Supporting Information

Long-Acting Human Interleukin 2 Bioconjugate Modified with Fatty Acids by Sortase A

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Contents

Reagents

8-[(9H-Fluoren-9-ylmethoxy)carbonylamino]-3,6-dioxa-n-octanoic acid (Fmoc-NH-PEG_2-CH_2COOH, Fmoc-AEEA-OH), (S)-2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-5-(tert-butoxy)-5-oxopentanoic acid (Fmoc-Glu(Otbu)-OH) and (S)-2-((((9H-Fuoren-9-yl)methoxy)carbonyl)amino)-6-(((allyloxy)carbonyl)amino)hexanoic acid (Fmoc-Lys(Alloc)-OH) were purchased from CS Biotechnology Co., Ltd. (Menlo Park, CA, US). Chloride resin (2-CTC resin), 1-hydroxybenzotriazole (HOBT), piperidine (PIP), N,N-diisopropylethylamine (DIEA) and 2,2-Dimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oic acid (Boc-Gly-Gly-Gly-OH) were obtained from GL Biochemical (Shanghai) Ltd. (Shanghai, China). N,N-Diisopropylcarbodiimide (DIC) and trifluoroacetic acid (TFA) were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). Dichloromethane (DCM) and N,N-dimethylformamide (DMF) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Phenylsilane and tetrakis (triphenylphosphine) palladium ($Pd(PPh_3)_4$) were purchased from Energy Chemical, (Shanghai, China). Sodium diethyldithiocarbamate was acquired from J&K Scientific Ltd. (Shanghai, China) and octadecanedioic acid, 1-(1,1-dimethylethyl) ester from Chia Tai Tianqing Pharmaceutical Group Co. Ltd. (Nanjing, China). Acetonitrile (MeCN) was purchased from Yunnan Xinlanjing Chemical Industry Co., Ltd. (Yuxi, China). All other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

Expression and Purification of IL-2 Analogs

Three recombinant IL-2 analogs, A1: IL-2-$(GGGGS)_3$-LPETGG-His_6, A2: IL-2-$(PA)_6$-LPETGG-His_6, and A3: IL-2-$(ED)_6$-LPETGG-His_6 were engineered via gene fusion. Among these, the amino acid sequence and structure of part of IL-2 are consistent with aldesleukin (125-L-serine-2-133-interleukin 2). First, codon DNA sequences were

synthesized. After amplification via polymerase chain reaction (PCR), sequences were cloned into a pET29a vector. Expression vectors were transformed into *E. coli* strain BL21 (DE3) for protein expression.

Expression was induced with 5 L fed-batch fermentation. Briefly, cells were grown at 37 ℃ to OD_{600} of ~ 60 and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 12 h. Cell pellets were harvested via centrifugation at 12,000 ×*g* for 20 min.

After washing twice in 25 mM Tris(hydroxymethyl) aminomethane-HCl (Tris-HCl) buffer at pH 8.0, the cell pellet was suspended in 25 mM Tris-HCl buffer, pH 8.0, and disrupted using ATS 100 (ATS Engineering Inc., Shanghai, China) operated at 800 bars at 4 ℃ three times to obtain inclusion bodies. Inclusion bodies were harvested via centrifugation at 12,000 ×*g* for 20 min and stored at -20 ℃ after washing in ddH_2O.

Inclusion bodies were suspended in 10 mM sodium phosphate buffer (pH 7.2) at a wet weight of 100 mg/mL. Sodium dodecyl sulfate (SDS) was added to a final concentration of 2% (w/v) for ultrasonic dissolution and the solution stirred for denaturation at room temperature for 2 h. After centrifugation at 12000 ×*g* for 10 min, denatured solution was obtained and dropped into renaturation solution (10 mM sodium phosphate, 0.5 M arginine, 10 mM $CuCl_2$, pH 9.5) at 1:25 (v/v) for renaturation at 4 ℃ overnight.

The renaturation solution was adjusted to pH 7.5 with 6 M HCl. Next, methanol was added to a final concentration of 5% (v/v) to terminate renaturation. After filtering with a 0.22 μm membrane, the sample was loaded onto a reversed-phase C4 preparative chromatography column (Fuji pro C4 30–75 μm, 10×250 mm, 300 Å, Toyama, Japan). The mobile phase comprised gradient elution with 0.1% TFA (v/v) water-acetonitrile solution (60:40, v/v, solution A) and 0.1% TFA (v/v) acetonitrile solution (solution B). The target protein was eluted under a gradient of 10–40% solution B buffer. After diluting with ddH_2O, the collected fractions containing target protein were further purified via reversed-phase C4 preparative chromatography (YMC park pro C4 30 μm, 10×250 mm, 300 Å, Kyoto, Japan). The mobile phase comprised gradient elution with

0.1% TFA (v/v) water-acetonitrile solution (60:40 v/v, solution A) and 0.1% TFA (v/v) acetonitrile solution (solution B). The target protein was under a gradient elution of 10–40% of solution B buffer. Pooled fractions were evaporated on a rotary evaporator and lyophilized overnight.

The target protein was reconstituted with PBS buffer and replaced three times in 3 kDa ultrafiltration centrifuge tubes (Millipore, Billerica, MA, US)， followed by SDS-PAGE, under reducing conditions (Coomassie brilliant blue (CBB) staining), reversed-phase HPLC (Agilent, Santa Clara, CA, US) and ESI-MS (Waters Xevo G2-XS QTof, Milford, Massachusetts, US). The protein concentration was determined using a bicinchoninic acid (BCA) assay kit (Sangon Biotech, Shanghai, China) with bovine serum albumin (BSA) as a standard.

IL-2-$(GGGGS)_3$-LPETGG-His_6 (A1), mass calculated 17652.15 Da, mass observed: 17651.65 Da.

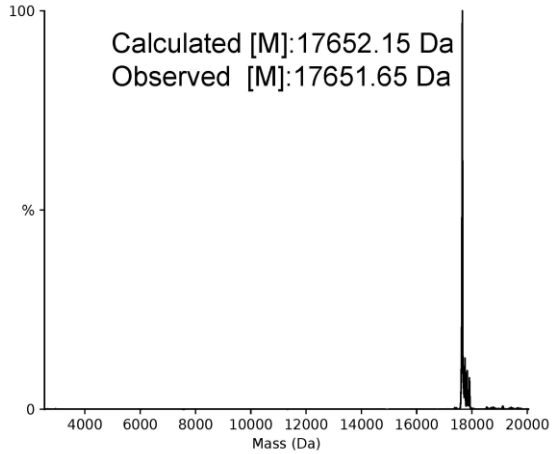


Figure S1. Deconvoluted ESI-MS spectra of A1

IL-2-$(PA)_6$-LPETGG-His_6 (A2), mass calculated 17715.47 Da, mass observed: 17714.97 Da.

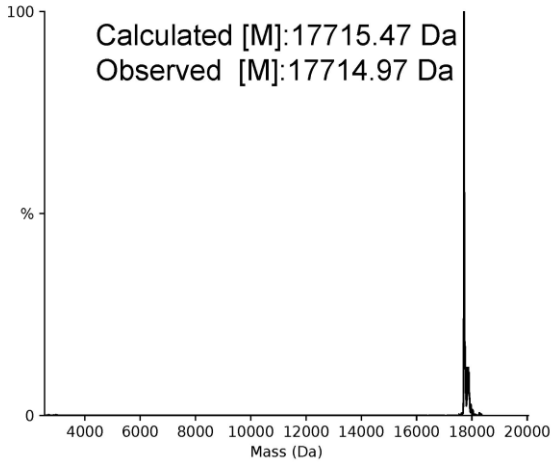


Figure S2. Deconvoluted ESI-MS spectra of A2

IL-2-$(ED)_6$-LPETGG-His_6 (A3), mass calculated 18171.52 Da, mass observed: 18170.99 Da.

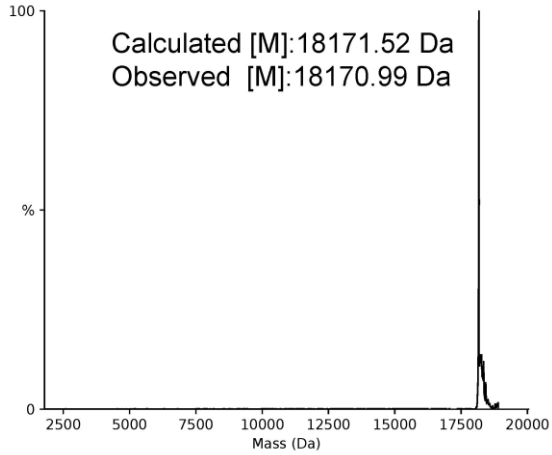
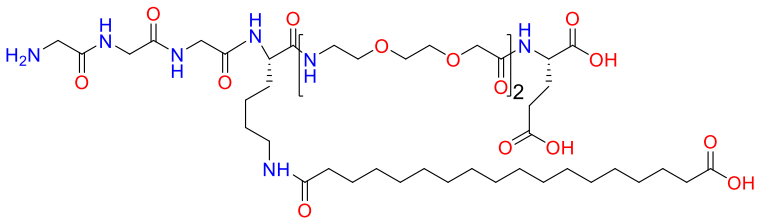


Figure S3. Deconvoluted ESI-MS spectra of A3

Synthesis and Preparation of Fatty Acid Moieties F1-F6

Preparation of Gly-Gly-Gly-Lys(Nε-C18-diacid)-2×AEEA-Glu (F6)



Fragments F6 was synthesized according to the following reaction scheme.

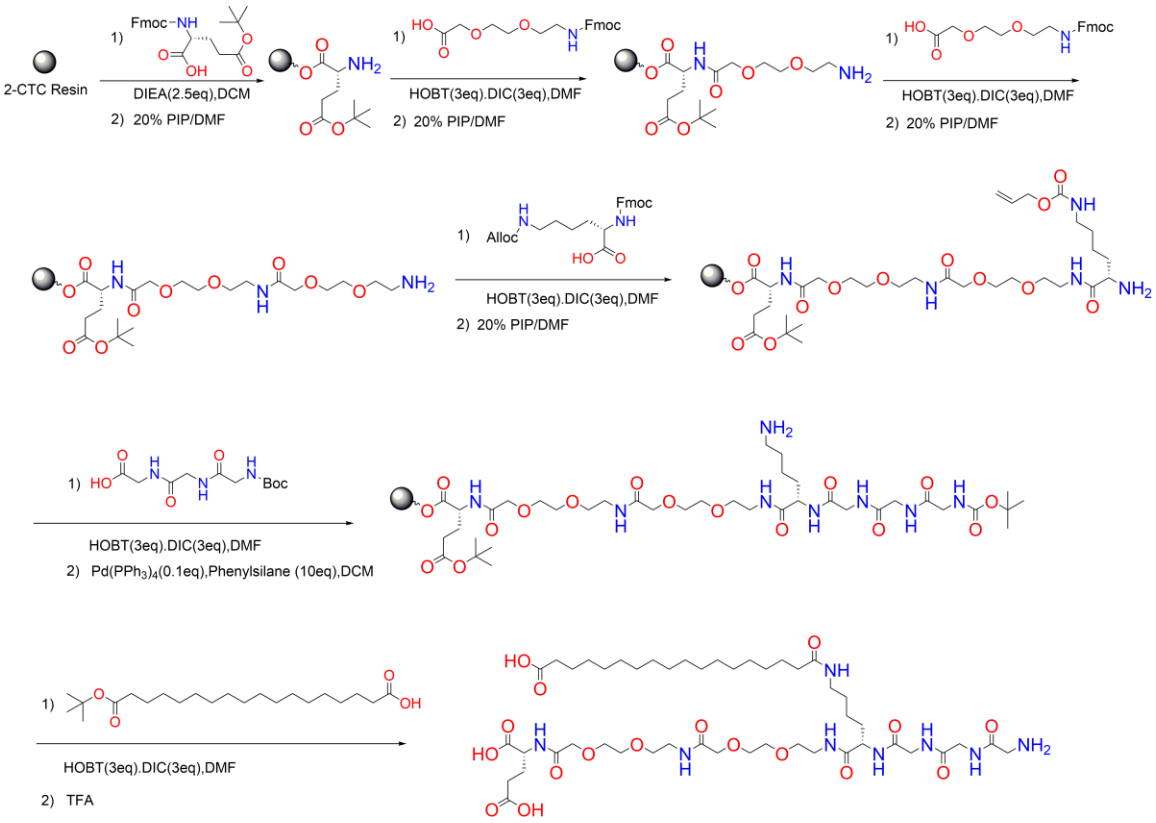


Figure S4. Reaction route of fatty acid moiety F6

2-Chlorotrityl chloride resin (2-CTC resin) and Fmoc-Glu(OtBu)-OH were used as the carrier and raw material, respectively. Fatty acid moieties were synthesized using a CS Bio solid-phase synthesizer (CS Bio, Menlo Park, CA, US).

First, 1 g of 2-CTC resin (degree of substitution (SD) = 1.08 mmol/g) was weighed and placed in a reactor. The resin was washed three times with N, N-dimethylformamide (DMF, 10 mL), swelled with DMF (10 mL) for 1 h, and the solvent emptied after completion.

A solution of Fmoc-Glu(OtBu)-OH (2.5 eq) and DIEA (2.5 eq) in 10 mL dichloromethane (DCM) was added, and the reaction stirred for 2 h. The resin was washed six times with DMF (10 mL), a small amount removed and vacuum-dried, and SD value measured (SD = 0.60 mmol/g).

For removal of the Fmoc-group, 10 mL of 20% piperidine (PIP)/DMF solution was added to the above resin, mixed for 10 min, and emptied. The procedure was repeated to process the resin. The resin was drained and washed six times with DMF (10 mL).

Fmoc-AEEA-OH (3 eq) was dissolved in 10 ml DMF and mixed with 1-hydroxybenzotriazole (HOBT, 3 eq) and N, N-diisopropylcarbodiimide (DIC, 3 eq). The mixture was added to drained resin, and the reaction was mixed at room temperature for 1 h. The resin was drained and washed six times with DMF (10 mL).

The Fmoc-group was removed as described above followed by addition of Fmoc-AEEA-OH (3 eq) activated by HOBT (3 eq) and DIC (3 eq) in 10 ml DMF. The reaction was mixed at room temperature for 1 h. The resin was drained and washed six times with DMF (10 mL).

The Fmoc-group was removed as described above followed by addition of Fmoc-Lys(Alloc)-OH (3 eq) activated by HOBT (3 eq) and DIC (3 eq) in 10 ml DMF. The reaction mixed at room temperature for 2 h. The resin was drained and washed six times with DMF (10 mL).

The Fmoc-group was removed as described above followed by addition of Boc-Gly-Gly-Gly-OH (3 eq) activated by HOBT (3 eq) and DIC (3 eq) in 10 ml DMF. The reaction mixed at room temperature for 2 h. The resin was drained and washed six times with DMF (10 mL).

For removal of the Alloc-group, 15 mL of tetrakis (triphenylphosphine) palladium ($Pd(PPh_3)_4$): phenylsilane (0.1:10) /DCM solution was added to resin, in the dark for 1 h, followed by draining of the resin and washed 6 times with DCM (10 mL). The resin was subsequently treated with 15 mL of sodium diethyldithiocarbamate (0.02 mol/L) /DCM

solution for three times, drained and washed six times with DMF (10 mL).

Octadecanedioic acid, 1-(1,1-dimethylethyl) ester (3 eq) was dissolved in 10 mL DMF and mixed with HOBT (3 eq) and DIC (3 eq). The mixture was added to drained resin, the reaction mixed at room temperature for 2 h. After completion of reaction, the resin was drained and washed six times with DMF (10 mL).

The resin was cleaved with a mixture of 95% TFA (9.5 mL) and 5% DCM (0.5 mL), shaken for 2 h at room temperature and filtered into cold diethyl ether (90 mL). The resulting precipitate was isolated via centrifugation at 900 ×*g* for 5 min followed by washing with diethyl ether twice and drying under vacuum.

After the molecular weight of the crude compound was confirmed through ESI-MS (Waters ACQUITY QDa, Milford, Massachusetts, US), the fatty acid moiety F6 was purified on prep-HPLC using reversed-phase C4 preparative chromatography column (YMC park pro C4 30 μm, 10×250 mm, 300 Å; Kyoto, Japan). The mobile phase comprised gradient elution with 0.1% TFA (v/v) water solution (solution A) and 0.1% TFA (v/v) acetonitrile solution (solution B). The target fragment was eluted under a gradient elution of 20–50% solution B buffer. Pooled fractions were evaporated on a rotary evaporator and lyophilised overnight.

ESI-MS: $[M+H]^+$ found: 1033.78, mass calculated 1032.60.

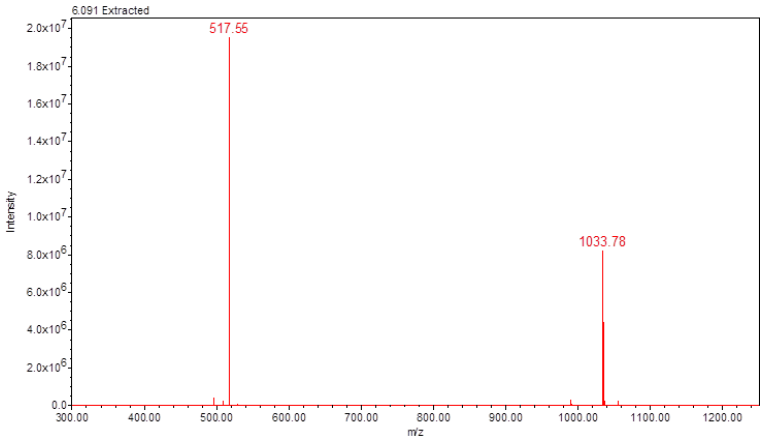
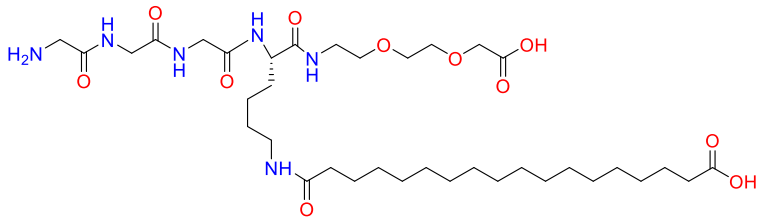


Figure S5. ESI-MS data for F6

Preparation of Gly-Gly-Gly-Lys(Nε-C18-diacid)-AEEA (F1)

In a similar way to fragments F6, F1 was prepared using Fmoc-AEEA-OH and 2-CTC resin.



ESI-MS: $[M+H]^+$ found: 759.68, mass calculated 758.48.

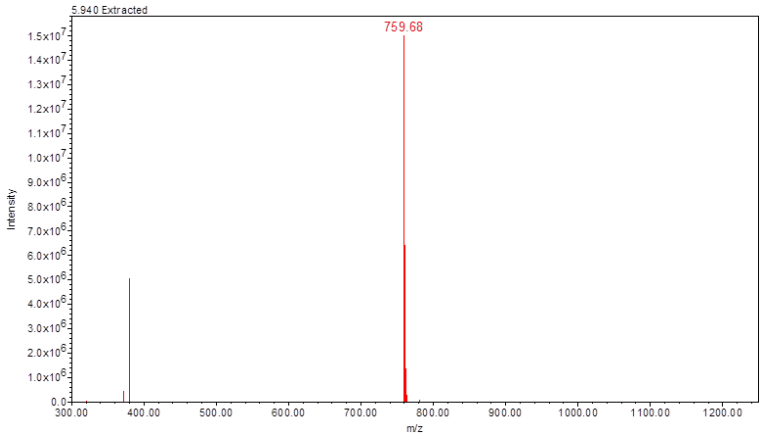
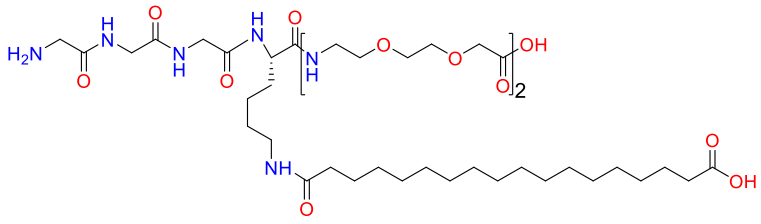


Figure S6. ESI-MS data for F1

Preparation of Gly-Gly-Gly-Lys(Nε-C18-diacid)-2×AEEA (F2)

In a similar way to fragments F6, F2 was prepared using Fmoc-AEEA-OH and 2-CTC resin.



ESI-MS: $[M+H]^+$ found: 904.65, mass calculated 903.55.

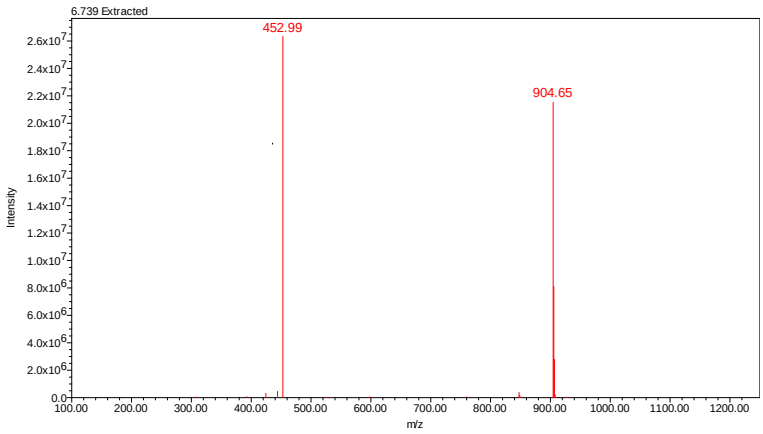
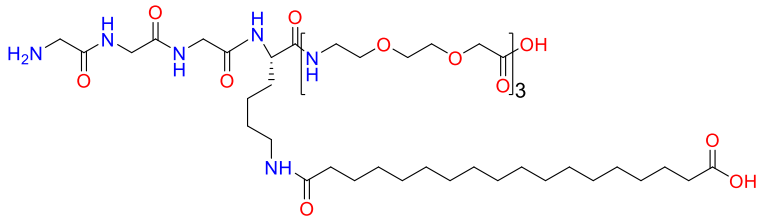


Figure S7. ESI-MS data for F2

Preparation of Gly-Gly-Gly-Lys(Nε-C18-diacid)-3×AEEA (F3)

In a similar way to fragments F6, F3 was prepared using Fmoc-AEEA-OH and 2-CTC resin.



ESI-MS: $[M+H]^+$ found: 1049.81, mass calculated 1048.63.

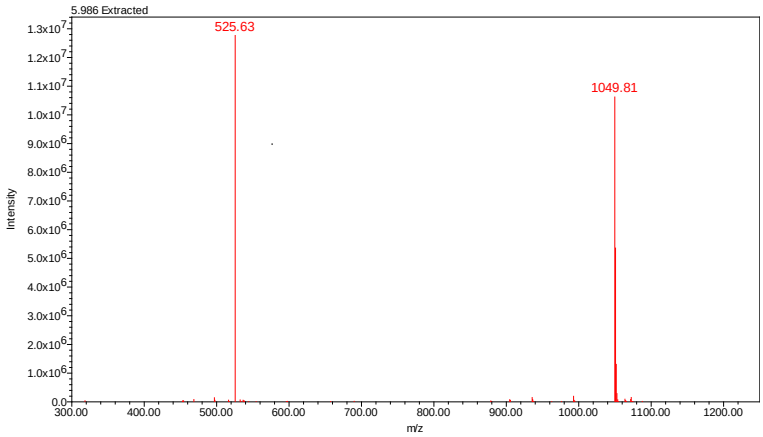
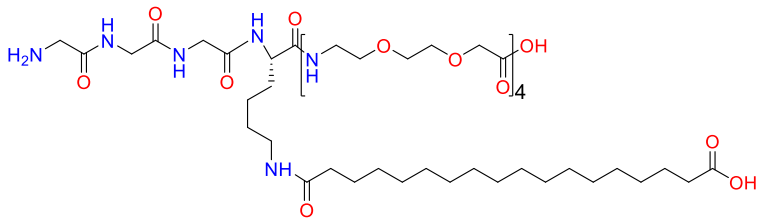


Figure S8. ESI-MS data for F3

Preparation of Gly-Gly-Gly-Lys(Nε-C18-diacid)-4×AEEA (F4)

In a similar way to fragments F6, F4 was prepared using Fmoc-AEEA-OH and 2-CTC resin.



ESI-MS: $[M+H]^+$ found: 1194.97, mass calculated 1193.70.

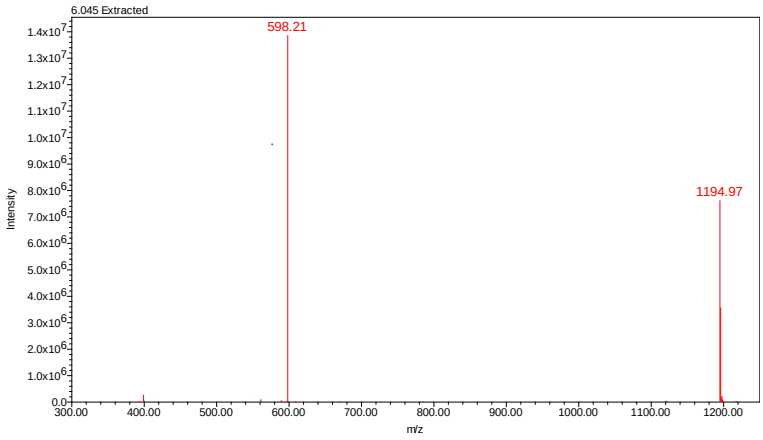
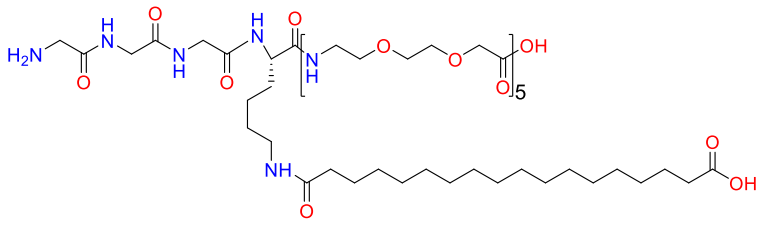


Figure S9. ESI-MS data for F4

Preparation of Gly-Gly-Gly-Lys(Nε-C18-diacid)-5×AEEA (F5)

In a similar way to fragments F6, F5 was prepared using Fmoc-AEEA-OH and 2-CTC resin.



ESI-MS: $[M+2H]^{2+}$ found: 670.82, mass calculated 1338.77.

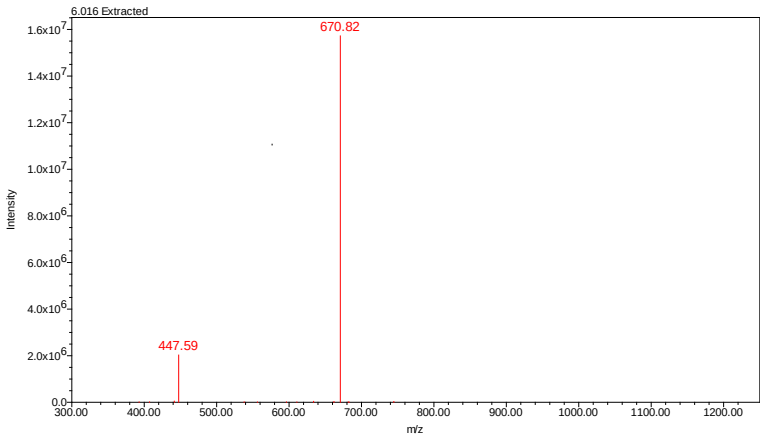
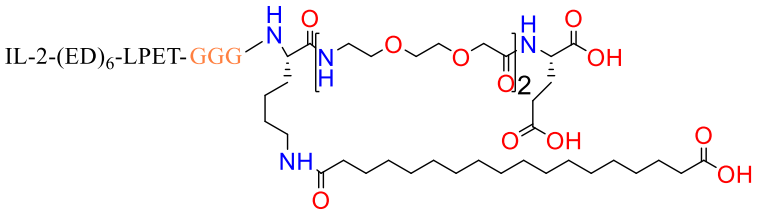


Figure S10. ESI-MS data for F5

Preparation and Purification of IL-2 Bioconjugates B1-B6

Preparation of IL-2-(ED)$_6$-LPET-GGG-Lys(Nε-C18-diacid)-2×AEEA-Glu (B6)



Bioconjugate B6 was conjugated according to the following reaction scheme.

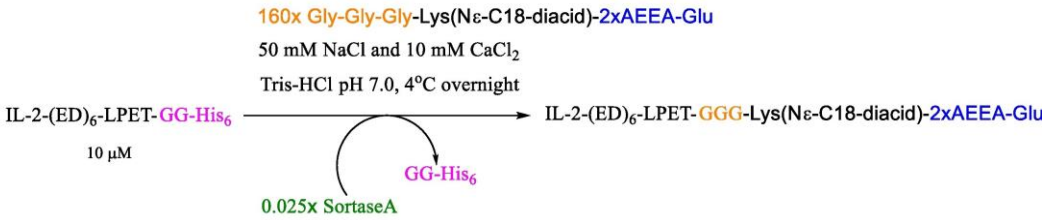


Figure S11. Reaction route of bioconjugate B6

IL-2-(ED)$_6$-LPETGG-His$_6$ (A3; 10 μM) was mixed with 0.25 μM SrtA, 160-fold excess fatty acid moiety (1600 μM), 50 mM NaCl and 10 mM $CaCl_2$ in Tris-HCl buffer at pH 7.0. The reaction was incubated overnight at 4 ℃.

The reaction was quenched by the addition of AcOH, pH adjusted to 3.0, and purified on a reversed-phase C4 preparative chromatography column (YMC park pro C4 30 μm,

10×250 mm, 300 Å, Kyoto, Japan) with a gradient of acetonitrile/0.1% TFA (15–45%) against 0.1% TFA in water-acetonitrile (60:40). Pooled fractions were evaporated on a rotary evaporator, pH adjusted to 7.4 with 1 M NaOH and replaced three times with PBS buffer in 3 kDa ultrafiltration centrifuge tubes (Millipore, Billerica, MA, US) for further use.

ESI-MS: mass calculated 18249.16 Da, mass observed: 18249.34 Da.

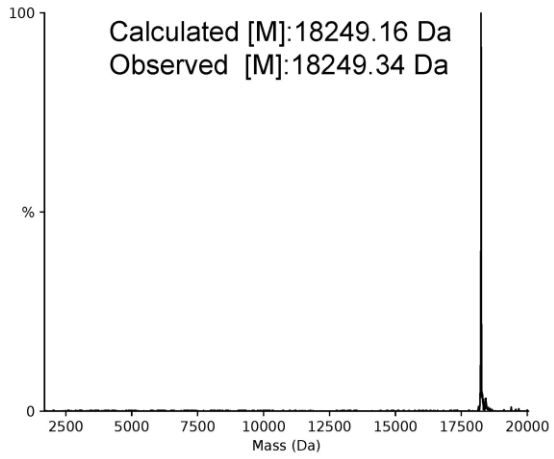
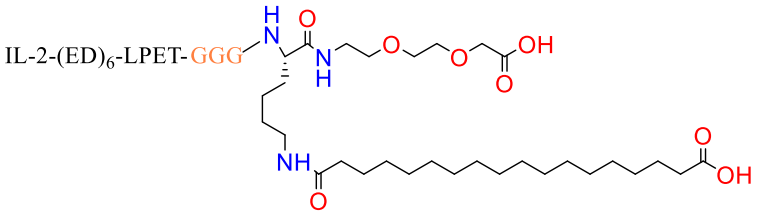


Figure S12. Deconvoluted ESI-MS spectra of B6

Preparation of IL-2-$(ED)_6$-LPET-GGG-Lys(Nε-C18-diacid)-AEEA (B1)

In a similar way to that described for bioconjugate B6, B1 was prepared using the IL-2 analog IL-2-$(ED)_6$-LPETGG-His_6 (A3) and fatty acid moiety F1.



ESI-MS: mass calculated 17975.04 Da, mass observed: 17975.07 Da.

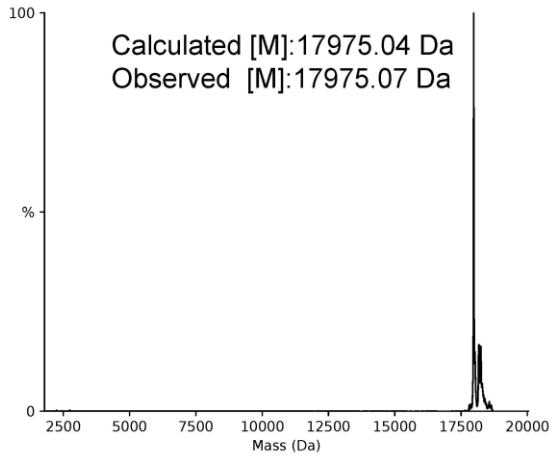
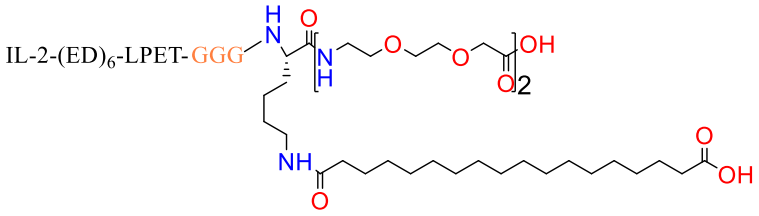


Figure S13. Deconvoluted ESI-MS spectra of B1

Preparation of IL-2-$(ED)_6$-LPET-GGG-Lys(Nε-C18-diacid)-2×AEEA (B2)

In a similar way to that described for bioconjugate B6, bioconjugate B2 was prepared using the IL-2 analog IL-2-$(ED)_6$-LPETGG-His_6 (A3) and fatty acid moiety F2.



ESI-MS: mass calculated 18120.11 Da, mass observed: 18120.26 Da.

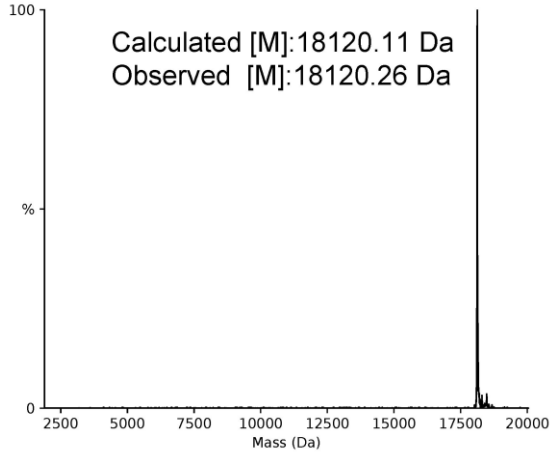
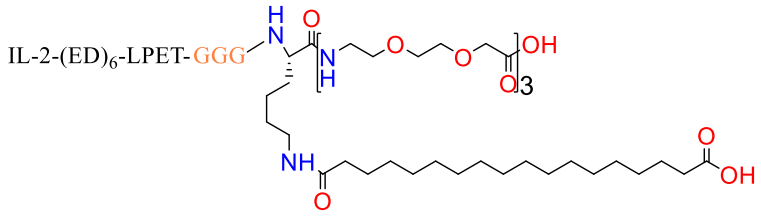


Figure S14. Deconvoluted ESI-MS spectra of B2

Preparation of IL-2-(ED)$_6$-LPET-GGG-Lys(Nε-C18-diacid)-3×AEEA (B3)

In a similar way to that described for bioconjugate B6, bioconjugate B3 was prepared using the IL-2 analog IL-2-(ED)$_6$-LPETGG-His$_6$ (A3) and fatty acid moiety F3.



ESI-MS: mass calculated 18265.19 Da, mass observed: 18265.14 Da.

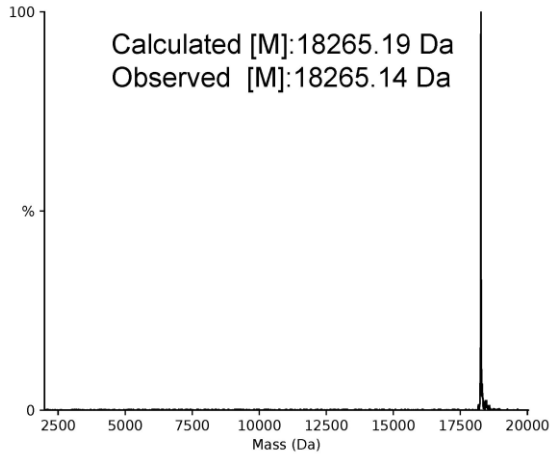
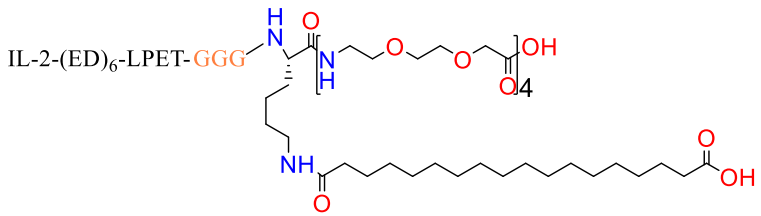


Figure S15. Deconvoluted ESI-MS spectra of B3

Preparation of IL-2-(ED)$_6$-LPET-GGG-Lys(Nε-C18-diacid)-4×AEEA (B4)

In a similar way to that as described for bioconjugate B6, bioconjugate B1 was prepared using the IL-2 analog IL-2-(ED)$_6$-LPETGG-His$_6$ (A3) and fatty acid moiety F4.



ESI-MS: mass calculated 18410.24 Da, mass observed: 18410.26 Da.

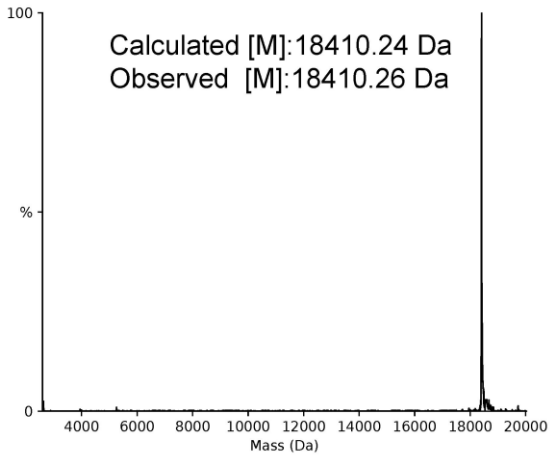
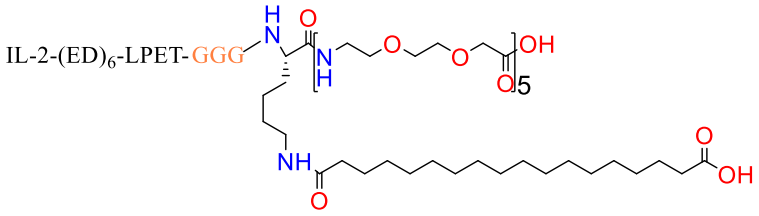


Figure S16. Deconvoluted ESI-MS spectra of B4

Preparation of IL-2-$(ED)_6$-LPET-GGG-Lys(Nε-C18-diacid)-5×AEEA (B5)

In a similar way to that described for bioconjugate B6, bioconjugate B5 was prepared using the IL-2 analog IL-2-$(ED)_6$-LPETGG-His_6 (A3) and fatty acid moiety F5.



ESI-MS: mass calculated 18555.33 Da, mass observed: 18555.48 Da.

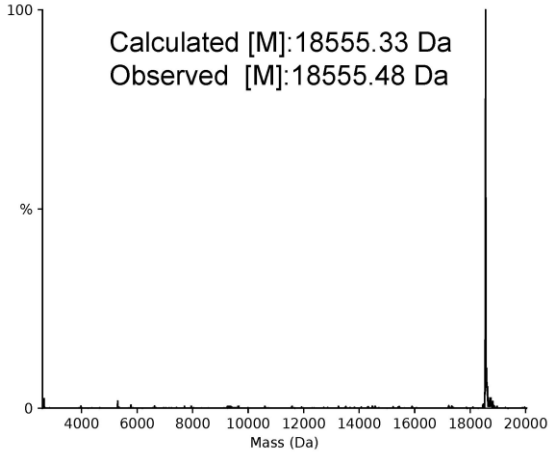


Figure S17. Deconvoluted ESI-MS spectra of B5

BLI binding analysis of IL-2Rα

An overlay plot of individual sensorgrams was generated using Prism software. The sensors were left to equilibrate for each phase as follows: baseline = 60 s, association = 300 s, dissociation = 600 s. All K_D values were obtained on BLItz (ForteBio, San Francisco, CA, US) equipment.

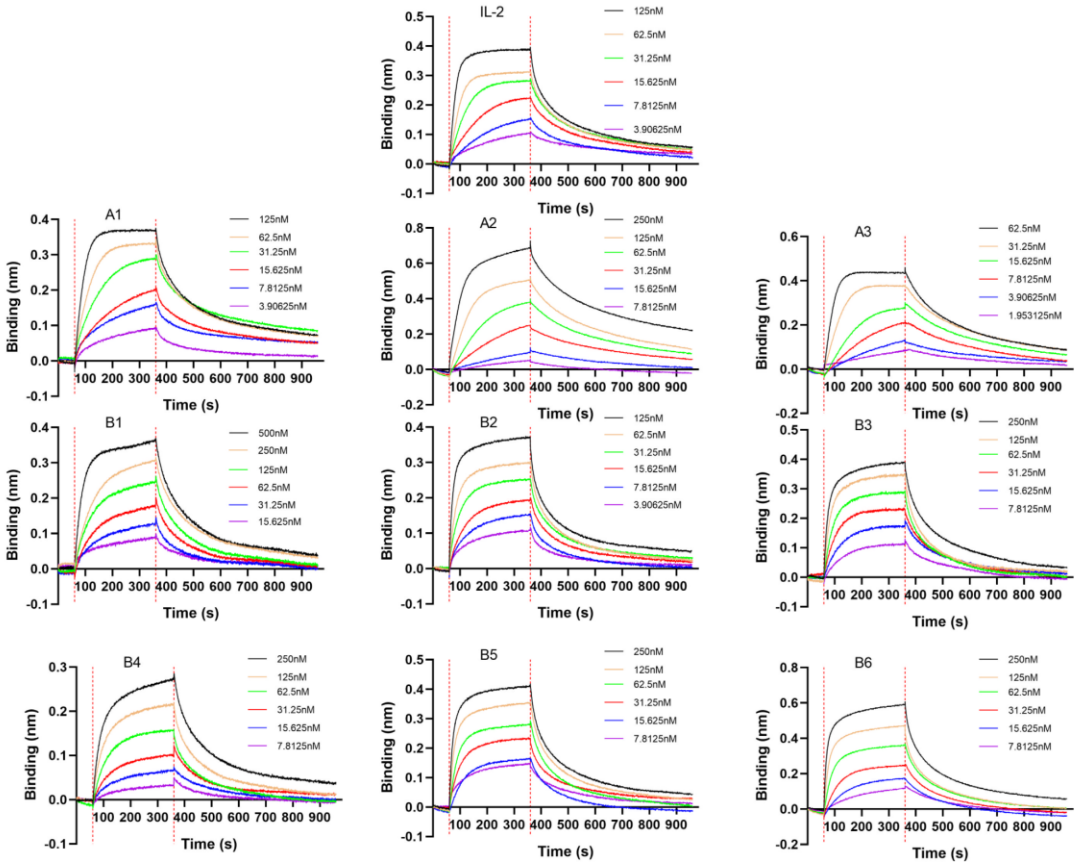


Figure S18. BLI binding analysis of IL-2Rα

Table S1. Summary of kinetic values (K_a, K_d, K_D) and R^2 obtained for IL-2 and bioconjugates

Compound	K_D (M)	K_a (1/Ms)	K_d (1/s)	R^2
IL-2	$(1.952 \pm 0.130) \times 10^{-8}$	$(3.317 \pm 0.093) \times 10^{5}$	$(6.147 \pm 0.563) \times 10^{-3}$	0.9901
B1	$(1.944 \pm 0.038) \times 10^{-7}$	$(4.322 \pm 0.038) \times 10^{4}$	$(8.398 \pm 0.088) \times 10^{-3}$	0.9851
B2	$(1.595 \pm 0.068) \times 10^{-8}$	$(9.282 \pm 1.049) \times 10^{5}$	$(1.473 \pm 0.104) \times 10^{-2}$	0.9867
B3	$(3.789 \pm 0.142) \times 10^{-8}$	$(2.487 \pm 0.013) \times 10^{5}$	$(9.407 \pm 0.291) \times 10^{-3}$	0.9843
B4	$(9.103 \pm 0.244) \times 10^{-8}$	$(9.478 \pm 0.123) \times 10^{4}$	$(8.403 \pm 0.123) \times 10^{-3}$	0.9869
B5	$(2.952 \pm 0.002) \times 10^{-8}$	$(3.041 \pm 0.052) \times 10^{5}$	$(8.980 \pm 0.158) \times 10^{-3}$	0.9907
B6	$(2.974 \pm 0.034) \times 10^{-8}$	$(2.657 \pm 0.097) \times 10^{5}$	$(7.905 \pm 0.376) \times 10^{-3}$	0.9910

Circular Dichroism (CD) Spectroscopy Measurements

The CD spectrum was measured by a Chirascan qCD Spectrometer (Applied Photophysics, UK) at 25 ℃ using a 0.1 cm cell. The IL-2, A3, and B6 three compounds were dissolved to a final concentration of 10 μg/mL in 10 mM phosphate buffer (PB; pH 7.4) The spectra were recorded in the wavelength range of 190−260 nm and averaged over 3 scans with a scan speed of 50 nm/min, a bandwidth of 1.0 nm, a response time of 1 s, and a resolution of 0.1 nm.

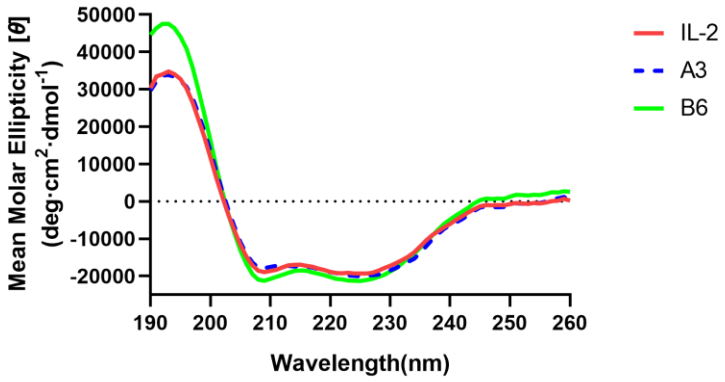
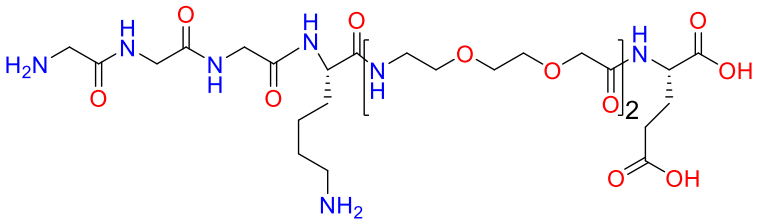


Figure S19. CD spectrum of IL-2, A3, and B6 in 10 mM phosphate buffer

Preparation and Purification of F6 control, B6 control and B7 (A2-F3)

Preparation of Gly-Gly-Gly-Lys-2×AEEA-Glu (F6 control)

In a similar way to fragments F6, F6 control was prepared using Fmoc-Glu(OtBu)-OH and 2-CTC resin.



ESI-MS: $[M-H]^-$ found: 735.52, mass calculated 736.36.

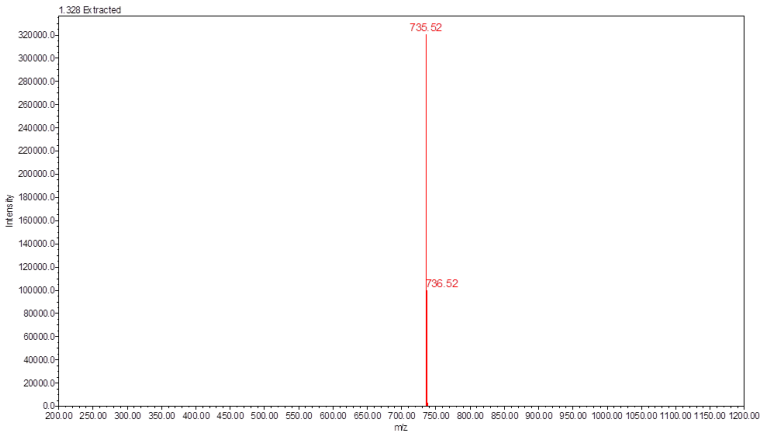
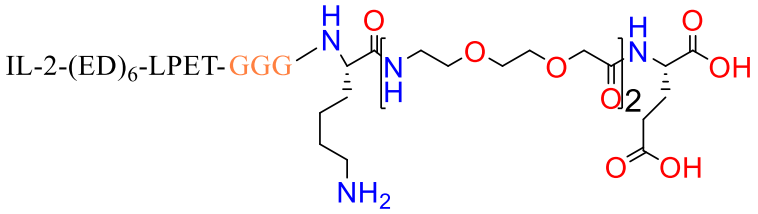


Figure S20. ESI-MS data for F6 control

Preparation of IL-2-$(ED)_6$-LPET-GGG-Lys-2×AEEA-Glu (B6 control)

In a similar way to that described for bioconjugate B6, B6 control was prepared using the IL-2 analog IL-2-$(ED)_6$-LPETGG-His_6 (A3) and F6 control.



ESI-MS: mass calculated 17952.92 Da, mass observed: 17952.66 Da.

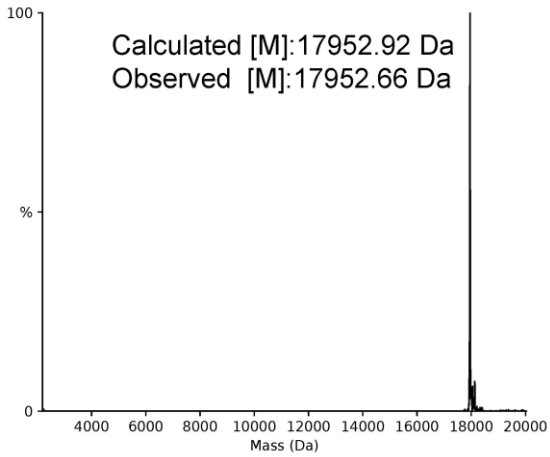
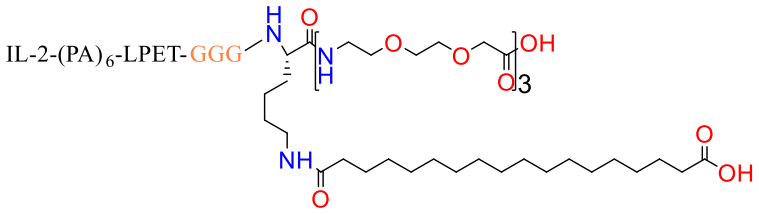


Figure S21. Deconvoluted ESI-MS spectra of B6 control

Preparation of IL-2-$(PA)_6$-LPET-GGG-Lys(Nε-C18-diacid)-3×AEEA (A2-F3, B7)

In a similar way to that described for bioconjugate B6, B7 (A2-F3) was prepared using the IL-2 analog IL-2-$(PA)_6$-LPETGG-His_6 (A2) and F3.



ESI-MS: mass calculated 17809.14 Da, mass observed: 17809.07 Da.

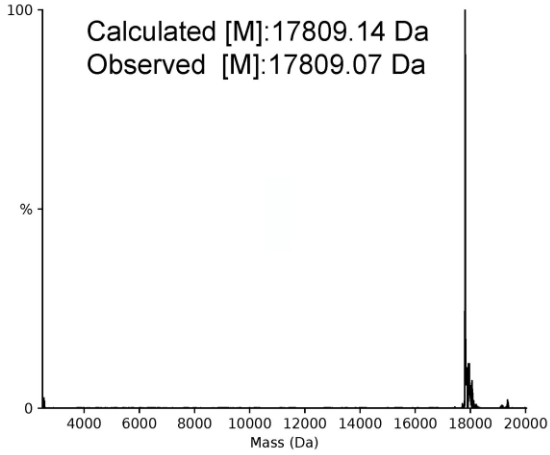


Figure S22. Deconvoluted ESI-MS spectra of B7

Assessment of B6 control and B7 in CTLL-2 Proliferation

In a similar way to that described for B1 to B6, the bioactivity of B6 control and B7 was assessed by the proliferation in CTLL-2 cells. The relative viability exposed to B6 control, B7, B3, and B6 compared to its parent analogs and IL-2 was shown in Table S2.

Table S2. Percentage relative viability of B6 control, B7, B3, and B6 compared to parent analog and IL-2

Bioconjugate	Parent analog	Peptide linker	Fatty acid moieties	Percentage relative viability compared to parent analog	Percentage relative viability compared to IL-2
B7	A2	$(PA)_6$	F3	38.34 ± 4.28%	49.72 ± 7.18%
B3	A3	$(ED)_6$	F3	54.82 ± 6.80%	120.65 ± 23.97%
B6	A3	$(ED)_6$	F6	71.70 ± 5.76%	160.80 ± 12.54%
B6 control	A3	$(ED)_6$	F6 control	58.97 ± 1.01%	128.55 ± 2.21%