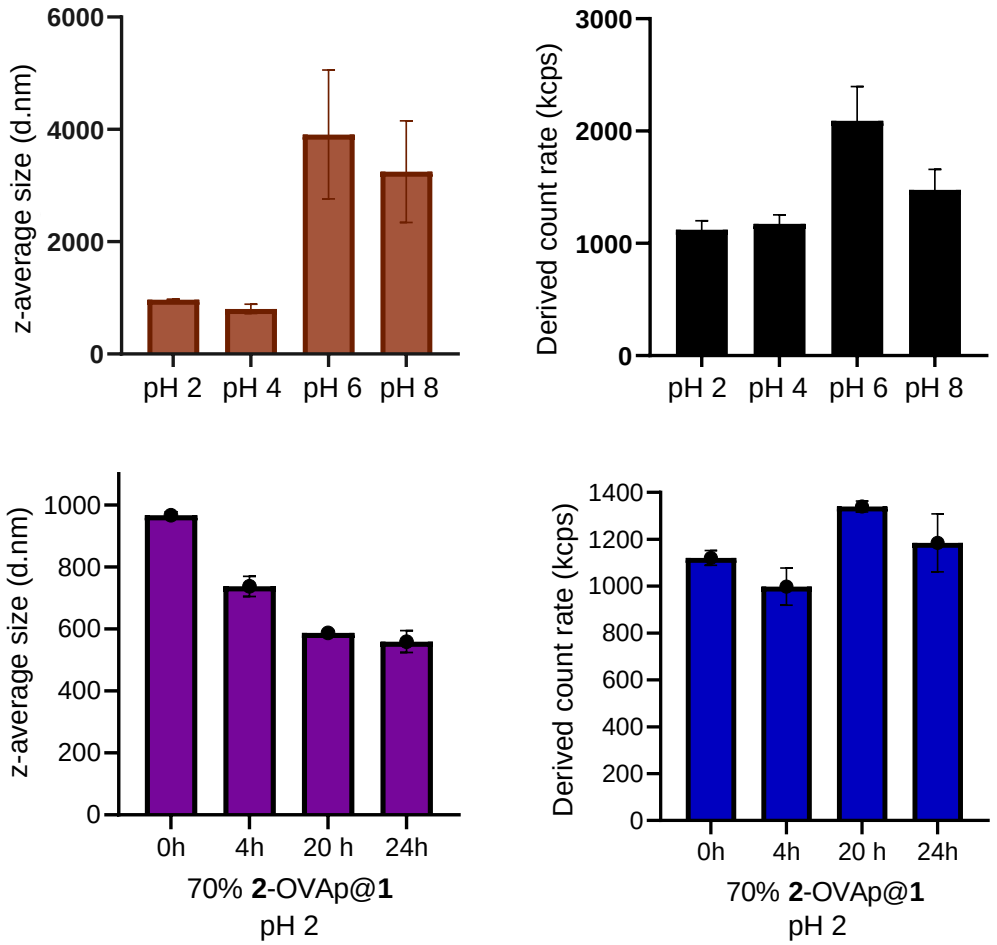


Fig. S7. The z-average sizes and derived count rates of 70% **2**-OVAp@**1** (30 µM) in phosphate buffers (pH 2-8) as measured by DLS.

Experimental procedure

Material and Methods

Biological reagents

Dulbecco's modified Eagle medium (DMEM) and PBS (pH 7.4) were purchased from Thermo Fisher Scientific. RPMI1640 medium and Penicillin-streptomycin mixed solution (P/S) were purchased from Nacalai Tesque Inc. Fetal bovine serum (FBS) was purchased from Biowest and Sigma-Aldrich. All RPMI and DMEM used in experiments were supplemented with 1% (v/v) Penicillin-Streptomycin. TNF-α and IL-6 ELISA kits were purchased from R&D Systems. Antibodies were purchased from BioLegend and described in each experiment.

Cell preparation and culture

RAW264.7 cells were purchased from ATCC and were cultured in DMEM containing 10% FBS and 1% P/S. C57BL/6 mice were purchased from CLEA Japan, Inc. BMDCs (bone marrow-derived dendritic cells) were prepared as previously described.[1] Briefly, Mice were sacrificed, and the bone marrow from the pelvis, femur, and tibia was collected and purified by treatment with ACK lysis buffer (for the elimination of red blood cells) to give bone marrow cells (BM cells). BM cells in RPMI1640 medium supplemented with 10% (v/v) FBS and β-mercaptoethanol were cultured for 10 days at 37°C at a density of 5×10^6 cells/mL in the presence of MGM-5 (provided by S. Nagata) as a source of granulocyte macrophage colony-stimulating factor (GM-CSF).

Co-assembly sample preparation

The stock solutions of conjugates and cholicamide were in DMSO at 10 mM. To prepare a co-assembly mixture with a fixed total final concentration of 10 mM, conjugates and cholicamide were mixed at 10 mM according to the following chart.

The molar percentage of conjugate in co-assembly	Conjugate (μL, 10 mM)	Conjugate (μL, 10 mM)
Cholicamide (**1**)	0	100
10% conjugate@**1**	10	90
20% conjugate@**1**	20	80
30% conjugate@**1**	30	70
40% conjugate@**1**	40	60
50% conjugate@**1**	50	50
70% conjugate@**1**	70	30
90% conjugate@**1**	90	10
conjugate	100	0

Dynamic light scattering (DLS) measurement

Each conjugate or mixture of the conjugate and cholicamide was dissolved in cell culture media (DMEM+ 10% FBS + 1% P/S) or phosphate buffers and incubated under room temperature for 10 min. The z-average sizes, derived count rates, and ζ-potential were measured by Malvern Dynamic Light Scattering Spectrophotometer (Zetasizer Nano 1600) at 50 μM (final concentration) at 25°C. All measurements were set to an auto-attenuation mode at 25°C, and data were analyzed from at least 10 acquisitions /measurement.

Confocal microscopic observation

Confocal microscopic images were captured with a CV1000-SP130 (Cell voyager CV1000, Yokogawa Electrical Corporation.) In Fig. 2B, conjugate **3**-Flag (50 or 100 μM) was dissolved in H_2O or PBS, and incubated overnight in a 96-well plate after adding Nile Red (15 μM) or fluorescent antibody of Flag (1 μL, 10 μg/mL, Sigma-Aldrich, A9594). For Fig. 3B, **4**-PEG-rhodamine@**1** was dissolved in H_2O and incubated overnight at room temperature before performing the confocal microscopic observation. In Fig. S5B, the co-assembly or cholicamide (**1**) (50 μM) was dissolved in H_2O in a 96-well plate, stained with Nile Red (15 μM), and incubated overnight at room temperature. Images were captured at 561 nm. For Fig. S6B, cholicamide (**1**) or **2**-OVAp@**1** was dissolved in DMEM containing 1.25%FBS. After 30 min, 1 μL FITC-streptavidin (Biolegend, 405202, 0.5mg/mL) was added to each sample. Images were captured after overnight incubation at 37°C.

Co-localization experiment

CellLight™ Early Endosomes-GFP or CellLight™ Late Endosomes-GFP (Invitrogen, C10586 and C10588) were added to HEK293 cells following the protocol provided by the manufacturer, respectively. After incubating at 37°C for 24 h, the cells were incubated with 20% **4**-PEG-rhodamine@**1** (50 μM) at 37°C. After 4 h, the cells were washed with PBS 3 times and stained with Hoechst 33342 (1:1000). Images were captured by confocal microscopy.

Solid-phase peptide synthesis

Peptides were prepared through solid-phase peptide synthesis using Rink Amide MBHA resin (Novabiochem, 431041-83-7). The resin was soaked in DCM at room temperature overnight before use. Fmoc protecting group was removed with 10% piperidine in DMF at room temperature shaking at 300 rpm for 30 min. After deprotection, the reaction solution was removed by vacuum, and resin was washed three times with DMF and DCM, respectively, after each step. Amino acids were dissolved in a 0.45 M coupling reagent (HBTU and HOBT in DMF) and 0.9 M DIPEA in NMP in a 1:1 v/v ratio. The resin was treated with an amino acid solution for 1 h at room temperature shaking at 300 rpm. After completion of the synthesis, the resin was dried under vacuum, transferred to a round-bottom flask, and treated with TFA for 1 h for cleaving the peptide. The resulting solution was filtered and evaporated for the removal of excess TFA. The peptide was precipitated with ice-cold Et_2O and centrifuged to the bottom of the centrifuge tube at 145,000 rpm at 4°C. The precipitates were dried under vacuum after removing the supernatants, purified with HPLC, and lyophilized to give a white powder.

Enzyme-linked immunosorbent assay (ELISA)

For the stimulation of RAW264.7 cells, 10 000 cells/well were seeded onto a 96-well tissue culture plate in DMEM containing 10% FBS at 37°C for 24 h. The medium was then replaced with a solution of compounds in DMEM containing 10% FBS (final concentration of DMSO: 1%). DMSO 1% (v/v) was used as the negative control. After 24 h, the media were centrifuged at 3000 rpm (4°C), collected, and stored at -30°C or directly assayed. The cytokines (IL-6 and TNF-α) in the supernatants were quantified by ELISA (R&D Systems). All assays were performed in triplicates or duplicates following the protocol provided by the manufacturer.

Antigen presenting efficiency assay

Antigen presenting efficiency was analyzed by flow cytometry. Flow cytometry was performed on a BD FACS Aria II flow cytometer, and the result was analyzed by FlowJo V10. BMDCs were seeded to a 6-well plate at $1x10^6$/well and incubated with compounds for 24 h at 37°C. For Fig. 5, the cells were treated with 30 μM **2**-OVAp-B@**1**(30%, 50%, 70%, or OVAp-B alone) or OVAp-B (9, 15, 21, or 30 μM) simply mixed with 30 μM cholicamide (indicated as 30-70% OVAp-B+**1**). The cells were collected by centrifugation at 1500 rpm, washed twice with PBS buffer, and incubated with 100 μL of 2.5 μg/mL FITC-streptavidin (Biolegend, 405202) in FACS buffer (PBS containing 2% FBS and 0.1% sodium azide) at room temperature for 30 min. After centrifugation at 1500 rpm, the cells were washed three times with the FACS buffer and analyzed by flow cytometry. Samples were triplicated and data were analyzed with FlowJo V10. For Fig. S2D, BMDCs were treated with 2 μg $OVA_{257-264}$, 20% **4**-$OVA_{257-264}$@**1** (50 μM or 100 μM), 20% **4**-PEG-$OVA_{257-264}$@**1** (50 μM or 100 μM), 30% **4**-$OVA_{257-264}$@**1** (30 μM), or 30% **4**-PEG-$OVA_{257-264}$@**1** (30 μM) for 24 h, collected by centrifugation at 1500 rpm, washed twice with PBS buffer, and incubated with 0.5 μg PE $H2K^b$ bound SIINFEKL antibody (BioLegend, 141603) in 100 μL FACS buffer.

Cryo-transmission electron microscopy (Cryo-TEM)

70% of **2**-OVAp@**1** was dissolved in H_2O at 50 μM and used for observation. Cryo-TEM images were obtained with a JEOL JEM-2100F (G5) microscope operated at the acceleration voltage of 200 kV and measured at liquid helium temperature (4.2 K).

Field Emission Scanning Electron Microscope (FESEM)

70% of **2**-OVAp@**1** was dissolved in H_2O at 50 μM. The resulting mixture was transferred to a culture dish and placed in the hood, and the water was then gently evaporated at 25°C. After evaporation, a white powder was scratched from the bottom of the culture dish, drop-casted over carbon stubs, and sputtered with gold/palladium for 180 s. Images were captured at a constant voltage of 15.0 kV (EI) using SEM JSM7500 (JEOL Ltd.).

Stability of assemblies in various pH phosphate buffers

Phosphate buffers at various pH were prepared according to the following procedure. Phosphoric acid (0.83

mL) and sodium hydrogen diphosphate (3.08 g) was dissolved in 50 mL H_2O, respectively. To prepare pH 2 buffer, 36.25 mL of the phosphoric solution and 13.75 mL of the sodium hydrogen diphosphate solution were mixed. For pH 4 buffer, 5.04 g disodium hydrogen phosphate and 3.01 g of potassium dihydrogen phosphate were dissolved in sufficient water to produce a 1000 mL solution. The pH of the solution was then adjusted with glacial acetic acid to pH 4. The pH 6 and 8 phosphate buffers were prepared by mixing 0.2 M sodium dihydrogen phosphate (solution A) and 0.2 M disodium hydrogen phosphate (solution B) as shown in the following chart.

pH	Solution A (mL)	Solution B (mL)
6	8.77	1.23
8	0.53	9.47

Chemical synthesis

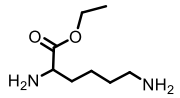
General procedure

The solvents and chemicals for chemical synthesis were used as purchased with no further purification. All solvents and chemicals for chemical synthesis were purchased from Wako Pure Chemicals, TCI, or Sigma-Aldrich. Analytical thin-layer chromatography (TLC) was performed on Merck 60G silica gel glass plates coated with fluorescent indicator F254. Silica gel column chromatography was performed using silica gel 60, spherical, neutra 75 μm (Nacalai Tesque). Preparative layer plates (PLC Silica gel 60 F254) were purchased from Merck, and flash column chromatography was employed with Silica gel 60 (spherical) purchased from Nacalai Tesque. Mass spectral data were recorded on a Shimadzu LCMS-2010EV quadrupole mass spectrometer, operating in both positive and negative electrospray ionization modes, m/z range of 200-2000 m/z. High-resolution mass spectra were obtained using a JEOL MStation JMS-700V in FAB mode. Solution ^{1}H and ^{13}C NMR spectra were collected by JEOL 300 or 600-MHz JNM-ECP NMR spectrometers or a Bruker 800-MHz NMR spectrometer. High-resolution mass spectra (HRMS) were obtained using a JEOL MStation JMS-700 in FAB mode. Mass spectral checkups were performed using a binary solvent system (A: H_2O with 0.1% TFA; B: CH_3CN with 0.1% TFA) with an eluting gradient of 10-95% B (0-5 min), UV detection at 254 nm, 220 nm, and 190 nm, and flow rate at 0.5 mL/min. High-performance liquid chromatography (HPLC) was performed with a Shimadzu LC-2010C.

General procedure for the amide condensation reaction

To a solution of amine (1.0 eq.) and acid (1.0 eq.) in DMF stirring in the ice bath, HATU (1.5 eq.) was added. After adding DIPEA (2.0 eq.) to the reaction mixture 1h later, the resulting reaction mixture was stirred overnight with temperature generally increasing to room temperature. The reaction mixture was quenched by adding water and extracted with ethyl acetate three times. The organic layer was combined, washed with brine, and dried with anhydrous sodium sulfate. The crude mixture was purified by flash column chromatography with gradient elution (DCM with 10-50% MeOH) to give purified compounds a white solid. The structure and purity of all products were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS-ESI.

L-Lysine ethyl ester (Intermediate **1**)

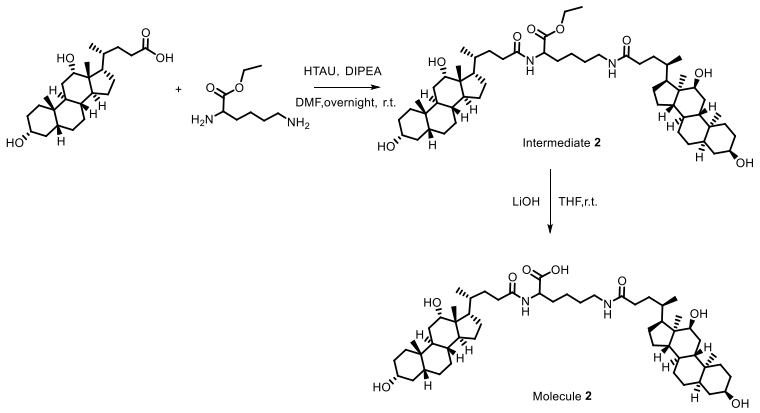


A round bottom flask was charged with lysine (1.8 g) and 13 mL EtOH, cooling with an ice bath. Thionyl chloride (2 mL) was added to the reaction mixture dropwise for 45 min. After completion, the resulting mixture was transferred to an oil bath at 80°C until it become transparent. Excess thionyl chloride and EtOH was removed by evaporation in *vacuo* to give a crude product as white solid. The crude product was used without further purification.

N^2, N^6-bis((R)-4-((3R,5R,8R,9S,10S,12S,13R,14S,17S)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoyl) lysine (Molecule **2**)

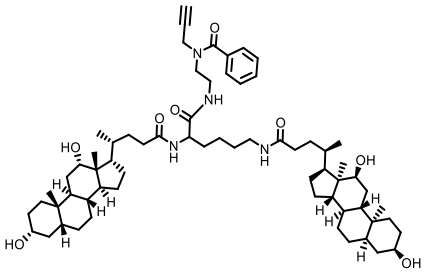
A round bottom flask was charged with intermediate **1** (1.44 g, 8 mmol) and deoxycholic acid (5.4 g, 16 mmol), and DMF (40 mL). HATU (4.5 g, 12 mmol) was added to the resulting solution at 0°C. After stirring for 1 h, DIPEA (3 mL, 16 mmol) was added to the reaction mixture to react overnight at room temperature. The reaction mixture was extracted with ethyl acetate and washed with brine. The organic layer was combined, dried with $MgSO_4$, and condensed by evaporation to give a crude product of intermediate **2**. The crude product was purified with flash chromatography with DCM: MeOH (from 100: 1 to 10:1). The corresponding elution fragment was checked with

LC-MS, concentrated under reduced pressure by rotary evaporation, and dried in vacuo to give a 73% yield of purified intermediate **2** (5.39 g, 5.8 mmol). ESI-LCMS (*m/z*): calc'd for $[C_{56}H_{95}N_2O_8]^+$ $[M+H]^+$: 924.71, found 924.67.



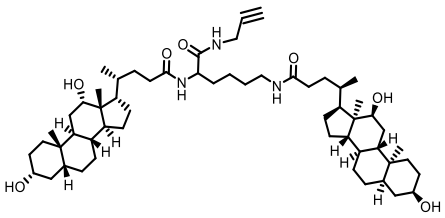
A round bottom flask was charged with intermediate **2** (100 mg, 0.108 mmol) and LiOH (10 mg, 0.432 mmol), and THF (10 mL). 1 mL water was added to the resulting solution at room temperature. The reaction was monitored by TLC staining with PMA to show the compound spot. After completion, THF was removed by evaporation to give a white oily solid. The solid was dissolved in 3 ml water, the pH of the resulting solution was adjusted to pH=2, and white precipitation appeared. Precipitation was collected by filtering to give the crude product of molecule **2** as a white solid (80 mg). ^{1}H-NMR (600 MHz, DMSO-*d*^{6}) δ: 7.9 (d, 2H, *J* = 1.8 Hz), 7.75 (t, 2H, 5.4 Hz), 4.07-4.11 (m, 2H), 3.78 (d, 2H, *J* =3.0 Hz), 3.33-3.38 (m, 2H), 2.93-3.03 (m, 2H), 1.91-2.24 (m, 4H), 1.72-1.82 (m, 7H), 1.62-1.66 (m, 7H), 1.44-1.57(m, 5H), 1.13-1.36 (m, 24H), 0.84-1.07 (m, 18H), 0.58(t, 6H, *J* = 3.6 Hz); ^{13}C-NMR (151 MHz, DMSO-*d*^{6}) δ: 12.33, 16.79, 16.98, 22.89, 23.40,25.98, 26.00, 26.86, 27.06, 27.09, 28.64,30.11, 30.03, 30.05, 30.62, 30.71, 31.55, 31.63, 32.11, 32.43, 32.80, 33.70, 34.86, 37.97, 38.43, 41.48, 45.85, 45.86, 45.87, 46.05, 46.15, 47.34, 51.54, 54.81, 69.82, 70.87, 70.89, 70.90, 172.30, 173.68, 174.83; ESI-LCMS (*m/z*): calc'd for $[C_{54}H_{91}N_2O_8]^+$ $[M+H]^+$:894.68, found 894.69.

(4R,4'R)-N,N'-(6-oxo-6-((2-(N-(prop-2-yn-1-yl)benzamido)ethyl)amino)hexane-1,5-diyl)bis(4-((3R,5R,8R,9S,10S,12S,13R,14S,17S)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamide) (Molecule **3**)



Molecule **3** was synthesized according to the general amine condensation reaction mentioned above. A round bottom flask was charged with N-(2-aminoethyl)-N-(prop-2-yn-1-yl) benzamide (17.5 mg, 0.1 mmol) and molecule **2** (95 mg, 0.1 mmol), and DMF (5 mL). HATU (40 mg, 0.15 mmol) was added to the resulting solution at 0^{o}C. After stirring for 1 h, DIPEA (39 µL, 0.2 mmol) was added to the reaction mixture to react overnight at room temperature. The reaction mixture was extracted with ethyl acetate and washed with brine. The organic layer was combined, dried with $MgSO_4$, and condensed by evaporation to give a crude product of molecule **3**. The crude product was purified with flash chromatography with DCM: MeOH (from 100: 1 to 10:1). The corresponding elution fragment was checked with LC-MS, concentrated under reduced pressure by rotary evaporation, and dried in vacuo to give a 37% yield of purified molecule **3** (40 mg). ^{1}H-NMR (600 MHz, DMSO-*d*^{6}) δ: 8.16 (s, 1H), 7.88 (s, 1H), 7.71 (t, 1H, *J* = 5.4 Hz), 7.44 (br, 3H), 7.36 (s, 1H), 4.47 (d, 2H, *J* = 3.0), 4.10-4.34 (m, 4 H), 4.00 (s, 1H), 3.78 (s, 2H), 3.16 (d, 1H, *J* = 4.2 Hz), 2.91-3.02 (m, 2H), 1.99-2.19 (m, 4H),1.7-1.82 (m, 7H), 1.44-1.66 (m, 12H), 0.8-1.4 (m, 42H), 0.58 (t, 6H, *J* = 8.4 Hz); ^{13}C-NMR (151 MHz, DMSO-*d*^{6}) δ: 12.32, 16.96, 16.99, 22.99, 23.40, 26.00, 26.87, 27.08, 28.50, 28.73, 30.11, 31.48, 31.63, 32.20, 32.43, 32.80, 33.70, 34.94, 35.02, 35.53, 36.17, 38.05 41.48, 45.85, 46.10, 47.34, 48.48, 69.82, 70.89, 126.44, 128.29, 129.61, 135.63, 170.31, 172.13, 172.26, 172.62; ESI-LCMS (*m/z*): calc'd for $[C_{66}H_{103}N_4O_8]^+$ $[M+H]^+$: 1079.77, found 1079.87.

(4R,4'R)-N,N'-(6-oxo-6-(prop-2-yn-1-ylamino)hexane-1,5-diyl)bis(4-((3R,5R,8R,9S,10S,12S,13R,14S,17S)-3,12-dihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamide) (Molecule **4**)

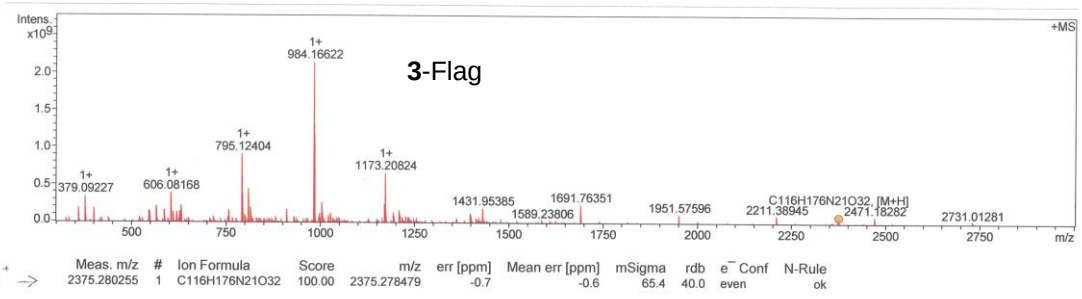


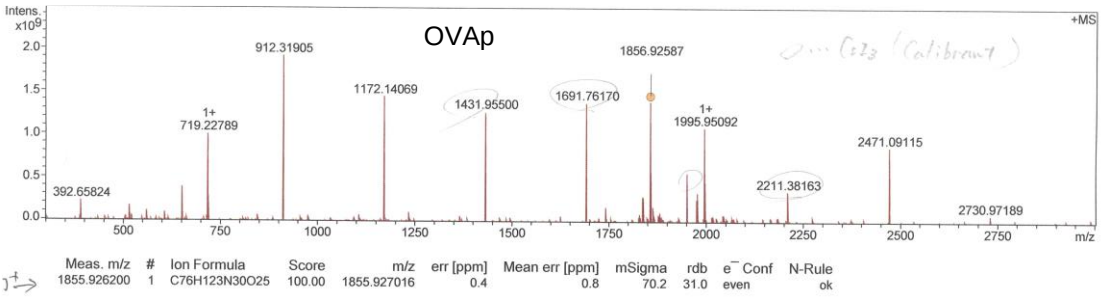
Molecule **4** was synthesized according to the general amine condensation reaction mentioned above. A round bottom flask was charged with propargyl amine (7.49 mg, 0.1293 mmol) and molecule **2** (100 mg, 0.1175 mmol), and DMF (5 mL). HATU (73 mg, 0.194 mmol) was added to the resulting solution at 0^{o}C. After stirring for 1 h, DIPEA (40 µL, 0.235 mmol) was added to the reaction mixture to react overnight at room temperature. The reaction mixture was extracted with ethyl acetate and washed with brine. The organic layer was combined, dried with $MgSO_4$, and condensed by evaporation to give a crude product of molecule **4**. The crude product was purified with flash chromatography with DCM: MeOH (from 100: 1 to 10:1). The corresponding elution fragment was checked with LC-MS, concentrated under reduced pressure by rotary evaporation, and dried in vacuo to give a 48.4% yield of purified molecule **4** (53 mg). ^{1}H-NMR (600 MHz, DMSO-*d*^{6}) δ: 8.31 (t, 1H, *J* = 5.4 Hz), 7.87 (d, 1H, *J* = 8.4 Hz), 7.719 (t, 1H, *J* = 5.4 Hz), 4.48 (dd, J_1 = 1.2 Hz, J_2 = 3.0 Hz), 4.14-4.22 (m, 3H), 3.82-3.83 (m, 3H), 3.786 (s, 1H), 3.090 (t, 1H, *J* = 5.4 Hz), 2.92-3.01(m, 2H), 1.90-2.23 (m, 4H), 1.74-1.825 (m, 8H), 1.44-1.66 (m, 14H), 1.13-1.36 (m, 26H), 0.84-1.0 (m, 19H), 0.58 (t, 6H, *J* = 8.4 Hz); ^{13}C-NMR (151 MHz, DMSO-*d*^{6}) δ: 12.32, 16.69, 17.00, 22.65, 22.99, 23.40, 26.00, 26.87, 27.09, 27.75, 28.49, 28.68, 30.11, 31.50, 31.53, 31.63, 32.23, 32.43, 32.80, 33.71, 34.95, 35.02, 35.54, 36.17, 38.06, 39.91, 41.48, 45.85, 45.86, 45.87, 46.05, 46.10, 47.34, 51.96, 69.82, 70.88, 72.82, 80.98, 171.65, 172.29, 172.62; ESI-LCMS (*m/z*): calc'd for $[C_{57}H_{94}N_3O_7]^+$ $[M+H]^+$: 933.71, found 933.75.

General procedure for copper-catalyzed azide-alkyne cycloaddition (CuAAC)

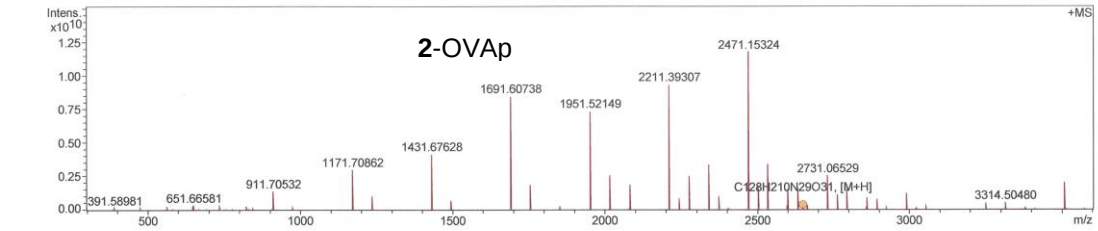
To a solution of azide (0.1 mmol), alkyne (0.1 mmol) in MeOH: THF (5:1, 10 mL), 1M aqueous $CuSO_4$ (0.3 mmol) and 1M aqueous sodium ascorbate (0.15 mmol) were added. The resulting mixture was stirred overnight. After reaction completion, precipitation was formed, collected by centrifugation in a centrifuge tube, and purified by HPLC.**4**-PEG-rhodamine^{1}H-NMR (600 MHz, DMSO-*d*^{6}) δ: 8.419 (d, 1H, *J* = 1.8 Hz), 8.343 (t, 1H, *J* = 5.4 Hz), 8.063 (t, 1H, *J* = 6.0 Hz), 7.771 (dd, 1H, J_1 = 1.8 Hz, J_2 = 6.0 Hz), 7.850 (d, 1H, *J* = 7.8 Hz), 7.834 (s, 1H), 7.703 (t, 1H, *J* = 6.0 Hz), 7.462 (d, 1H, *J* = 7.8 Hz), 7.04 (dd, 2H, J_1 = 6.0 Hz, J_2 = 9.6 Hz), 6.982 (s, 1H), 6.966 (s, 1H), 6.937 (d, 2H, *J* = 2.4 Hz), 4.476 (t, 2H, *J* = 5.4 Hz), 4.271 (d, 1H, *J* = 5.4 Hz), 3.785 (t, 2H, *J* = 5.4 Hz), 3.673-3.603 (m, 8H), 3.521-3.456(m, 10H), 3.02(q, 2H, *J* = 5.4 Hz), 2.976-2.955 (m, 2H), 2.180 (t, 1H, *J* = 7.2 Hz), 1.817-1.462 (m, 10H), 1.350-0.835 (m,70H), 0.573 (d, 6H, *J* =4.2 Hz); ^{13}C-NMR (151 MHz, DMSO-*d*^{6}) δ: 12.30, 13.85, 16.992, 16.956, 21.99, 22.40, 22.71, 22.98, 23.33, 23.40, 24.37, 26.00, 26.87, 27.08, 28.428, 28.483, 28.594, 28.624, 28.702, 28.789, 28.8668, 28.893,28.092, 28.921, 28.935, 30.11, 31.502, 31.541, 31.625, 32.24, 32.61, 32.80, 33.54, 33.70, 34.10, 34.913, 34.956, 35.016, 35.033, 35.35, 35.54, 36.17, 38.06, 39.93, 40.89, 41.48, 42.34, 45.14, 45.84, 46.08, 46.09, 47.32, 49.18, 52.20, 68.66, 69.01, 69.412, 69.530, 69.554, 69.564, 69.817, 70.89, 70.96, 79.38, 113.35, 113.53, 122.94, 125.58, 126.48, 130.47, 132.55, 132.88, 141.47, 144.60, 147.84, 154.90, 156.99, 157.34, 157.70, 157.94, 158.19, 158.34, 171.33, 171.83, 172.27, 172.64, 174.10.

MS spectrum

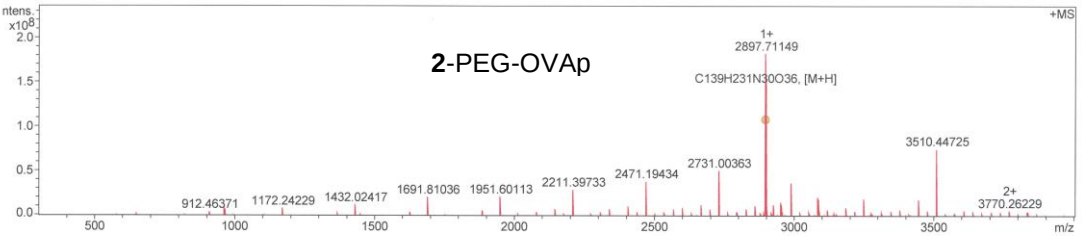




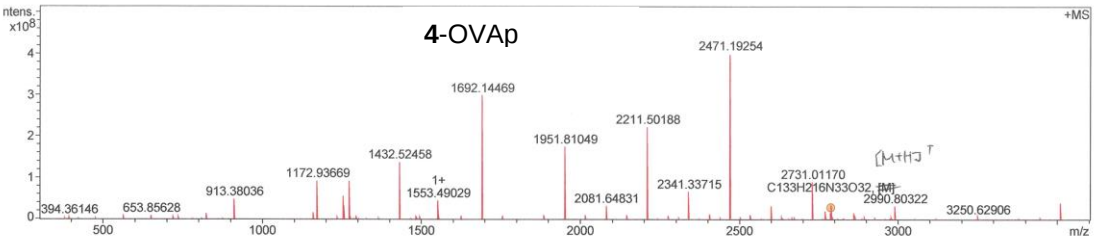
Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
1855.926200	1	C76H123N30O25	100.00	1855.927016	0.4	0.8	70.2	31.0	even	ok



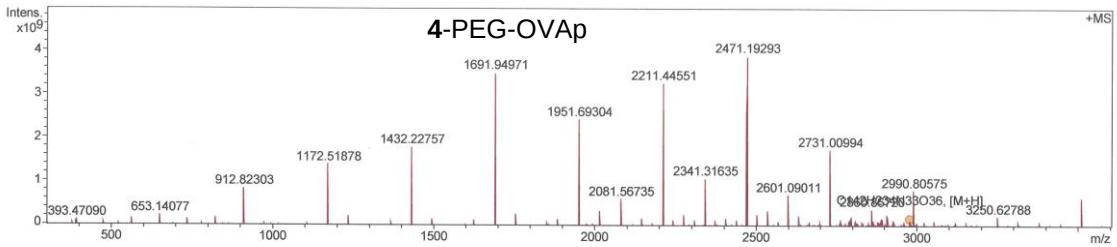
Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
2649.574580	1	C128H210N29O31	100.00	2649.574208	-0.1	0.1	166.6	39.0	even	ok



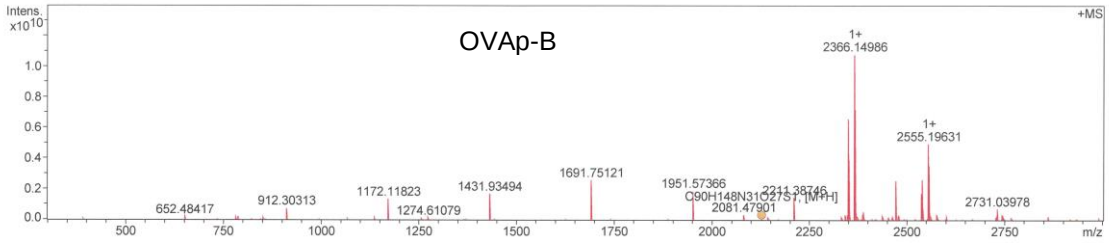
Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
2896.720072	1	C139H231N30O36	100.00	2896.716180	-1.3	-0.5	559.3	40.0	even	ok



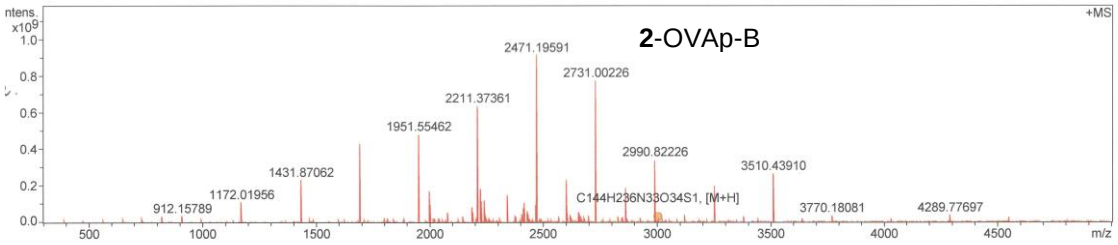
Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
2787.623333	1	C133H216N33O32	100.00	2787.628368	1.8	2.3	136.2	43.0	even	ok



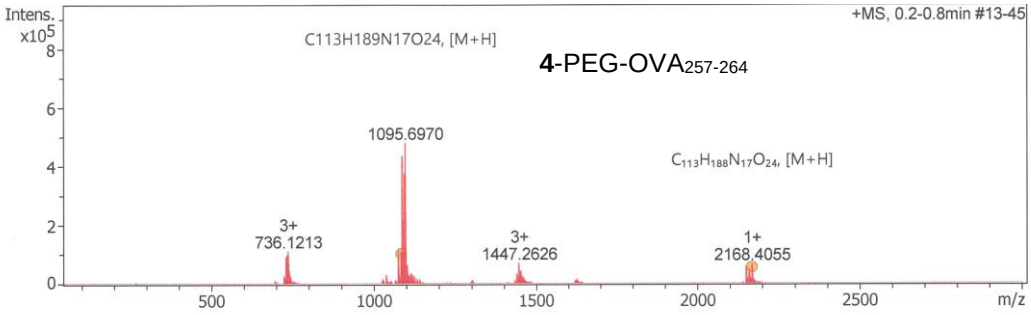
Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
2977.727051	1	C142H234N33O36	100.00	2977.748877	7.3	5.2	109.9	43.0	even	ok



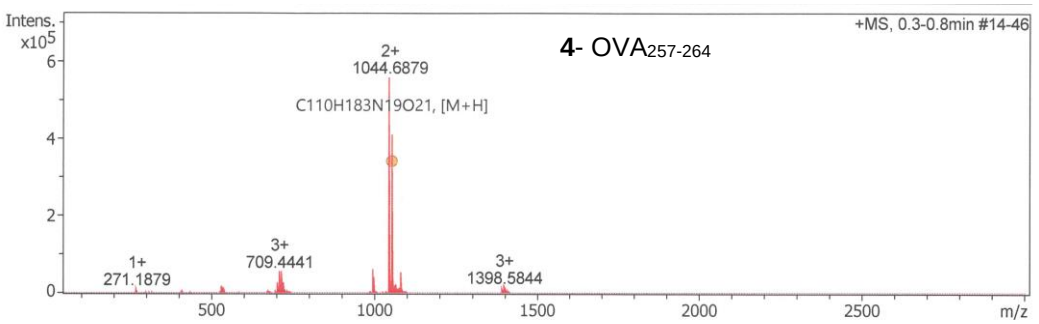
Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
2127.090178	1	C90H148N31O27S	100.00	2127.087616	-1.2	-1.4	66.6	33.0	even	ok



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
3003.745959	1	C144H236N38O34S	100.00	3003.746769	0.3	-0.4	132.4	44.0	even	ok



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
1084.2047	1	C113H189N17O24	1084.2040	-0.6	76.0	1	100.00	29.0	even	ok
2167.4020	1	C113H188N17O24	2167.4008	-0.6	104.2	1	100.00	29.0	even	ok



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
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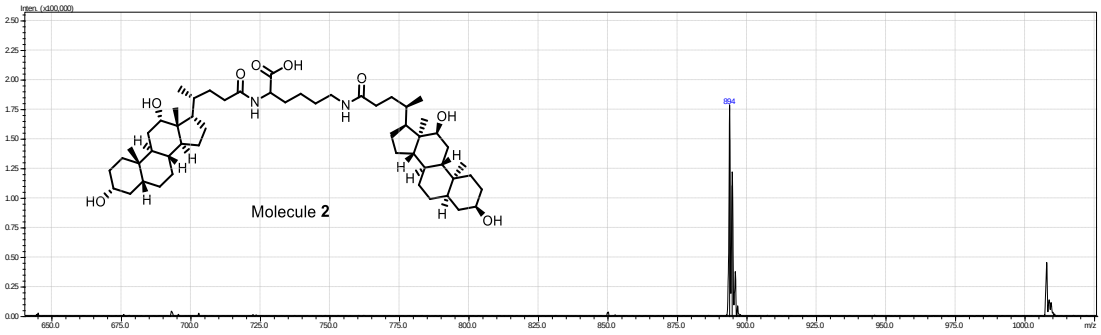
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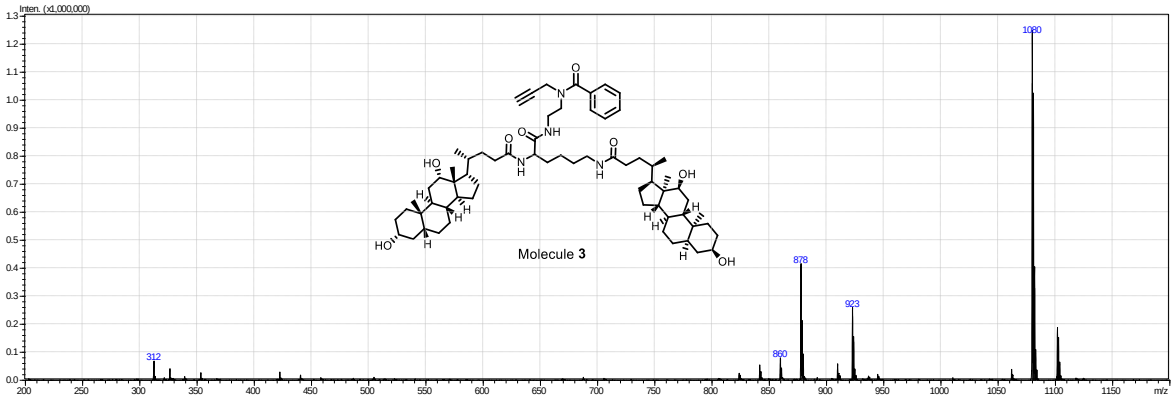
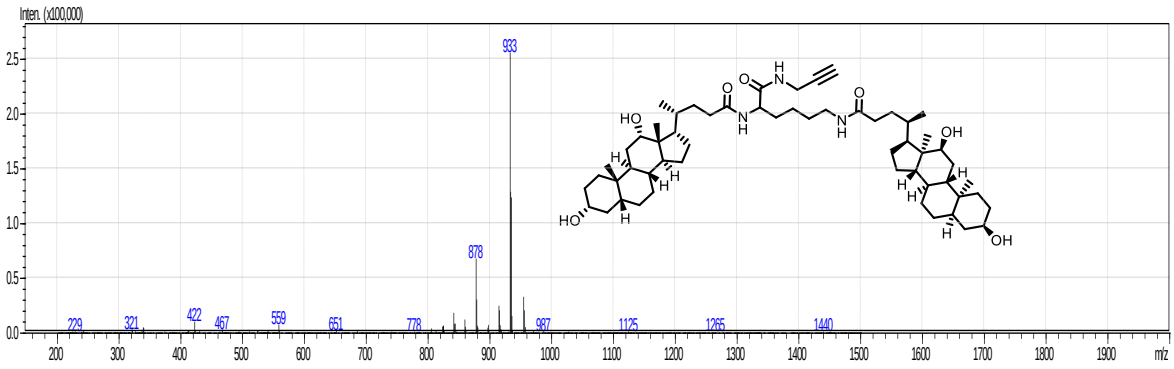
Molecule 2
894
m/z


Inten. (x1,000,000)
Molecule 3
1080
878
923
860
312
m/z


Inten. (x100,000)
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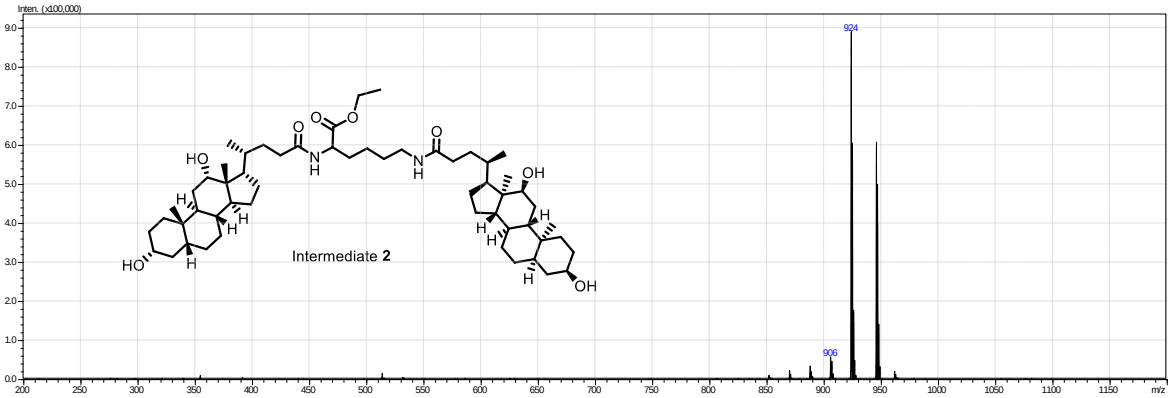
559
651
778
987
1125
1265
1440
m/z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Intermediate 2
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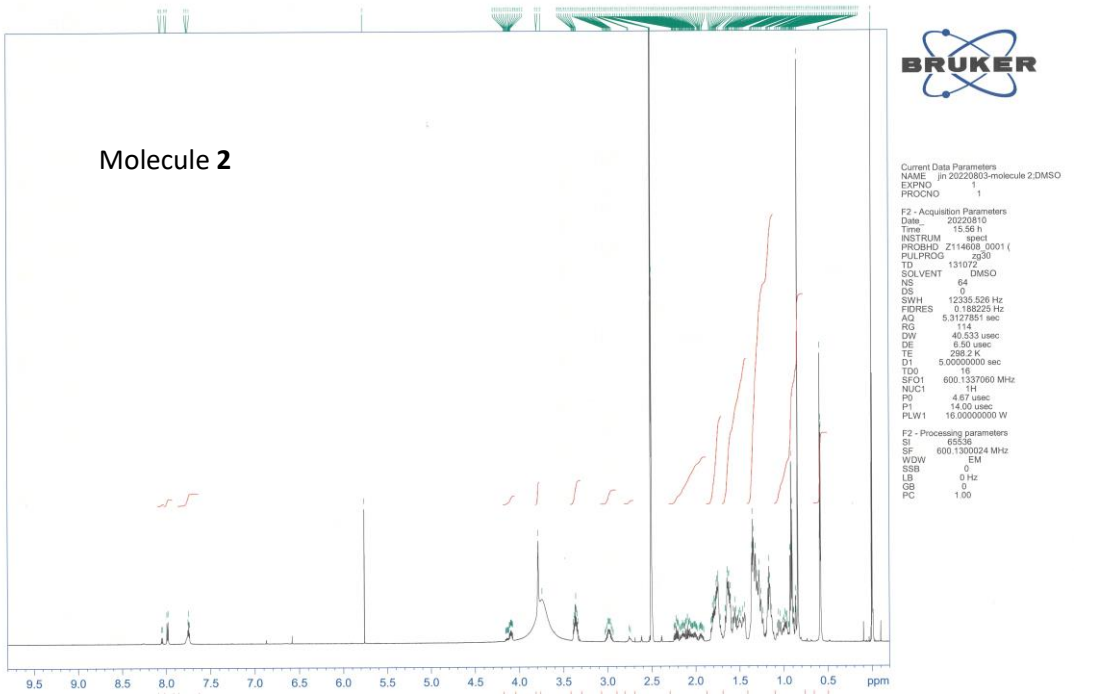
906
m/z

NMR spectra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DE 6.50 usec
TE 298.2 K
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TD0 16
SFO1 600.1337060 MHz
NUC1 1H
P0 4.67 usec
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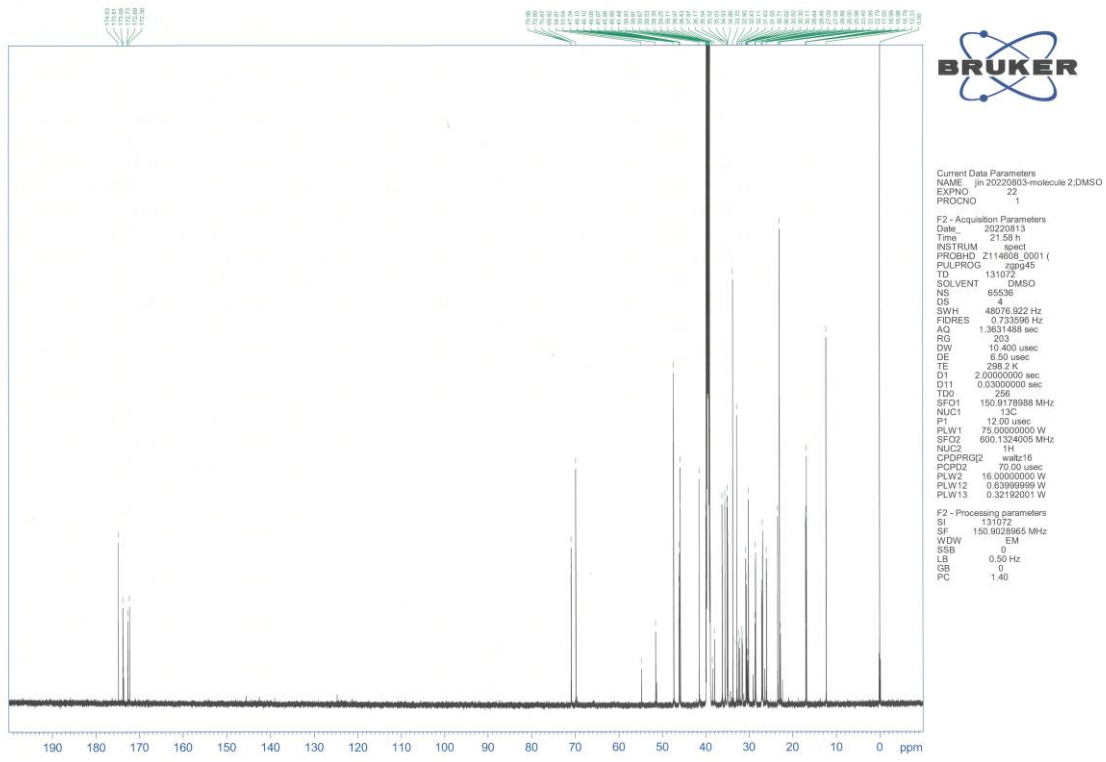
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RG 203
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D1 2.00000000 sec
D11 0.03000000 sec
TD0 256
SFO1 150.9178988 MHz
NUC1 13C
P1 12.00 usec
PLW1 75.00000000 W
SFO2 600.1324005 MHz
NUC2 1H
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PLW12 0.63999999 W
PLW13 0.32192001 W
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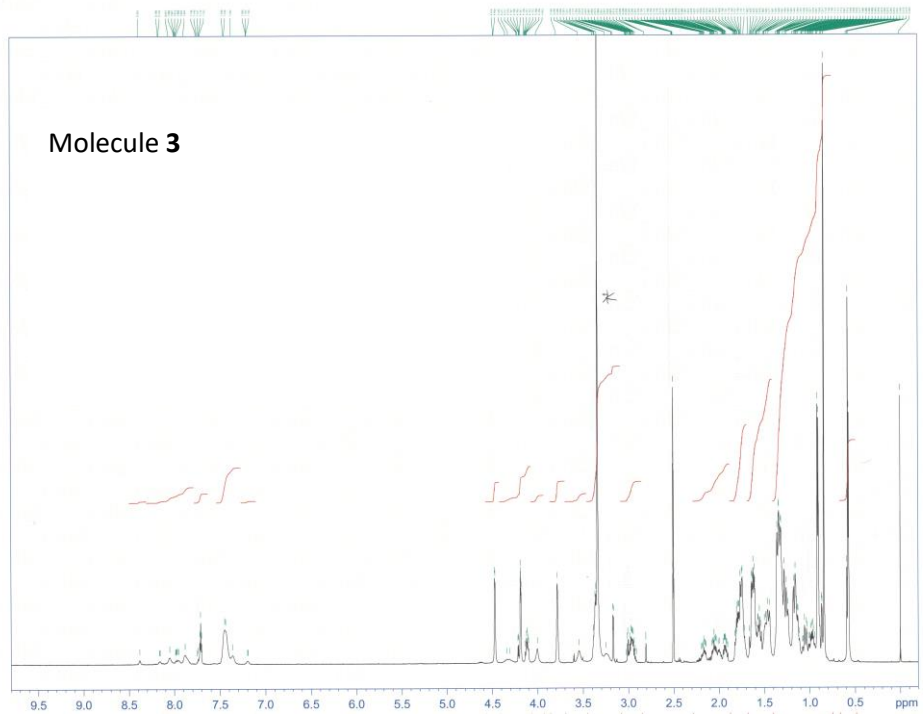
Molecule **3**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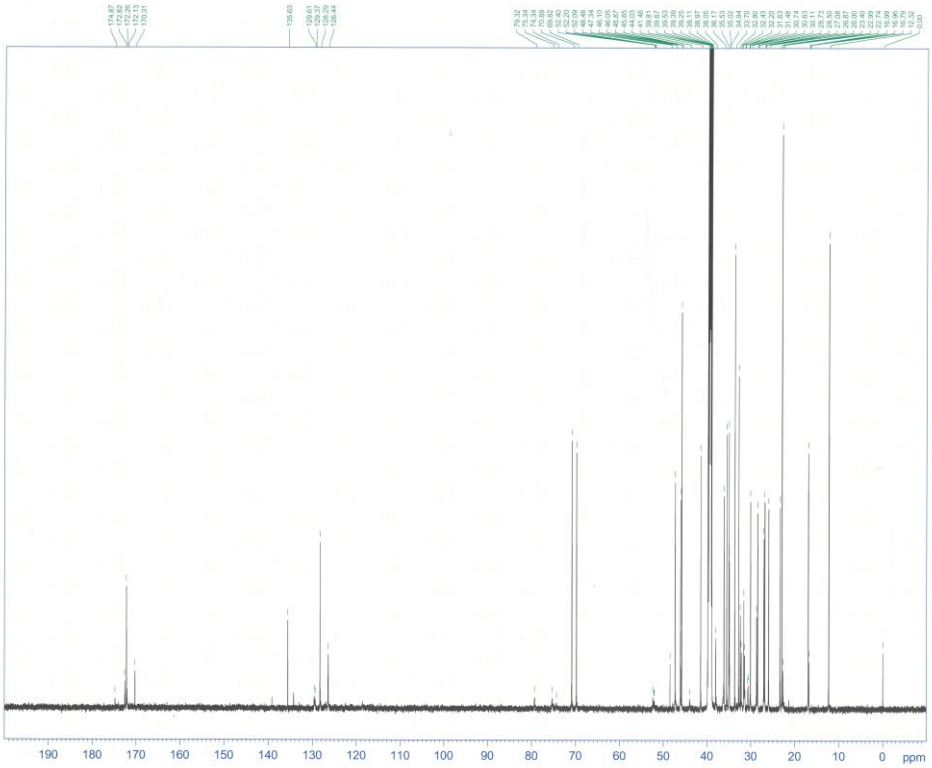
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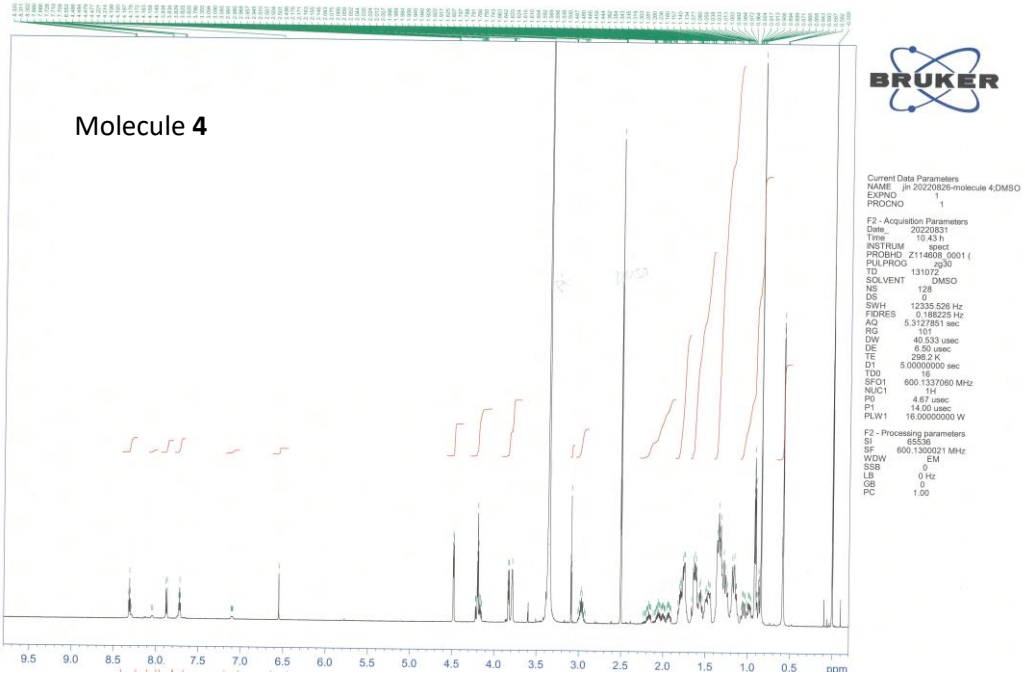
Molecule **4**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TE 298.2 K
D1 5.00000000 sec
TD0 16
SFO1 600.1337060 MHz
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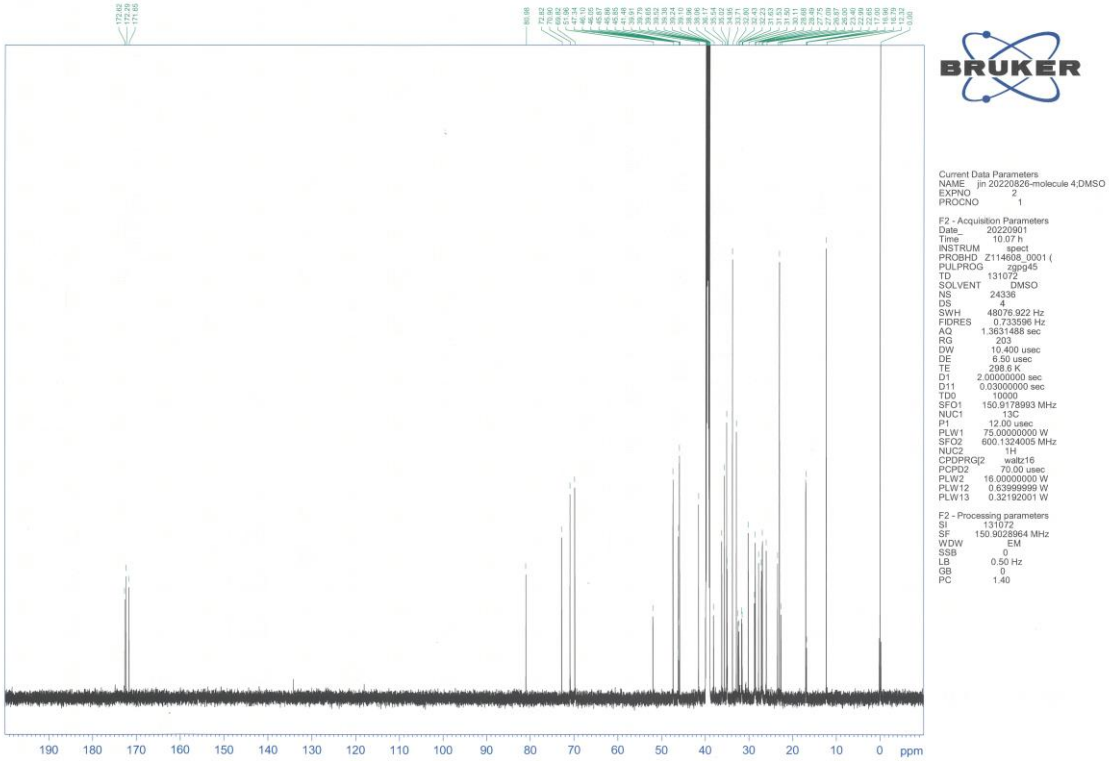
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DW 10.400 usec
DE 6.50 usec
TE 298.6 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 10000
SFO1 150.9178993 MHz
NUC1 13C
P1 12.00 usec
PLW1 75.00000000 W
SFO2 600.1324005 MHz
NUC2 1H
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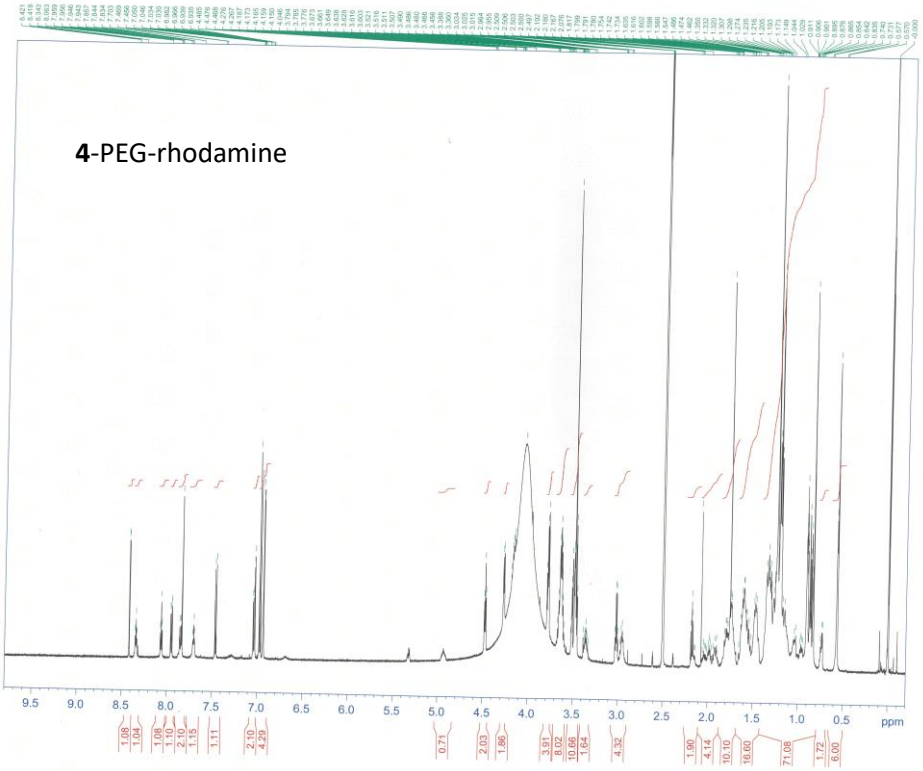
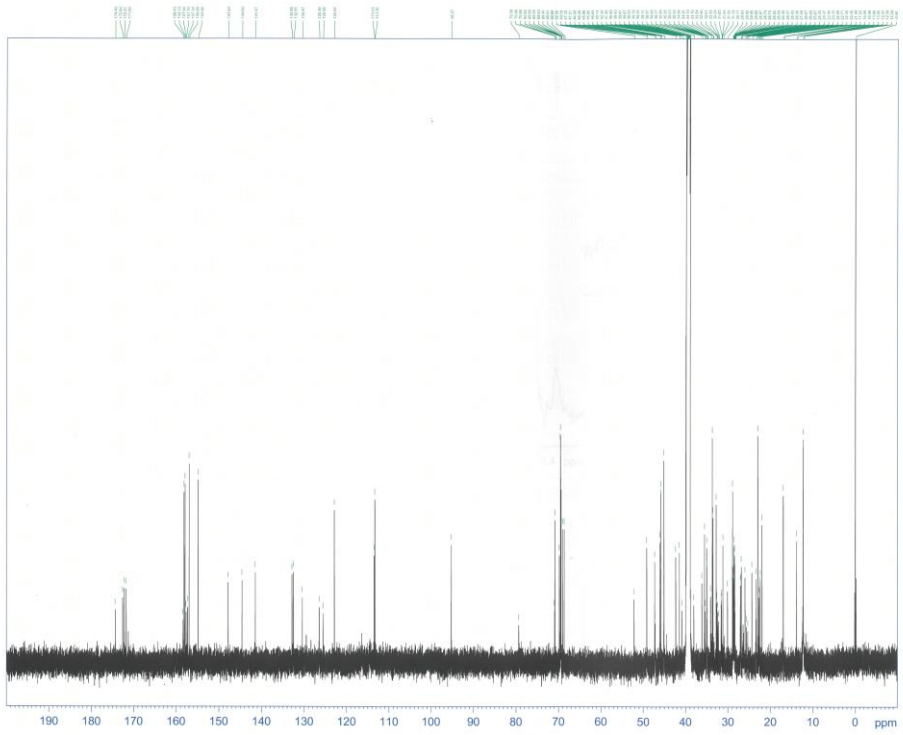
4-PEG-rhodamine





Purity analysis

Molecule **2**

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サンプルID	:20221003 Molecule 2-0		
データファイル	:20221006 Molecule 2-1.lcd		
メソッドファイル	:Cholicamide Derivatives _project purity_check.lcm		
バッチファイル	:		
バイアル番号	:-1	サンプルタイプ	:未知
注入量	:50 uL		
分析日時	:2022/10/06 18:36:43	分析者	:System Administrator
解析日時	:2022/10/06 19:23:25	解析者	:System Administrator

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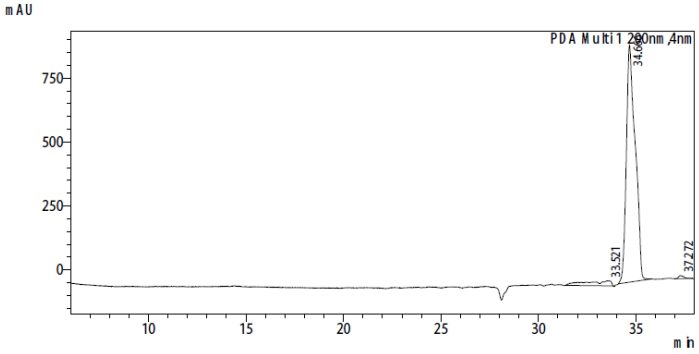

<ピークレポート>

PDA Ch1 200nm

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3	37.272	241045	12309	0.765
合計		31500730	958169	100.000

Molecule **3**

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サンプルID	:Molecule 3		
データファイル	:Molecule 3		
メソッドファイル	:Cholicamide Derivatives _project purity_check.lcm		
バッチファイル	:		
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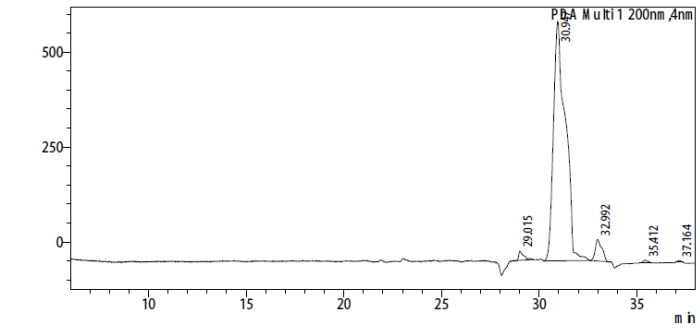

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PDA Ch1 200nm

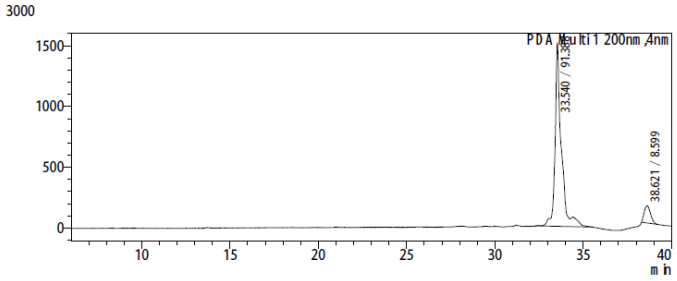
ピーク#	保持時間	面積	高さ	濃度	面積%
1	29.015	426153	24173	1.476	1.476
2	30.947	26931994	631125	93.260	93.260
3	32.992	1349854	56562	4.674	4.674
4	35.412	119286	7124	0.413	0.413
5	37.164	51050	4136	0.177	0.177
合計		28878337	723119		100.000

Molecule **4**

〈サンプル情報〉

サンプル名	20221006 Molecule 4		
サンプルID	:20221006 Molecule 4		
データファイル	:20221006 Molecule 4		
メソッドファイル	:Cholicamide Derivatives_project purity_check.lcm		
バッチファイル	:		
バイアル番号	:-1	サンプルタイプ	:未知
注入量	:50 uL		
分析日時	:2022/10/06 10:07:11	分析者	:System Administrator
解析日時	:2022/10/06 11:05:31	解析者	:System Administrator

〈クロマトグラム〉



〈ピークレポート〉

PDA Ch1 200nm

ピーク#	保持時間	面積	高さ	面積%
1	0.141	3490	781	0.009
2	0.267	1861	636	0.005
3	33.540	37065225	1504895	91.388
4	38.621	3487455	141389	8.599
合計		40558030	1647702	100.000

Molecule **4**-PEG-rhodamine

〈サンプル情報〉

サンプル名	20221007 4-PEG-rhodamine		
サンプルID	:20221007 4-PEG-rhodamine		
データファイル	:20221007 4-PEG-rhodamine		
メソッドファイル	:Cholicamide Derivatives_project purity_check.lcm		
バッチファイル	:		
バイアル番号	:-1	サンプルタイプ	:未知
注入量	:50 uL		
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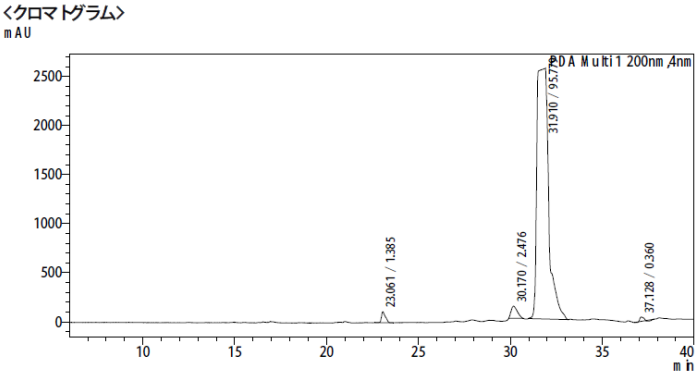
解析日時	:2022/10/07 13:06:10	解析者	:System Administrator

〈クロマトグラム〉



〈ピークレポート〉

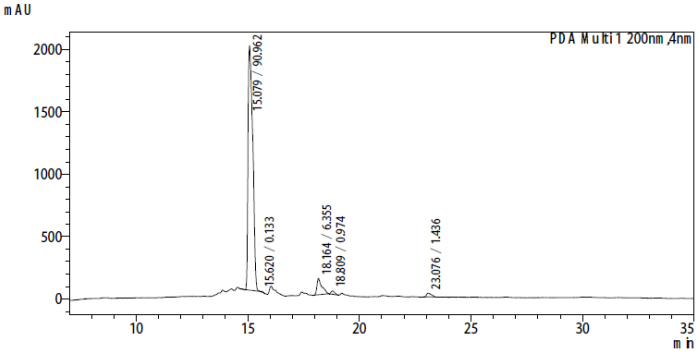
PDA Ch1 200nm

ピーク#	保持時間	面積	高さ	面積%
1	23.061	1632606	111501	1.385
2	30.170	2919184	124673	2.476
3	31.910	112918492	2555138	95.779
4	37.128	424322	43608	0.360
合計		117894605	2834921	100.000

2-OVAp

サンプル名	:20221007 2-OVAp		
サンプルID	:20221007 2-OVAp		
データファイル	:20221007 2-OVAp1.lcd		
メソッドファイル	:Cholicamide Derivatives_project purity_check.lcm		
バッチファイル	:		
バイアル番号	:-1	サンプルタイプ	:未知
注入量	:50 uL		
分析日時	:2022/10/07 16:27:43	分析者	:System Administrator
解析日時	:2022/10/07 17:09:33	解析者	:System Administrator

<クロマトグラム>



<ピークレポート>

PDA Ch1 200nm

ピーク#	保持時間	面積	高さ	面積%
1	0.240	44369	6510	0.140
2	15.079	28746112	1956828	90.962
3	15.620	42027	6018	0.133
4	18.164	2008352	129951	6.355
5	18.809	307652	27446	0.974
6	23.076	453871	29821	1.436
合計		31602382	2156574	100.000

1. T. Ishikawa, F. Itoh, S. Yoshida, S. Saijo, T. Matsuzawa, T. Gonoi, T. Saito, Y. Okawa, N. Shibata, T. Miyamoto and S. Yamasaki, *Cell Host Microbe*, 2013, **13**, 477-488.