Design and biological evaluation of Triagonist GLP-1R/GCGR/GIPR Peptides as Potential Therapeutic Agents for Diabetes and Obesity

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SUPPLEMENTARY INFORMATION

1. Experimental

Solid-phase syntheses were carried out on a Kamush® LP360AMP shaker and in peptide synthesis reactors with capacities of 16 ml, 25 ml or 50 ml.
The obtained linkers and conjugates were purified using preparative HPLC (Shimadzu or Waters). Reaction monitoring and preliminary determination of purity and identity of the synthesized compounds, as well as development of analytical methods, were carried out on a Shimadzu LCMS LC2040 with an electrospray (ES) mass detector.
The obtained crosslinkers and modified peptides were lyophilized with Christ Alpha 1-2 Laboratory freeze-dryer.

Synthesis procedure with analytical data for the linker L1

The synthesis procedure of linker L1 (Figure 1) consists 9 steps according to Scheme 1.

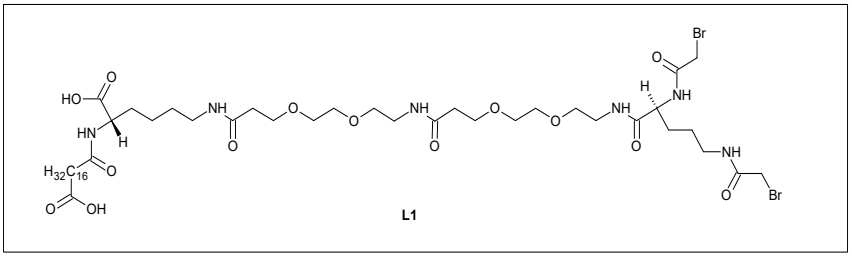


Figure 1. Structure of L1

Step 1: Preparation of Fmoc-Lys(ivDde)-2-chlorotrityl resin (1)
2-Chlorotrityl resin (2-CTC, loading 0.6mmol/g, 0.2 mmol) was swollen in dry DCM (5 ml) for 1 h and then dried under vacuum. Fmoc-L-Lys(ivDde)-OH (3 eq) and DIPEA (9 mmol) were dissolved in DCM (5 ml) and were added to the prepared gel. The reaction was carried out in Kamush vessels on a LP360AMP shaker. After the reaction was complete, the resin was washed with DCM (3x). In order to remove unreacted chlorotrityl residues, a mixture of MeOH:DCM:DIPEA (8:1:1) was added to the resin and shaken for 30 min. Then, the unreacted reagents were filtered off, washed with DCM (3x) and dried on a vacuum pump. Stored under vacuum.

Step 2: Preparation of Lys(ivDde)-2-chlorotrityl resin (2)
Fmoc group was removed using a 20% piperidine solution in dry DMF (5 ml). The reaction was shaken for 30 min, the solution was filtered off and the procedure was repeated. The completeness of the reaction was confirmed by the chloranil test. Then, the unreacted reagents were filtered off and the resin was washed with DMF (3x) and DCM (3x).

Step 3: Preparation of C18-diacid-Lys(ivDde)-2-chlorotrityl (3)
The compound 2 was coupled with octadecanedioic acid mono-t-butyl ester (4 eq), using HATU (4 eq) as a coupling reagent and DIPEA (8 eq) in dry DMF (5ml). The mixture was shaken for 20-24 h, until the chloranil test was negative. After the reaction was complete, the resin was washed with DMF (3x) and DCM (3x).

Step 4-6: Reaction with Fmoc-(PEG)2-propionic acid
Deprotection of ivDde group from compound 3 was carried out with 5% solution of hydrazine monohydrate in DMF (5 ml). The reaction was shaken for 15 min. Then, the solution was filtered off and the procedure was repeated 4-5 times. The completeness of the reaction was confirmed by the chloranil test. The unreacted reagents were then filtered off and the resin was washed with DMF (3x) and DCM (3x). The resin was then treated with Fmoc-(PEG)2-propionic acid (4 eq), using HATU (4 eq) and DIPEA (8 eq) in DMF (5 ml). The mixture was shaken for 4 h, until the chloranil test was negative. After the reaction was complete, the resin was washed with DMF (3x) and DCM (3x). Fmoc deprotection was carried out using a 20% piperidine solution in dry DMF (5 ml). The reaction was shaken for 30 min. The solution was filtered off and the procedure was repeated. Then, unreacted reagents were filtered off and the resin was washed with DMF (3x) and DCM (3x). The resin was treated again with Fmoc-(PEG)2-propionic acid (4 eq), using HATU (4 eq) and DIPEA (8 eq) in DMF (5ml) for 20-24h, until the chloranil test was negative. The resin was washed with DMF (3x) and DCM (3x).

Step 7: Reaction with Fmoc-(Orn)-Fmoc-OH

The Fmoc group was removed and washing steps were repeated as describe above for 2. The resin was treated with Fmoc-Orn(Fmoc)-OH (4 eq), using HATU (4 eq) and DIPEA (8 eq) in DMF (5 ml) for 20-24h, until the chloranil test was negative. After the reaction was complete, the resin was washed with DMF (3x) and DCM (3x).

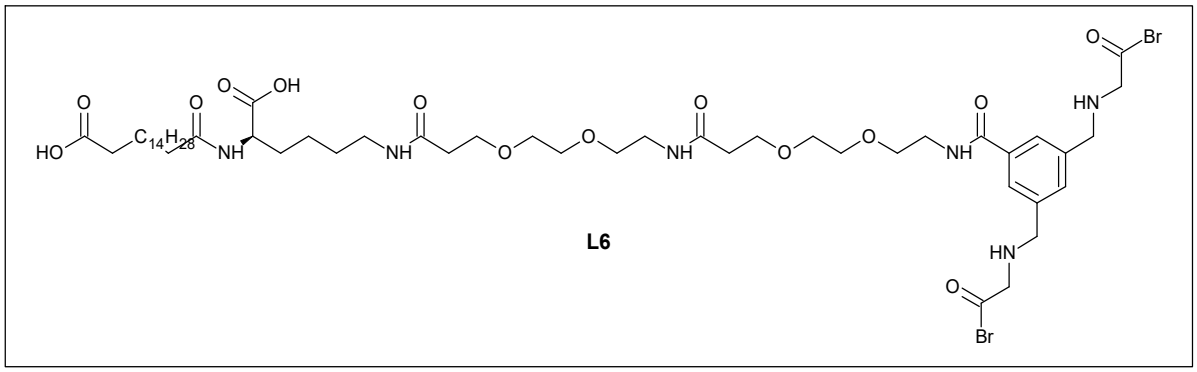
Step 8: Reaction with bromoacetic anhydride

The Fmoc group was removed and washing steps were repeated as describe above for 2. Then, the resin was treated with bromoacetic anhydride (4 eq), DIPEA (8 eq) in DCM (5 ml) for 1 h. After completion of the reaction, the resin was washed with DCM (3x).

Step 9: 17-{[(1R)-5-[3-(2-{2-[3-(2-{2-[2,5-bis(2-bromoacetamido)pentanamido]ethoxy}ethoxy)propanamido] ethoxy}ethoxy)propanamido]-1-carboxypentyl]carbamoyl} heptadecanoic acid (L1)

The product was cleaved from the resin using TFA:H2O:TIPS (8:1:1, 4.5 ml : 0.25 ml : 0.25 ml). The reaction was stirred at room temperature for 1 h, the resin was filtered and washed with DCM. The crude L1 was purified using preparative HPLC on Gemini-NX column (5µm, 150x21.2mm) from Phenomenex with gradient A: H2O+TFA 0.1%, B: ACN (0.0–1.0 min 15% B, 1.0–6.0 min 60% B, 6.0–7.0 90% B, 7.0–9.0 min 90% B, 9.0–10.0 min 15% B, 12.0 min stop) at flow rate 20 ml/min. The fractions containing products were collected and then lyophilized to obtain as a powder with 99.1% purity and in 42.1% yield. ESI-MS: calculated expected mass 1116.4 Da; found [M+1]+ 1117.5 Da. ESI-MS: expected mass 1116.44 Da; found [M+1]+ 1117.5 Da. The identity and purity of L1 was confirmed by LC-MS. Analytical HPLC was performed using an LC-MS Shimadzu 2060 with Gemini-NX column (3µm, 100x2mm) from Phenomenex with gradient A: H2O+TFA (0.1%), B: ACN (0.0–2.0 min 15% B, 2.0–9.5 min 99% B, 9.5–12.0 min 99% B, 12.0–13.0 min 15% B, 15.0 min stop) at a flow rate 0.3 ml/min with UV detection wavelength set at Max Plot 190-400nm.

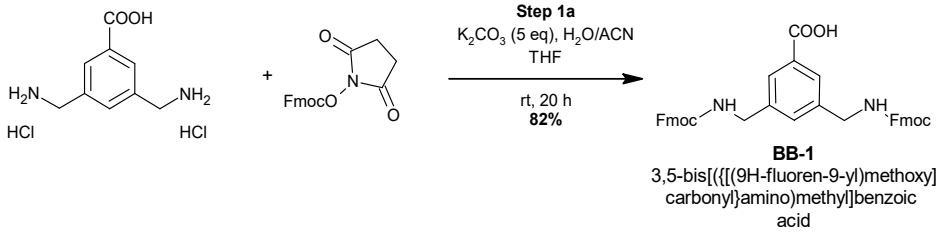
Synthesis procedure with analytical data for the linker L6



L6

Figure 2. Structure of L6

Step 1a: Synthesis of 3,5-bis[({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)methyl] benzoic acid (BB-1)



BB-1
3,5-bis[({[(9H-fluoren-9-yl)methoxy] carbonyl}amino)methyl]benzoic acid

Scheme 1. Synthesis of BB-1

3,5-Bis(aminomethyl)benzoic acid; dihydrochloride (1.77 mmol, 1 eq) in THF (20 ml), N-Fmoc-imide of succinic acid (Fmoc-OSu, 2.5 eq) was dissolved in a mixture of H2O:ACN (15 ml: 15 ml). The reaction was carried out at room temperature for 20 hours. A saturated solution of sodium bicarbonate was added, and the aqueous phase was washed with diethyl ether. Then, the aqueous phase was acidified to pH 1 with 3M HCl. The resulting precipitate was filtered on a Schott funnel. 918 mg of the

compound **BB-1** was obtained with a purity of 97.5% and a yield of 82%. ESI-MS: expected mass 624.22 Da; found $[M+1]^+$ 625.09 Da.

Steps 1–6 were carried out analogously to the synthesis of linker L1 (Scheme 1). The synthesis was carried out on chlorotrityl resin at a scale of 0.25 mmol.

Step 7: Reaction with BB-1
The Fmoc group was removed and washing steps were repeated as describe above for **2**. The resin was treated with BB-1 (4 eq), using HATU (4 eq) and DIPEA (8 eq) in DMF (5 ml) for 20-24h, until the chloranil test was negative. After the reaction was complete, the resin was washed with DMF (3x) and DCM (3x).

Step 8: Reaction with bromoacetic anhydride
Then the Fmoc group was removed and washing steps were repeated. Then the resin was treated with bromoacetic anhydride (4 eq), DIPEA (8 eq) in DCM (5 ml) for 1 h. After completion of the reaction, the resin was washed with DCM (3x).

Step 9: 17-{[(1R)-5-(3-{2-[2-(3-{2-[2-({3,5-bis[(2-bromoacetamido)methyl]phenyl}formamido)ethoxy]ethoxy} propanamido)ethoxy]ethoxy}propanamido)-1-carboxypentyl]carbamoyl} heptadecanoic acid (L6)
The product was cleaved from the resin using TFA:H2O:TIPS (8:1:1, 4.5 ml : 0.25 ml : 0.25 ml). The reaction was stirred at room temperature for 1 h, the resin was filtered and washed with DCM. The crude 6 was purified using preparative HPLC on Gemini-NX column (5µm, 150x21.2mm) from Phenomenex with gradient A: H2O+TFA 0.1%, B: ACN (0.0–2.0 min 20% B, 2.0–18.0 min 62% B, 18.0–19.0 99% B, 19.0–20.0 min 99% B, 20.0–20.5 min 20% B, 23.0 min stop) at flow rate 20 ml/min. The fractions containing products were collected and then lyophilized to obtain 70 mg as a powder with 99% purity and in 24% yield. ESI-MS: expected mass 1164.44 Da; found $[M+1]^+$ 1165.52 Da.

Synthesis procedure with analytical data for the linker L9

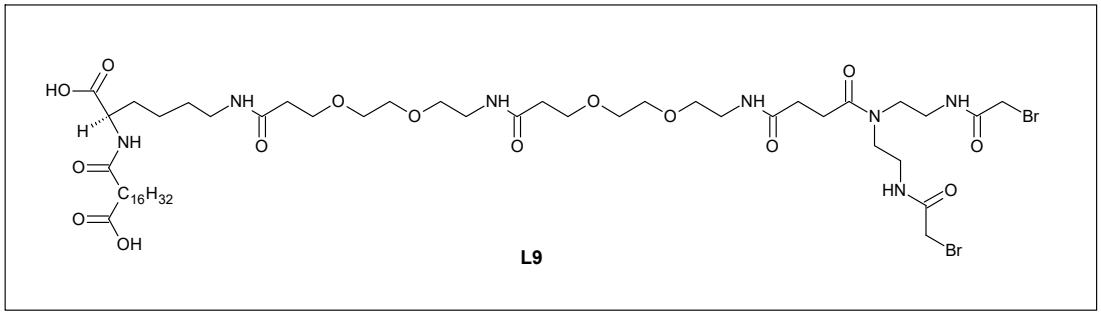
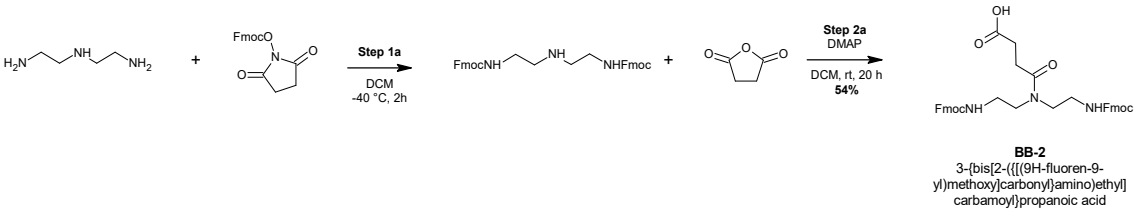


Figure 3. Structure of L9

Synthesis of 3-{bis[2-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)ethyl]carbamoyl} propanoic acid (BB-2)



Scheme 2. Synthesis of BB-3

The planned linker required the prior synthesis of **BB-2** (Scheme 2).

Step 1a: The mixture of Fmoc-ONSu (6.4 g, 19 mmol) in DCM (20 ml) was added dropwise to a solution of diethylenetriamine (1.05 ml, 9.5 mmol) in DCM (10 ml) at -40 °C under argon. The reaction was carried out for 2 hours until the substrate was no longer detectable by LC/MS. The crude mixture was used in the next step without purification.
Step 2a: To the mixture from step 1a, DMAP (237 mg, 1.92 mmol) and succinic anhydride (3.4 g, 33.98 mmol) were added. The reaction was carried out at room temperature for 18 hours. After this time, 3M HCl was added to the mixture until pH 1

was reached and stirred for 15 minutes. The phases were separated, and the organic phase was washed with water and brine. The product was extracted from the aqueous phase using DCM (3x). The combined organic phases were dried over anhydrous magnesium sulfate. The product was purified by column chromatography on silica gel using DCM:MeOH and ethyl acetate:MeOH as a mobile phase. **BB-2** was obtained in amount of 3.5 g with a purity 96% and yield 54%. ESI-MS: expected mass 647.26 Da; found $[M+1]^+$ 648.35 Da.

Steps 1–6: were performed in the same manner as described for the synthesis of linker L1 (see Scheme 1). The synthesis was carried out on chlorotrityl resin at a scale of 0.29 mmol.

Step 7: Reaction with BB-2

The Fmoc group was removed and washing steps were repeated as describe above for **2**. The resin was treated with **BB-2** (4 eq), using HATU (4 eq) and DIPEA (8 eq) in DMF (5 ml) for 20-24 h, until the chloranil test was negative. After the reaction was complete, the resin was washed with DMF (3x) and DCM (3x).

Step 8: Reaction with bromoacetic anhydride

Then the Fmoc group was removed and washing steps were repeated. Then, the resin was treated with bromoacetic anhydride (4 eq), DIPEA (8 eq) in DCM (5 ml) for 1 h. After completion of the reaction, the resin was washed with DCM (3x).

Step 9: 17-{[(1R)-5-(3-{2-[2-(3-{2-[2-(3-{bis[2-(2-bromoacetamido)ethyl]carbamoyl} propanamido)ethoxy] ethoxy}propanamido)ethoxy]ethoxy}propanamido)-1-carboxypentyl] carbamoyl} heptadecanoic acid (L9)

The product was cleaved from the resin using TFA:H2O:TIPS (8:1:1, 4.5 ml : 0.25 ml : 0.25 ml). The reaction was stirred at room temperature for 1 h, the resin was filtered and washed with DCM. The crude L9 was purified using preparative HPLC on Gemini-NX column (5µm, 150x21.2mm) from Phenomenex with gradient A: H2O+TFA 0.1%, B: ACN B: ACN (0.0–2.0 min 15% B, 2.0–6.0 min 60% B, 6.0–9.0 90% B, 9.0–11.0 min 90% B, 11.0–13.0 min 15% B, 15.0 min stop) at flow rate 20 ml/min. The fractions containing products were collected and then lyophilized to obtain 100 mg **L9** as a powder with 98% purity and in 29% yield. ESI-MS: expected mass 1145.47 Da; found $[M+1]^+$ 1146.48 Da.

Synthesis procedure with analytical data for the linker L10

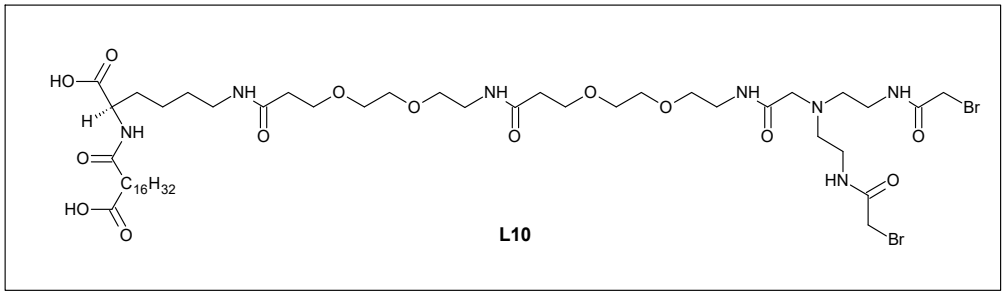
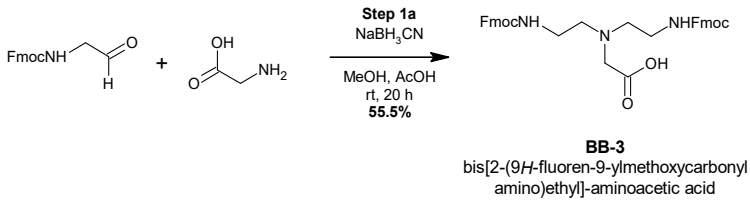


Figure 4. Structure of L10

Step 1a: Preparation of bis[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)ethyl]-aminoacetic acid (BB-3)



Scheme 3. Synthesis of BB-3

The synthesis of BB-3 was carried out in a single step using reductive amination of the aldehyde with glycine in the presence of $NaBH_3CN$ (Scheme 2).

9H-fluoren-9-ylmethyl-N-(2-oxoethyl)carbamate (2 g, 7.1 mmol) was dissolved in a mixture of methanol and acetic acid (34 ml: 6 ml). Glycine (216.8 mg, 2.86 mmol) and sodium cyanoborohydride (470 mg, 7.1 mmol) were then added. The reaction was carried out at room temperature for 20 hours. After this time, 30 ml of diethyl ether was added to the mixture

and the resulting precipitate was filtered off and washed with a small portion of ether. Next, the precipitate was dissolved in 10% NaOH, then acidified to pH 2. The compound was extracted with ethyl acetate. The combined organic phases were dried over anhydrous magnesium sulphate. After evaporating the solvent, 1 g of product with 95.4% purity (yield 55.5%) was obtained, which was used for further synthesis. ESI-MS: expected mass 605.25 Da; found $[M+1]^+$ 606.28 Da.

Steps 1–6: were carried out analogously to the synthesis of linker **L1** (Scheme 1). The synthesis was carried out on chlorotrityl resin at a scale of 0.29 mmol.

Step 7: Reaction with BB-3

The Fmoc group was removed and washing steps were repeated as describe above for **2**. The resin was treated with **BB-3** (4 eq), using HATU (4 eq) and DIPEA (8 eq) in DMF (5 ml) for 20-24 h, until the chloranil test was negative. After the reaction was complete, the resin was washed with DMF (3x) and DCM (3x).

Step 8: Reaction with bromoacetic anhydride

Then the Fmoc group was removed and washing steps were repeated. Then the resin was treated with bromoacetic anhydride (4 eq), DIPEA (8 eq) in DCM (5 ml) for 1 h. After completion of the reaction, the resin was washed with DCM (3x).

Step 9: 17-{[(1R)-5-(3-{2-[2-(3-{2-[2-(2-{bis[2-(2-bromoacetamido)ethyl]amino}acetamido)ethoxy]ethoxy} propanamido)ethoxy]ethoxy}propanamido)-1-carboxypentyl]carbamoyl}heptadecanoic acid (L10)

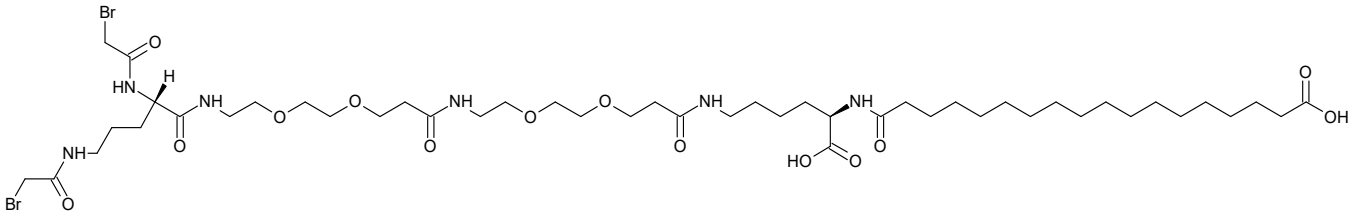
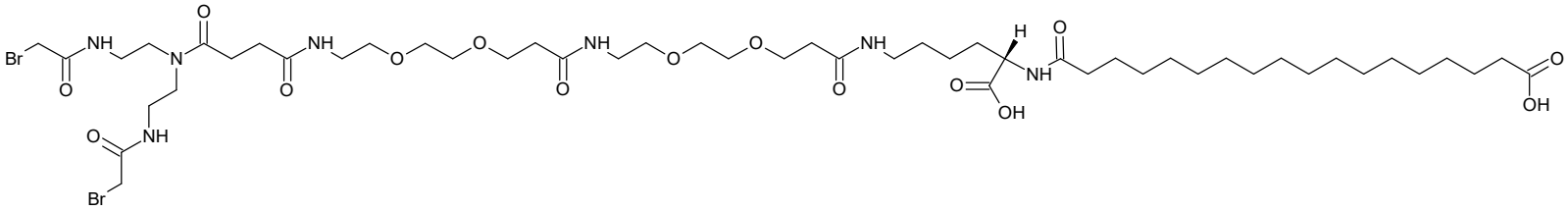
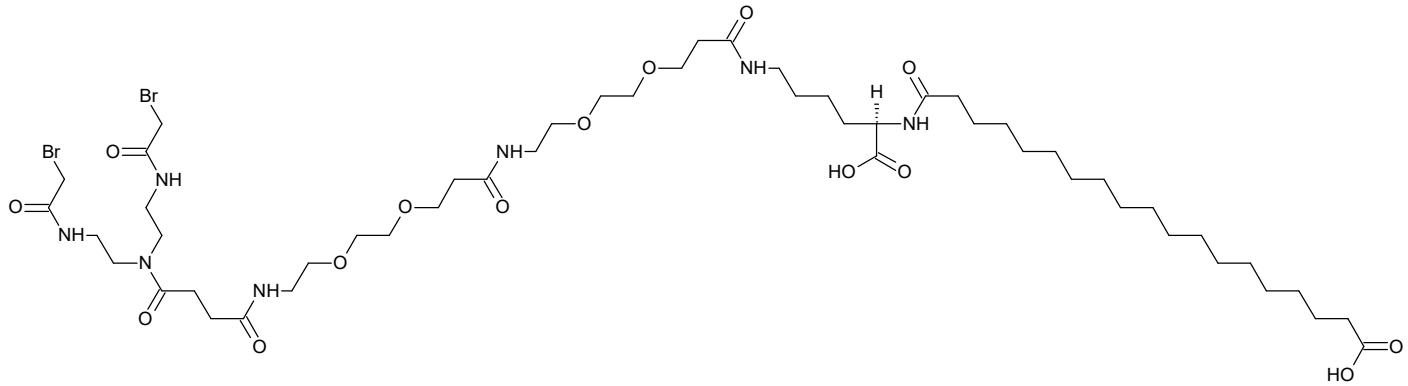
The product was cleaved from the resin using TFA:H2O:TIPS (8:1:1, 4.5 ml : 0.25 ml : 0.25 ml). The reaction was stirred at room temperature for 1 h, the resin was filtered and washed with DCM. The crude L10 was purified using preparative HPLC on Gemini-NX column (5µm, 150x21.2mm) from Phenomenex with gradient A: H2O+TFA 0.1%, B: ACN (0.0–2.0 min 15% B, 2.0–6.0 min 60% B, 6.0–9.0 90% B, 9.0–11.0 min 90% B, 11.0–13.0 min 15% B, 15.0 min stop) at flow rate 20 ml/min. The fractions containing products were collected and then lyophilized to obtain 153 mg **L10** as a powder with 99.9% purity and in 44% yield. ESI-MS: expected mass 1187.48 Da; found $[M+1]^+$ 1188.56 Da.

General procedure for alkylation peptide with cross-linkers

Peptide (1 eq) was subjected to alkylation reaction with cross-linker **L1-L14** (1.1-1.5 eq) in the presence of DIPEA base (8-12 eq). The reaction was carried out in ACN:H2O 1:1 (v/v) for 1-20 h at room temperature. The crude modified peptide was purified by preparative HPLC on Gemini-NX column (5µm, 150x21.2mm) from Phenomenex with gradient 15-90% of B (A: H2O+TFA 0.05–0.1%, B: ACN) at flow rate 20 ml/min. The fractions containing products were collected and then lyophilized to obtain as a powder with >91% purity and in 22-75% yield. The identity and purity of final conjugates was confirmed by HPLC-MS. Analytical HPLC was performed using an HPLC Prominence Shimadzu with CHROMSHELL C18 Plus column (2.6 µm, 100x2.1 mm) from Chromservis with gradient A: H2O+TFA (0.2%), B: ACN (0.0–0.5 min 25% B, 0.5–50.0 min 50% B, 50.0–53.0 95% B, 53.0–56.0 min 95% B, 56.0–58.0 min 25% B, 75.0 min stop) at a flow rate 0.3 ml/min with UV detection wavelength set at 200 nm.

The remaining conjugates were synthesized following the procedure described analogously above.

2. Chemical structures and abbreviations of crosslinkers used in the design of stapled peptides.

Name	Structure
L1	
L2	
L3	

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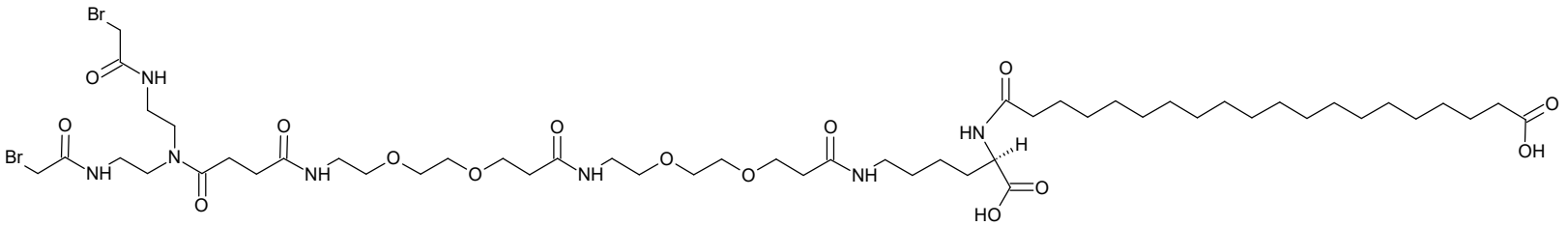

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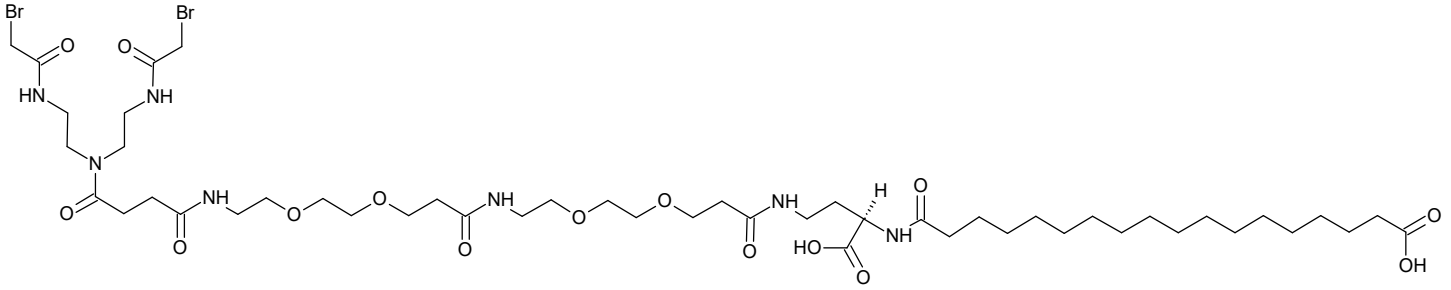
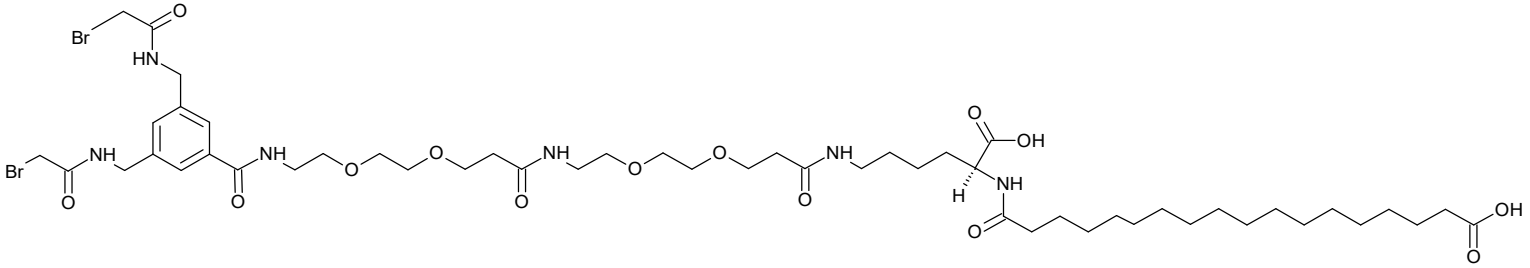


L6



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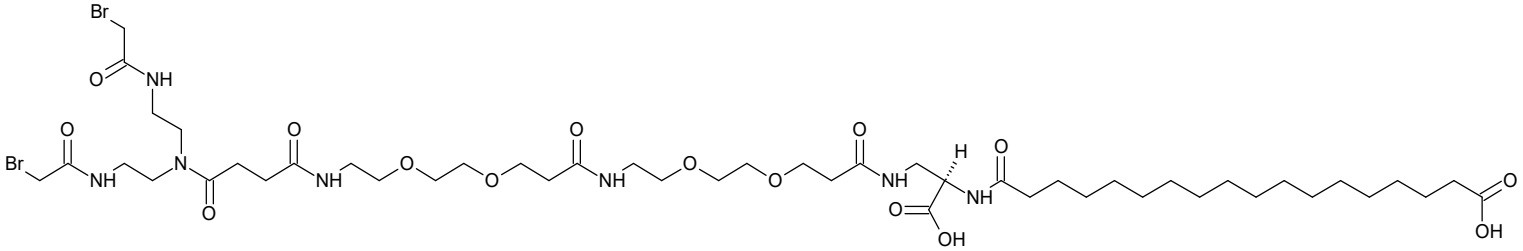

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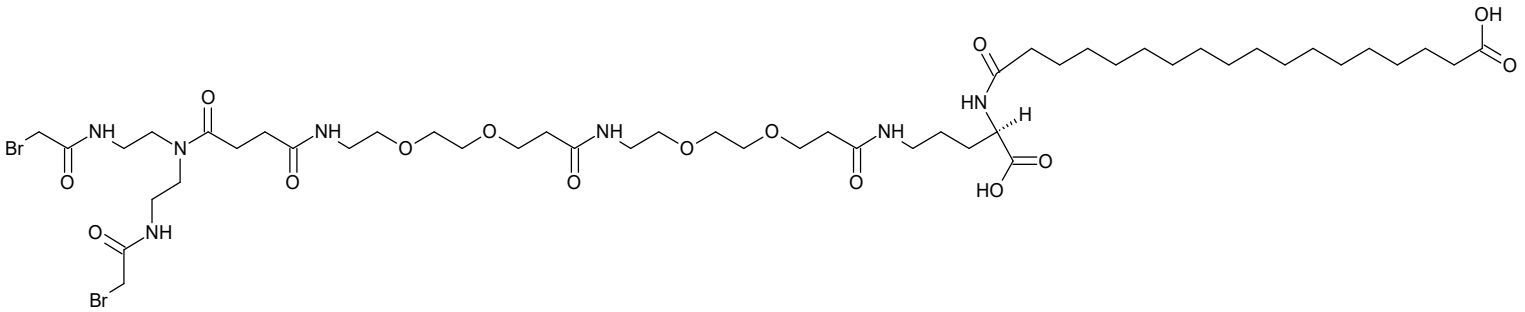
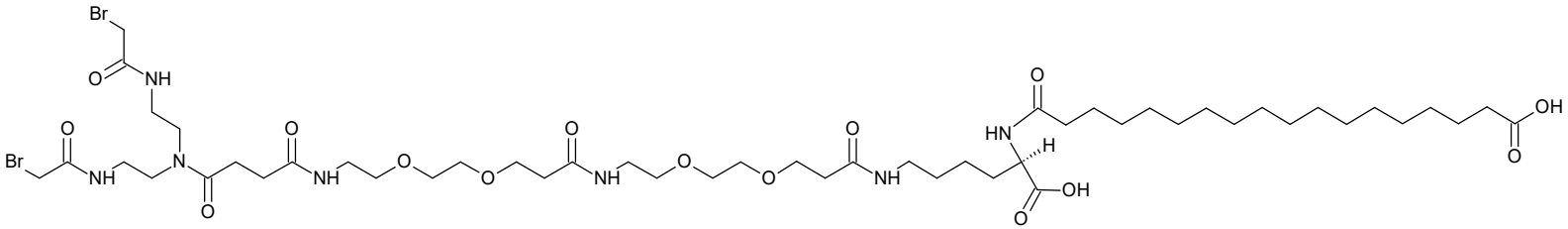


L9



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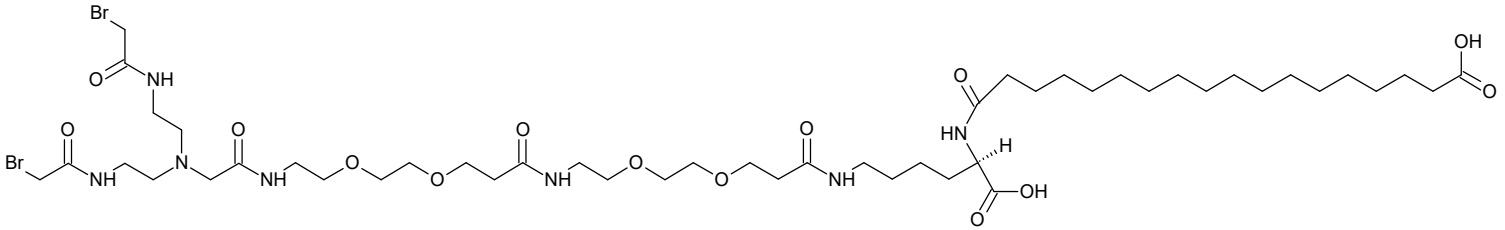

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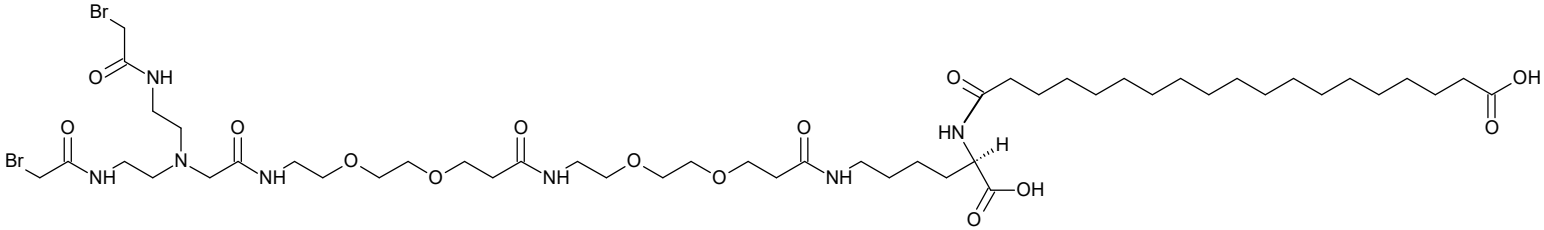

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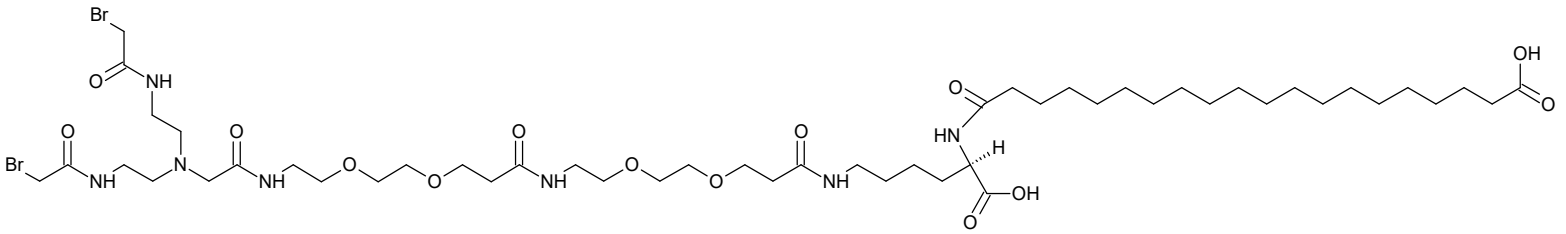

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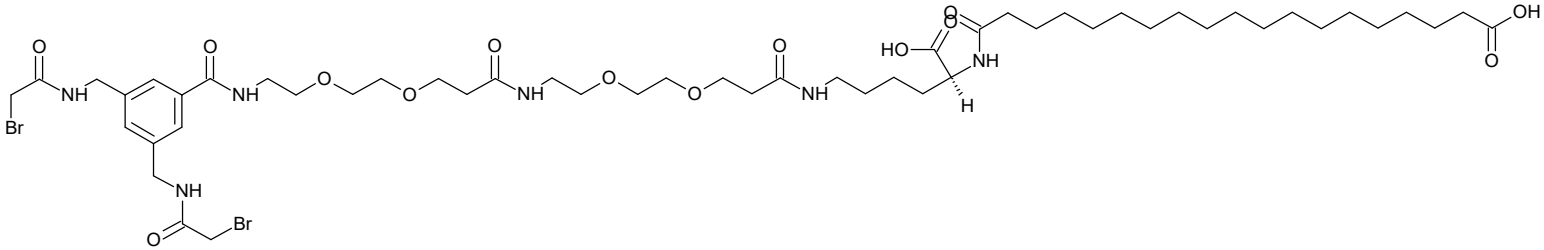
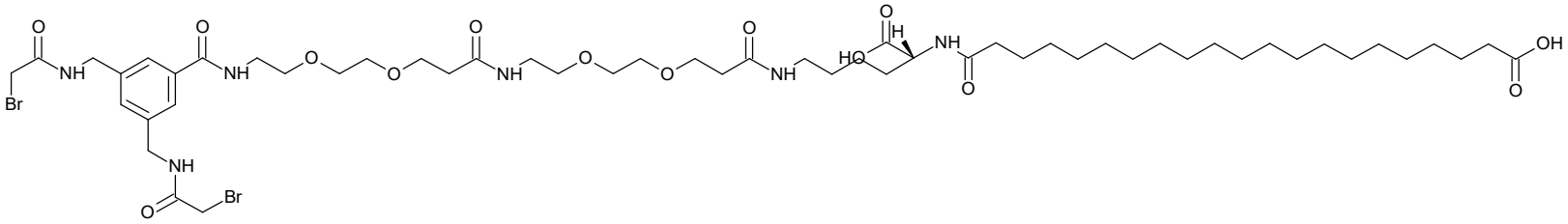


L14



3. **In vitro GLP-1R activity results for designed peptides, including individual replicate values and geometric mean EC_{50} Assays were performed under serum replacement (SR) conditions and additionally under fetal bovine serum (FBS) conditions.**

Name	FBS EC50 rep.1 [nM]	FBS EC50 rep.2 [nM]	FBS EC50 rep.3 [nM]	FBS EC50 geom avg [nM]	SR EC50 rep.1 [nM]	SR EC50 rep.2 [nM]	SR EC50 rep.3 [nM]	SR EC50 geom avg [nM]
P1-L1	29.230	23.890	28.930	**27.235**	n.d.	14.880	16.030	**15.444**
P2-L2	114.600	289.00	155.400	**172.654**	22.800	26.310	8.333	**17.098**
P2-L1	38.640	27.570	10.200	**22.149**	6.821	6.003	5.614	**6.126**
P2-L3	140.500	181.600	77.750	**125.650**	11.360	16.290	6.402	**10.581**
P2-L4	95.140	170.700	82.380	**110.189**	6.623	8.436	2.090	**4.888**
P2-L5	66.280	111.100	52.920	**73.042**	25.830	15.020	7.687	**14.394**
P2-L6	112.300	133.200	43.510	**86.661**	38.730	33.730	54.480	**41.441**
P2-L7	69.430	122.200	42.350	**71.093**	14.420	19.220	8.637	**13.377**
P2-L8	62.010	191.600	53.840	**86.163**	25.390	18.760	7.032	**14.962**
P2-L9	52.500	124.100	52.410	**69.895**	25.110	13.690	10.870	**15.518**
P2-L10	57.130	62.260	23.530	**43.742**	21.000	14.020	17.210	**17.176**
P3-L1	6.311	6.735	6.149	**6.394**	2.300	3.067	3.280	**2.850**
P3-L11	32.410	34.120	13.760	**24.780**	6.415	3.705	7.220	**5.557**
P3-L12	37.600	4.825	14.550	**13.820**	5.132	2.636	3.371	**3.573**
P3-L10	18.060	18.730	7.202	**13.456**	9.982	5.865	6.734	**7.333**
P4-L1	n.d.	17.130	22.470	**19.619**	n.d.	5.718	11.000	**7.931**
P4-L10	n.d.	20.760	25.260	**22.900**	n.d.	6.984	15.470	**10.394**
P5-L1	n.d.	90.210	138.600	**111.817**	n.d.	18.320	46.900	**29.312**
P6-L1	1.410	1.028	n.d.	**1.204**	0.574	0.864	0.714	**0.707**
P7-L1	530.000	452.200	n.d.	**489.557**	241.100	241.100	n.d.	**241.100**
P8-L13	2.323	1.221	n.d.	**1.684**	2.782	8.434	n.d.	**4.844**
P8-L14	5.215	3.400	n.d.	**4.211**	6.074	18.670	n.d.	**10.649**
Tirzepatide	13.47	13.12	15.71	**14.055**	2.564	1.850	5.229	**2.916**
d-ala GIP	NA	NA	NA	**NA**	NA	NA	NA	**NA**
GCG	NA	NA	NA	**NA**	NA	NA	NA	**NA**
GLP-1	0.2635	0.3447	0.6227	**0.384**	2.394	1.645	2.621	**2.177**
Semaglutide	4.124	7.890	3.026	**4.618**	0.935	1.321	0.851	**1.017**

NA – nonactive
n.d. — not determined; for selected compounds. EC_{50} values are the mean of two replicates.
SR – serum-replacement conditions using 10% Panexin CD (albumin-free assay conditions)
FBS: fetal bovine serum–containing assay conditions, including serum proteins such as albumin

4. **In vitro GCGR activity results for designed peptides, including individual replicate values and geometric mean EC_{50}. Assays were performed under serum replacement (SR) conditions and additionally under fetal bovine serum (FBS) conditions.**

Name	FBS EC50 rep.1 [nM]	FBS EC50 rep.2 [nM]	FBS EC50 rep.3 [nM]	**FBS EC50 geom avg [nM]**	SR EC50 rep.1 [nM]	SR EC50 rep.2 [nM]	SR EC50 rep.3 [nM]	SR EC50 geom avg [nM]
P1-L1	257.700	202.600	216.000	**224.252**	n.d.	53.800	48.000	**50.830**
P2-L2					0.740	1.440	0.690	**0.904**
P2-L1	7.097	6.343	7.185	**6.864**	1.260	1.090	1.740	**1.336**
P2-L3					0.520	0.780	0.560	**0.612**
P2-L4					0.680	0.780	0.370	**0.579**
P2-L5					1.350	1.070	0.540	**0.923**
P2-L6					3.100	3.190	6.190	**3.943**
P2-L7					0.500	0.950	0.610	**0.664**
P2-L8					1.840	1.060	0.600	**1.055**
P2-L9					0.850	0.790	0.650	**0.759**
P2-L10					1.740	1.260	1.870	**1.601**
P3-L1	0.689	1.121	0.659	**0.798**	0.750	0.250	0.400	**0.421**
P3-L11					1.160	0.320	0.620	**0.614**
P3-L12	4.706	3.252	n.d	**3.912**	0.810	0.450	0.690	**0.632**
P3-L10	1.409	0.967	1.431	**1.249**	1.760	0.420	0.950	**0.889**
P4-L1	1.032	0.800	n.d	**0.909**	n.d.	0.260	0.400	**0.326**
P4-L10	1.113	0.789	n.d	**0.937**	n.d.	0.250	0.410	**0.323**
P5-L1	491.000	712.200	n.d	**591.346**	n.d.	43.500	118.000	**71.490**
P6-L1					8187.000	3599.000	8498.000	**6302.910**
P7-L1	312.4	189.9	n.d	**243.567**	54.980	42.710	n.d.	**48.458**
P8-L13					817.800	1142.000	n.d.	**966.399**
P8-L14					1039.000	2033.000	n.d.	**1453.371**
Tirzepatide					NA	NA	NA	**NA**
d-ala GIP					NA	NA	NA	**NA**
GCG	0.183	0.096	0.052	**0.097**	0.080	0.100	0.070	**0.080**
GLP-1					NA	NA	NA	**NA**
Semaglutide					NA	NA	NA	**NA**

NA – nonactive
n.d. — not determined; for selected compounds. EC_{50} values are the mean of two replicates.
SR – serum-replacement conditions using 10% Panexin CD (albumin-free assay conditions)
FBS: fetal bovine serum–containing assay conditions, including serum proteins such as albumin

5. In vitro GIPR activity results for designed peptides, including individual replicate values and geometric mean EC_{50}. Assays were performed under serum replacement (SR) conditions and additionally under fetal bovine serum (FBS) conditions.

Name	FBS EC50 rep.1 [nM]	FBS EC50 rep.2 [nM]	FBS EC50 rep.3 [nM]	FBS EC50 geom avg [nM]	SR EC50 rep.1 [nM]	SR EC50 rep.2 [nM]	SR EC50 rep.3 [nM]	SR EC50 geom avg [nM]
P1-L1	84.900	62.550	87.730	77.522	n.d.	80.600	49.700	**63.240**
P2-L2					5.600	26.100	16.200	**13.333**
P2-L1	19.510	14.680	18.800	17.527	6.400	9.040	7.440	**7.550**
P2-L3					1.930	5.980	12.400	**5.228**
P2-L4					1.790	4.460	4.250	**3.236**
P2-L5					4.910	9.370	12.400	**8.282**
P2-L6					6.400	9.040	7.440	**7.550**
P2-L7					3.160	19.100	17.400	**10.165**
P2-L8					4.520	10.100	12.600	**8.315**
P2-L9					5.170	10.200	18.500	**9.923**
P2-L10					16.300	13.900	9.820	**13.066**
P3-L1	4.488	1.784	1.566	2.323	4.080	3.820	3.480	**3.787**
P3-L11					10.200	4.990	6.010	**6.726**
P3-L12	2.802	4.096		3.388	6.810	4.190	4.160	**4.912**
P3-L10	13.42	2.771	3.233	4.936	15.200	8.200	6.060	**9.105**
P4-L1	1.858	2.831	n.d	2.293	n.d.	1.300	3.190	**2.034**
P4-L10	1.646	2.597	n.d	2.068	n.d.	1.520	3.430	**2.281**
P5-L1	0.456	0.509	n.d	0.519	n.d.	0.230	0.730	**0.411**
P6-L1	0.056	0.045	n.d	0.050	0.091	0.120	0.117	**0.109**
P7-L1	0.533	0.451	n.d	0.490	0.345	0.566	n.d	**0.441**
P8-L13	804.500	200.000	n.d	401.123	90.090	51.720	n.d	**68.260**
P8-L14	746.900	339.100	n.d	503.263	239.900	110.200	n.d	**162.595**
Tirzepatide	0.012	0.0122	0.022	0.015	0.040	0.010	0.070	**0.031**
d-ala GIP	0.005	0.003	0.004	0.004	0.150	0.080	0.120	**0.115**
GCG					NA	NA	NA	**NA**
GLP-1					NA	NA	NA	**NA**
Semaglutide					NA	NA	NA	**NA**

NA – nonactive
n.d. — not determined; for selected compounds. EC_{50} values are the mean of two replicates.
SR – serum-replacement conditions using 10% Panexin CD (albumin-free assay conditions)
FBS: fetal bovine serum–containing assay conditions, including serum proteins such as albumin

List of peptides collected from patents and scientific publications

Publikacja/Patent	Peptides	Amount	Sequence based	Modifications	Staple	Activity
Madsen et al., Structure−Activity and Protraction Relationship of Long-Acting Glucagon-like Peptide-1 Derivatives: Importance of Fatty Acid Length, Polarity, and Bulkiness, J. Med. Chem. 2007	GLP-1 agonists	44	GLP-1 analogs		K20 modified with different fatty chains	GLP-1R EC_{50}: 8.6 - 4440 pM/L
Day et al., A new glucagon and GLP-1 co-agonist eliminates obesity in rodents, Nat. Chem. Bio. 2009	GLP-1R/GCGR dual agonists	33	GCG based analogues	sequence modifications (Aib, A in position 2)	lactam bridge in position 16-20	GLP-1R EC_{50}: 0.011-1.1 nM GCGR EC_{50}: 0.046 - >1000 nM
Murage et al., Development of potent glucagon-like peptide-1 agonists with high enzyme stability via introduction of multiple lactam bridges, J. Med. Chem. 2010	GLP-1R agonists	12	GLP-1 analogs		lactam bridges in positions 16-20, 18-22, 22-26, 30-34; double and triple lactam bridges	GLP-1R EC50: 0.6 - 7.0 nM
Santoprete et al., DPP-IV-resistant, long-acting oxyntomodulin derivatives, J. Pept. Sci. 2011	GLP-1R/GCGR dual agonists	49	OXM analogs	sequence modifications in position 2,3 and C/N-terminus	3 peptides with K -γGlu-CO-C15 (positions: 12, 14, 40)	GLP-1R EC50: 0.2 -770 nM GCGR EC50: 2.6 - >1000 nM
Gault et al., A Novel Glucagon-like Peptide-1 (GLP-1)/Glucagon Hybrid Peptide with Triple-acting Agonist Activity at Glucose-dependent Insulinotropic Polypeptide, GLP-1, and Glucagon Receptors and Therapeutic Potential in High Fat-fed Mice, J. Biol. Chem. 06.2013	GLP-1R/GCGR/GIPR triagonists	12	GLP1 and GCG analogs	with dA/dS in 2 position		
Finan et al., Unimolecular Dual Incretins Maximize Metabolic Benefits in Rodents, Monkeys, and Humans, Science Translational Medicine. 10.2013	GLP-1R/GCGR/GIPR triagonists	21	GLP-1 and GIP analogs	sequence modifications; extended C-terminus K40 and K10 modified	lactam bridges in positions 16-20	GLP-1R EC50: 0.005 - 3527 nM GCGR EC50: 0.005 - 2000 nM GIPR EC50: 0.003 - 1200 nM

Publikacja/Patent	Peptides	Amount	Sequence based	Modifications	Staple	Activity
Finan et al., A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents, Nat. Med. 2015	GLP-1R/GCGR/GIPR triagonists	32	GCG based analogues	sequence modifications in position 2, 3, 16, 17, 18, 20;	K10 modified with γGlu-C16 fatty chain	GLP-1R EC50: 0.002-100.48 nM GCGR EC50: 0.003-162.4 nM
van Witteloostujin et al., Neoglycolipids for Prolonging the Effects of Peptides: Self-Assembling Glucagon-like Peptide 1 Analogues with Albumin Binding Properties and Potent in Vivo Efficacy, Mol. Pharmaceutics. 01.2016	GLP-1R agonists	11	GLP-1 analogs		K20 glycolipidation with 1-2 mucic acid, γGlu and lipid substituent C16, C18 or C20	GLP-1R EC50: 13.4 – 118.8 pM
Muppidi et al., Design of Potent and Proteolytically Stable Oxyntomodulin Analogs, ACS Chem. Biol. 02.2016	GLP-1R/GCGR dual agonists	15	OXM analogs		Cys alkylated with cross linkers CL-1-CL-5	GLP-1R EC50: 0.070 – 2000 nM GCGR EC50: 0.180 – 1000 nM
Yang et al., Engineering a long-acting, potent GLP-1 analog for microstructure-based transdermal delivery, Proc. Natl. Acad. Sci. U.S.A. 04.2016	GLP-1R agonists	16	exendin-4 analogs		cross-linkers modifications (cysteins), substituted in positions: 17-24, 13-24, 10-24	GLP-1R EC50: 16-340 pM
Evers et al., Design of Novel Exendin-Based Dual Glucagon-like Peptide 1 (GLP-1)/Glucagon Receptor Agonists, J. Med. Chem. 05.2017	GLP-1R/GCGR dual agonists	24	Exendin-4 and OXM analogs	sequence modifications: positions 2, 10, 16-18		GLP-1R EC50: 0.7 - 55.8 pM GCGR EC50: 1.9 - >5.7e08 pM
Zhou et al., A novel glucagon-like peptide-1/glucagon receptor dual agonist exhibits weight-lowering and diabetes-protective effects, Eur. J. Med. Chem. 09.2017	GLP-1R/GCGR dual agonists	28	GCG analogues	sequence modifications Cys in positions 19-30	C22/C23/C25 modified with different maleimide fatty chains	GLP-1R EC50: 0.86-112.03 nM GCGR EC50: 0.46- 122.57 nM
Han et al., Xenopus GLP-1-inspired discovery of novel GLP-1 receptor agonists as long-acting hypoglycemic and insulinotropic agents with significant therapeutic potential, Biochemical Pharmacology. 10.2017	GLP-1R agonists	42	Xenopus GLP-1 and hGLP-1 analogs		Cys-PEGylated conjugations in positions 12,14, 17 and 26)	GLP-1R EC50: 0.6 - 43.2 nM

Publikacja/Patent	Peptides	Amount	Sequence based	Modifications	Staple	Activity
Demartis et al., Polypharmacy through Phage Display: Selection of Glucagon and GLP-1 Receptor Co-agonists from a Phage-Displayed Peptide Library, Scientific Reports. 01.2018	GLP-1R/GCGR dual agonists	35	GCG analogs			GLP-1R EC50: 0.007 – 78.32 nM GCGR EC50: 0.0019 – 8.45 nM
Evers et al., Dual Glucagon-like Peptide 1 (GLP-1)/Glucagon Receptor Agonists Specifically Optimized for Multidose Formulations, J. Med. Chem. 07.2018	GLP-1R/GCGR/GIPR triagonists	15	exendin-4 analogs	sequence modifications: positions 2, 27, 18	K-ɣGlu-ɣGlu -C16/ K-ɣGlu-C18 in position 14	GLP-1R EC50: 1.4 - >100000000 pM GCGR EC50: 1.6 - >100000000 pM GIPR EC50: 39.7 - >10000000 pM
Han et al., Lithocholic Acid-Based Peptide Delivery System for an Enhanced Pharmacological and Pharmacokinetic Profile of Xenopus GLP-1 Analogs, Mol. Pharmaceutics. 07.2018	GLP-1R agonists	26	Xenopus GLP-1 analogs		K20//K28 modified with lithocholic acid conjugation	GLP-1R EC50: 0.12 - 6.35 nM
Han et al., Xenopus-derived glucagon-like peptide-1 and polyethylene-glycosylated glucagon-like peptide-1 receptor agonists: long-acting hypoglycaemic and insulinotropic activities with potential therapeutic utilities, British Journal of Pharmacology. 2018	GLP-1R agonists	42	Xenopus glucagon and GLP-1 analogs		Cys-PEGylated conjugations in positions 12,14, 17 and 26)	GLP-1R EC50: 0.12 – 22.1 nM
Han et al., Lipidation and conformational constraining for prolonging the effects of peptides: Xenopus glucagon-like peptide 1 analogues with potent and long-acting hypoglycemic activity, European Journal of Pharmaceutical Sciences. 09.2018	GLP-1R agonists	40	Xenopus glucagon and GLP-1 analogs		K -ɣGlu-CO-C11/C15/C17 in position 20, 28, 29	GLP-1R EC50: 1.7 – 28.6 nM
Fremaux et al., Peptide-oligourea hybrids analogue of GLP-1 with improved action in vivo, Nature Communications. 12.2019	GLP-1R agonists	24	GLP-1 analogs	ureido residues		GLP-1R EC50: 0.10 – 10000 nM

Publikacja/Patent	Peptides	Amount	Sequence based	Modifications	Staple	Activity
Han et al., The chronic administration of two novel long-acting Xenopus glucagon-like peptide-1 analogs xGLP159 and XGLP296 potently improved systemic metabolism and glycemic control in rodent models, The FASEB Journal. 2019	GLP-1R agonists	4	Xenopus GLP-1 analogs		K -ɣGlu-CO-C15/C17 in position 20	GLP-1R EC50: 0.6 – 2.3 nM
Cai et al., Novel glucagon- and OXM-based peptides acting through glucagon and GLP-1 receptors with body weight reduction and anti-diabetic properties, Bioorganic Chemistry. 01.2020	GLP-1R/GCGR dual agonists	24	OXM and exendin-4 analogues		K16 modified with maleimide fatty chains	GLP-1R EC50: 0.3 - 133.2 pM GCGR EC50: 1.6 - 128.0 pM
Lear et al., Recombinant Expression and Stapling of a Novel Long-Acting GLP-1R Peptide Agonist, Molecules. 01.2020	GLP-1R agonists	6	exendin-4 analogs	in position 14 (M)	C17-C24 stapled and lapidated with fatty chains	GLP-1R EC50: 39 – 260 pM
Jones et al., Impact of N-terminally substituted glucagon family receptor agonists on signal bias, trafficking and downstream responses, bioRxiv. 04.2020	GLP-1R/GCGR/GIPR triagonists	15	GLP-1, GCG and GIP analogs	in positions 1,2 and 3		GLP-1R EC50: 0.316 – 3.160 nM GCGR EC50: 0.158 – 7.943 nM GIPR EC50: 0.100 – 25.110 nM
Bird et al., Hydrocarbon-Stitched Peptide Agonists of Glucagon-Like Peptide-1 Receptor, ACS Chem. Biol. 06.2020	GLP-1R agonists	42	GLP-1 analogs		hydrocarbon stapled (i-i+7)	
Zhang et al., A novel thrombin-based triagonist with diabetes-protective and weight-lowering potential, Life Sciences. 09.2020	GLP-1R/GCGR/GIPR triagonists	26	GLP-1, GCG and GIP analogs	positions 2,3, 7, 10, 12		GLP-1R EC50: 0.031 – 1784.326 nM GCGR EC50: 0.027 – 2514.229 nM GIPR EC50: 0.078 – 1642.008 nM
Chen et al., Stapled and Xenopus Glucagon-Like Peptide 1 (GLP-1)-Based Dual GLP-1/Gastrin	GLP-1R agonists	68	Xenopus GLP-1		in positions: 14-21; 21-28 (Cys stapled with 4 fatty acid	GLP-1R EC50: 0.022 – 0.23

Publikacja/Patent	Peptides	Amount	Sequence based	Modifications	Staple	Activity
Receptor Agonists with Improved Metabolic Benefits in Rodent Models of Obesity and Diabetes, J. Med. Chem. 11.2020			analogs		modifications)	nM
Cui et al., Rational design of a GLP-1/GIP/Gcg receptor triagonist to correct hyperglycemia, obesity and diabetic nephropathy in rodent animals, Life Sciences. 11.2020	GLP-1R/GCGR/GIPR triagonists	13	GIPR/GCGR dual agonist with exendin-4 analogs	K7/K16/K31 modified with fatty chain maleimide (C12, C14 and C16)		GLP-1R EC50: 0.12 -1.42 nM GCGR EC50: 0.68 - 2.79 nM GIPR EC50: 0.33 - 3.75 nM
Evers et al., Multiparameter Peptide Optimization toward Stable Triple Agonists for the Treatment of Diabetes and Obesity, Advanced Therapeutics. 2020	GLP-1R/GCGR/GIPR triagonists	14	exendin-4 analogs	(K10, positions 34, 20, 2)	K-ɣGlu-ɣGlu -C16 in position 14	GLP-1R EC50: 0.8 - 1.8 pM GCGR EC50: 0.3 - 33.3 pM GIPR EC50: 0.9 - 2890 pM
Nestor et al., Design and characterization of a surfactant-conjugated, long-acting, balanced GLP-1/glucagon receptor dual agonist, Peptide Science. 2020	GLP-1R/GCGR dual agonists	22	GLP-1 and GCG analogs	Aib in position 2	lactam bridges in positions 16-20 K17 and K20 modified with different fatty chains (and glycolipid linkers)	GLP-1R EC50: 5 - 52 pM GCGR EC50: 15 – 884 pM
Liu et al., Discovery of a novel GLP-1/GIP dual receptor agonist CY-5 as long-acting hypoglycemic, anti-obesity agent, Bioorganic Chemistry. 01.2021	GLP-1R/GCGR/GIPR triagonists	16	GLP-1 analogs	in positions 2 (Aib), 13, 16-20	K -Peg2-Peg2-ɣGlu-CO-C16 (positions: 10, 13-20)	GLP-1R EC50: 0.29 - 30.91 nM GCGR EC50: 9.71 - >100 nM GIPR EC50: 0.75 - 18.9 nM
Longwell et al., Identification of N-Terminally Diversified GLP-1R Agonists Using Saturation Mutagenesis and Chemical Design, ACS Chem. Biol. 01.2021	GLP-1R agonists	10	GLP-1 analogs	N-terminal (5 aminoacids)		GLP-1R EC50: 0.090 – 160 nM
Jiang et al., Design of novel Xenopus GLP-1-based dual glucagon-like peptide 1 (GLP-1)/glucagon receptor agonists, Eur. J. Med. Chem. 02.2021	GLP-1R/GCGR dual agonists	19	Xenopus GLP-1, OXM and exendin-4 analogs		3 peptides with K -ɣGlu-CO-C15 (positions: 12, 14, 40)	GLP-1R EC50: 0.031 – 3.8 nM GCGR EC50: 0.079 – 2.7 nM

Publikacja/Patent	Peptides	Amount	Sequence based	Modifications	Staple	Activity
Tinsley et al., Synthesis, Optimization, and Biological Evaluation of Corrinated Conjugates of the GLP-1R Agonist Exendin-4, J. Med. Chem. 03.2021	GLP-1R agonists	18	exendin-4 analogs		K12 and K40 corinated	GLP-1R EC50: 10.7 - 105.6 pM
Han et al., Stapled, Long-Acting Xenopus GLP-1-Based Dual GLP-1/Glucagon Receptor Agonists with Potent Therapeutic Efficacy for Metabolic Disease, Mol. Pharmaceutics. 07.2021	GLP-1R/GCGR dual agonists	39	Xenopus GLP-1 analogs	Cys in positions 11-30	C cross-linkers (bismaleimide amide) with fatty chains	GLP-1R EC50: 0.022 - 2.32 nM GCGR EC50: 0.082 -1.92 nM
WO2012088379 – Marcadia: Methods for Treating Metabolic Disorders and Obesity with Gip and Glp-1 Receptor-Active Glucagon-Based Peptides	GLP-1R/GCGR/GIPR triagonists	401	GIP analogs	position 2 (Aib), 20 (Aib), 40 (substituted C and K)	lactam bridges in positions 16-20	GLP-1R EC50: 0.002 – 4548.90 nM GCGR EC50: 0.007 – 23313.00 nM GIPR EC50: 0.001 – 148.630 nM
US20140213513A1 – Sanofi: Exendin-4 Derivatives as dual GLP1/GIP or trigonal GLP1/GIP/Glucagon Agonists	GLP-1R/GCGR/GIPR triagonists	66	Exendin-4 analogs	Aib in position 2	K20 modified with different fatty chains	GLP-1R EC50: 0.002 – 0.074 nM GCGR EC50: 0.001 - 1000 nM GIPR EC50: 0.001 – 0.189 nM
WO2015000942 – Novo Nordisk: Derivatives of Glp-1 Like Peptides, and Uses Thereof	GLP-1R agonists	29	GLP-1 analogs	Aib in position 2	double K42 and K18/23/27/31/36/38 modified with different fatty chains	GLP-1R EC50: 3.4 - 381 pM
WO2015086731 – Sanofi: Exendin-4 Peptide Analogues as Dual Glp-1/Glucagon Receptor Agonists	GLP-1R/GCGR dual agonists	23	Exendin-4 analogs	in position 3	K20 modified with different fatty chains	GLP-1R EC50: 0.8 – 8.5 pM GCGR EC50: 4.7 – 77.5 pM
WO2016083499 – Novo Nordisk: Glp-1 Derivatives and Uses Thereof	GLP-1R agonists	35	GLP-1 analogs	Aib in position 2	K31 modified with different fatty chains	GLP-1R EC50: 0.001 – 0.041 nM

Publikacja/Patent	Peptides	Amount	Sequence based	Modifications	Staple	Activity
WO2016198604 – Sanofi: Exendin-4 Derivatives as Dual Glp-1 /Glucagon Receptor Agonists	GLP-1R/GCGR dual agonists	20	Exendin-4 analogs	positions 2 (Aib), 21	K20 modified with different fatty chains	GLP-1R EC50: 0.6 - 5.3 pM GCGR EC50: 2.1 - 13.4 pM GIPR EC50: 10.8 - 1120 pM
WO2017074798 – Merck: Long-Acting Co-Agonists of the Glucagon and Glp-1 Receptors	GLP-1R/GCGR dual agonists	61		positions 2, 16	K21 and K20 modified with different fatty chains	GLP-1R EC50: 0.02 - 1.89 nM GCGR EC50: 0.01 - 1.98 nM
CN107266557A – Tianjin Institute: A kind of polyethyleneglycol modified glucagon-like peptide 1 analog	GLP-1R agonists	20	GLP-1 analogs	position 2,3		GLP-1R EC50: 0.06 - 0.200 nM
WO2017149070 – Novo Nordisk: Glp-1 Derivatives and Uses Thereof	GLP-1R agonists	30	GLP-1 analogs	position 2 (W, X)	double K20 and K30/31/32 modified with different fatty chains	GLP-1R EC50: 0.53 – 70.5 pM
WO2018100135 – Sanofi: New Compounds as Peptidic Glp1/Glucagon/Gip Receptor Agonists	GLP-1R/GCGR/GIPR triagonists	18		Aib in position 2	K14 modified with different fatty chains	GLP-1R EC50: 0.001 – 0.017 nM GCGR EC50: 0.016 – 0.108 nM GIPR EC50: 0.002 – 0.025 nM
US10131702 – Zealand Pharma: Glucagon - GLP-1 - GIP Triple Agonist Compounds	GLP-1R/GCGR/GIPR triagonists	58	GCG analogs	Aib in position 2	K17 modified with different fatty acid chains	GLP-1R EC50: 0.007 - 10 nM GCGR EC50: 0.010 - 10 nM GIPR EC50: 0.004 - 10 nM
WO201912592 – Elli Lilly: Incretin Analogs and Uses Thereof	GLP-1R/GCGR/GIPR triagonists	9		positions 2,3, 13, 17, 20	K17 modified with different fatty chains	GLP-1R EC50: 0.015 -0.115 nM GCGR EC50: 0.012 – 0.096 nM GIPR EC50: 0.021 – 0.154 nM

Publikacja/Patent	Peptides	Amount	Sequence based	Modifications	Staple	Activity
WO2019125938 – Elli Lilly: Incretin Analogs and Uses Thereof	GLP-1R/GCGR/GIPR triagonists	23		positions 2, 13, 17, 19, 20, 28, 29, 34	K17 modified with different fatty chains	GLP-1R EC50: 0.063 – 0.253 nM GCGR EC50: 0.012 – 0.205 nM GIPR EC50: 0.036 – 0.154 nM
US20190218269A1 – Hanmi Pharmaceuticals: Glucagon, Composition Comprising Glp-1 Receptor and Gip Receptor Dual Agonist and Therapeutic Use Thereof	GLP-1R/GCGR/GIPR triagonists	104	GLP-1, GCG and GIP analogs	Aib in position 2	lactam bridges in positions 16-20	GLP-1R EC50: 0.002 - 4 nM GCGR EC50: 0.004 - 10 nM GIPR EC50: 0.003 – 3.80 nM
WO2020019813 – China Pharmaceutical University: Long-Acting Oxyntomodulin Hybrid Peptide, Preparation Method Therefor, and Application Thereof	GLP-1R/GCGR dual agonists	61	OXM analogs	positions 2	K16 modified with different fatty chains	GLP-1R EC50: 0.001 – 0.154 nM GCGR EC50: 0.001 - 1 nM
WO2020103729 – Tianjin: Glucagon-Derived Peptide and Use Thereof	GLP-1R/GCGR dual agonists	41			K20 and K10 modified with different fatty chains	GLP-1R EC50: 0.011 - 20.75 nM GCGR EC50: 0.014 -6.25 nM

Aminoacid analysis of natural and designed GLP-1, GCG and GIP sequences derived from patents and scientific publications

Sequence position	GLP-1	GCG	GIP	Role	Comment	Possible mutations
1	H	H	Y	• GLP-1R: part of van der Waals and polar interactions network • GCGR: part of van der Waals and polar interactions network	• to GIP native tyrosine decreases activity on GLP-1R by a factor of 3 while increasing activity on GCGR and GIPR 5 and 4 times	Y
2	A	S	A	• main purpose of mutating Alanine is to decrease the binding and cleavage by proteolytic enzyme DPP-IV. • GLP-1R: L388; Aib2 interacts with (L384 7.39, E387 7.42). • GCGR: E387/D385/E369 (7.42b) • A2S mutant of GLP-1 restores binding of the GLP-1R mutant • A is said to stabilize GIP-TMD interface (together with F6 and Y10) through nonpolar network with (L136 1.36b, L137 1.39b, Y141 1.43b, L7347.39b, I3787.43b).	• Aib can even increase binding to GLP-1R by a factor of 1.5. • not found any amino acid that would be better than Serine in terms of hydrogen bonding	Aib
3	E	Q	E	• form van der Waals and polar interactions with receptor. • GLP-1R: Y152(1.47b) (hydrogen bonds); R190(2.60) (salt bridge)	• Q looks like the best fit for position 3 • Q native to GCG can boost GCGR activity GCGR even 100 times • E3Q mutation can boost GCGR activity without strongly decreasing GLP-1R and GIPR activity	Q
4	G	G	G	• conserved residues essential for activity across all three receptors	• Conserved G seems not to be involved in any protein-ligand interactions • literature we do not find any better substitutions to native G. In sequences studied V (1.5nm), A(0.290nM), M(0.15nM) (GLP-1R) in Longwell suggested there thiol group or a longer sidechain might be more beneficial in this place than branched methyl groups	G
5	T	T	T	• conserved residues essential for activity across all three receptors	• no single point mutation data about this position, particularly any beneficial substitutions for native T. From violin plots it can be seen that M5 containing peptides perform worse than native GLP-	T

				• GLP-1R T: D372(ECL3)/D/E	1 on GLP-1R	
6	F	F	F	• conserved residues essential for activity across all three receptors • forms a hydrophobic patch important for activity • GLP-1R: close to L141(1.36), L144(1.39) and Y148(1.43) might be forming hydrophobic interactions with these residues and Y148 hydroxyl group might be forming interaction with delocalized charge on phenylalanine ring.	• no single point mutation data on this mutation. • peptides containing 6Y perform worse than native GCG on GCGR. • not find any beneficial substitution for native F	F
7	T	T	I	• GLP-1R: K197(2.67)(salt bridge)	• I7T mutation boosts GLP-1R (up to 100 times) activity at the expense of GIPR and (surprisingly) GCGR activity (up to 10 times drop in activity) • I7 containing peptides do not reach activity superior to GLP-1	T
8	S	S	S	• conserved residues essential for activity across all three receptors • GLP-1R: N300(ECL2)/N298/N290	• not find any single point mutations	S
9	D	D	D	• conserved residues essential for activity across all three receptors • GLP-1R: R380(7.35)/ R378/R370 (salt-bridge) • GCGR: Q374(ECL3)	• D9E reduces GCG potency but maintains binding affinity (effect on helix VII conformational change) • not find any substitutions that would improve peptide binding	D

10	V	Y	Y	• forms a hydrophobic patch important for activity • GCGR: Y138(1.36b), L198(2.71b) – hydrophobic interactions • GIPR: Q138 (part of A2, F6,Y10 non polar network with L134, L137, Y141, L374, I378)(1.40b)	• mutation from tyrosine native to GCG to leucine decreases activity towards GCGR and GIPR several times while increasing GLP-1R activity several (up to ten) times on average • mutation from cysteine to lysine decreases GLP-1R activity several-fold • peptides with 10V activity on GIPR is lower than that of native GCG peptide while 10Y peptides on GLP-1R do not have such limitations and perform almost as well as 10V • Y is best residue for this position	Y L
11	S	S	S	• conserved residues essential for activity across all three receptors • form van der Waals and polar interactions with receptor • GLP-1R: T298, R299 (ECL2) – hydrogen bonds, Y205 - hydrogen bonds	• mutation of threonine to cysteine does not affect activity while mutating to alanine can have varying effects from decreasing GLP-1R activity more than 10 times to increasing activity more than 10 times • serine is incomparably best amino acid in this position	S
12	S	K	I		• lysine reduces activity on GIPR and boosts activity on GLP-1R and GCGR several times • GIPR peptides containing S12 is always perform worse than native GIP • For GIPR GIP relative activity for I can reach up to 1000 while K rises a little above 100. • maximum potency for any of the receptors or GIPR in particular our choice would be I12. • K can achieve relative potency of at least 10 for each of the receptors, also offers additional benefit as a target for lipidation.	I, K
13	Y	Y	A	• forms a hydrophobic patch important for activity • Y/Y/A: binds to F/F/Q (1.29b) (on receptors) • GLP-1R: E138 (1.33)	• α-tyrosine native to GLP-1 and GCG results vary depending on context from several-fold decrease to more than 10 times increase • mutation from leucine to glutamine shifts the activity several-fold from GCGR and GIPR towards GLP-1R • mutating leucine to α-methylo-leucine increases activity on GIPR • peptides containing 13A have limited relative potency (<1.0) on	Q, L

					GLP-1R and GCGR. • to produce good GIPR agonist with 13Y	
14	L	L	M	• forms a hydrophobic patch important for activity • GLP-1R: Y13 and L14 form a hydrophobic patch important for mediating binding affinity in GCGR • GCGR: Y202(2.75b)(hydrophobic interactions) (Y202A is knockout for GCG binding)	• L is best amino acid for position 14	L, K
15	E	D	D	• GLP-1R: S31(ECD) and R299 (ECL2) and Y205 (2.75) (h-bond) L32 (backbone; L32) • GCGR: V28(backbone) • GIPR: V28(backbone)	• mutations from aspartic acid native to GCG and GIP to glutamic acid can increase activity on GLP-1, mean activity does not change • D is best amino acid at position 15	D, E
16	G	S	K	• GIPR: E122 (salt bridge)	• mutations from glycine native to GLP-1 to glutamic acid can have varying effect on GLP-1R activity ranging from several-fold increase to several-fold decrease • G to lysine native to GIP increases GIPR activity more than 10-fold while decreasing GCGR activity and slight increase in GLP-1R activity • mutations from lysine native to GIP to serine native to GCG can slightly shift from GIPR and GCGR towards GLP-1R activity • S to glutamic acid can shift activity from GCGR activity to GIPR activity (more) and GLP-1R (less) • S -> E increases activity (-0.699/-1.574/-) • G->E has positive effect on GLP-1R activity	Q, K, E

					• K-> E we find mostly improvement in GLP-1R and GIPR. Compensated however by decrease in GCGR activity • E16 perform better than any of the native variants	
17	Q	R	I	• GCGR: Q131	• glutamine at position 18 allows for the greatest relative potency on each receptor	C
18	A	R	H	• GCGR: W215 (ECL1) (arginine -π interaction) (important W215L is knockout mutation), Y202 (2.75b) (hydrophobic interactions) (Y202A is a knockout mutation) • GIPR: Y36 (polar contacts)	• possible to develop potent agonists for each receptor using Alanine on position 18 • most of the peptides studied with 18R had relative GCGR potency < 1.0. Single point mutation radar show that native residues are best candidates for this position with at least some specificity • Alanine native to GLP-1 to arginine native to GCG increases (shifts to) GCGR activity up to tenfold, while decreasing GIPR activity several-fold, effect on GLP-1R can vary from several-fold decrease to tenfold increase	A, R
19	A	A	Q		• alanine is the best amino acid for position 19 • mutations from A -> Q in Eve20-05-06 we see 0.921 on GCG (decrease) and -0.464 (increase) with GIPR which would and -0.4 (increase) in triagonists claiming that Q is actually better here	A, Q, K
20	K	Q	Q	• GLP-1R: E128 • GIPR: N124 (polar contacts)	• lysine can be better than Q even for GCGR and have similar potency to 20Q on GIPR • K can still have polar contacts with asparagine	K, Q
21	E	D	D		• peptides with 21E can be better than peptides containing 21D (and other amino acids) not only on GLP-1R but also on GCGR and as potent as latter on GIPR • E21D mutation improves GLP-1R and GCGR activity without hurting GIPR activity	A, E, D, Q

22	F	F	F	• conserved residues essential for activity across all three receptors • GLP-1R: W214 (stacking) (F22, V23, L26, L27) occupy hydrophobic binding groove (L35, Y36, W39, M67, Y68, Y87, L88, P89, W90). • GIPR: L35, Y36, L88, Y87 (hydrophobic)	• native phenylalanine is definitely the best known option for position 22	F, Y
23	I	V	V	• GIPR: observed contact L88, W90	• peptides having valine or isoleucine can reach similar relative potencies on all three receptors • I24V single point mutation study shows that such mutation can increase GIPR effect up to 100 fold without sacrificing GLP-1R and GCGR activity	C, V, I
24	A	Q	N		• single point mutation does not show any preferred native amino acid on position 24. • possible to substitute N with C with little effect on activity • alanine is the best option for triagonism	C
25	W	W	W	• conserved residues essential for activity across all three receptors • GLP-1R: close to W39 (π-π stacking), W214 (ECL-3) (π-π stacking) • GIPR: proximity to W39, Y87	• mutation from tryptophane native to all native peptides (GLP-1, GCG, GIP) to alanine has varying effect on GLP-1R from tenfold decrease to several-fold increase, averaging to slight increase • native tryptophane seems the best option for position 25	W
26	L	L	L	• conserved residues essential for activity across all three receptors	• native leucine is the best choice for aminoacid at this position	L
27	V	M	L	• GLP-1R: Y69 (ECD) • GIPR: Y68	• from isoleucine to lysine decreases activity on GLP-1R several fold • L seems the best and safest option for position 27	L, I, K

28	K	N	A	• GLP-1R: water mediated salt bridge with own E (stability)	• from asparagine native to GCG to glutamine can increase all activities several-fold • alanine seems to be the best option for position 28 • from alanine native to GIP to glutamic acid slightly increases activity on GIPR and GLP-1R	N, D, A
29	G	T	Q	• GIPR: M67 (proximity)	• glycine seems best amino acid variant for position 29	G, H, A

Aminoacid analysis of natural and designed GLP-1, GCG and GIP sequences derived from patents and scientific publications – raw data

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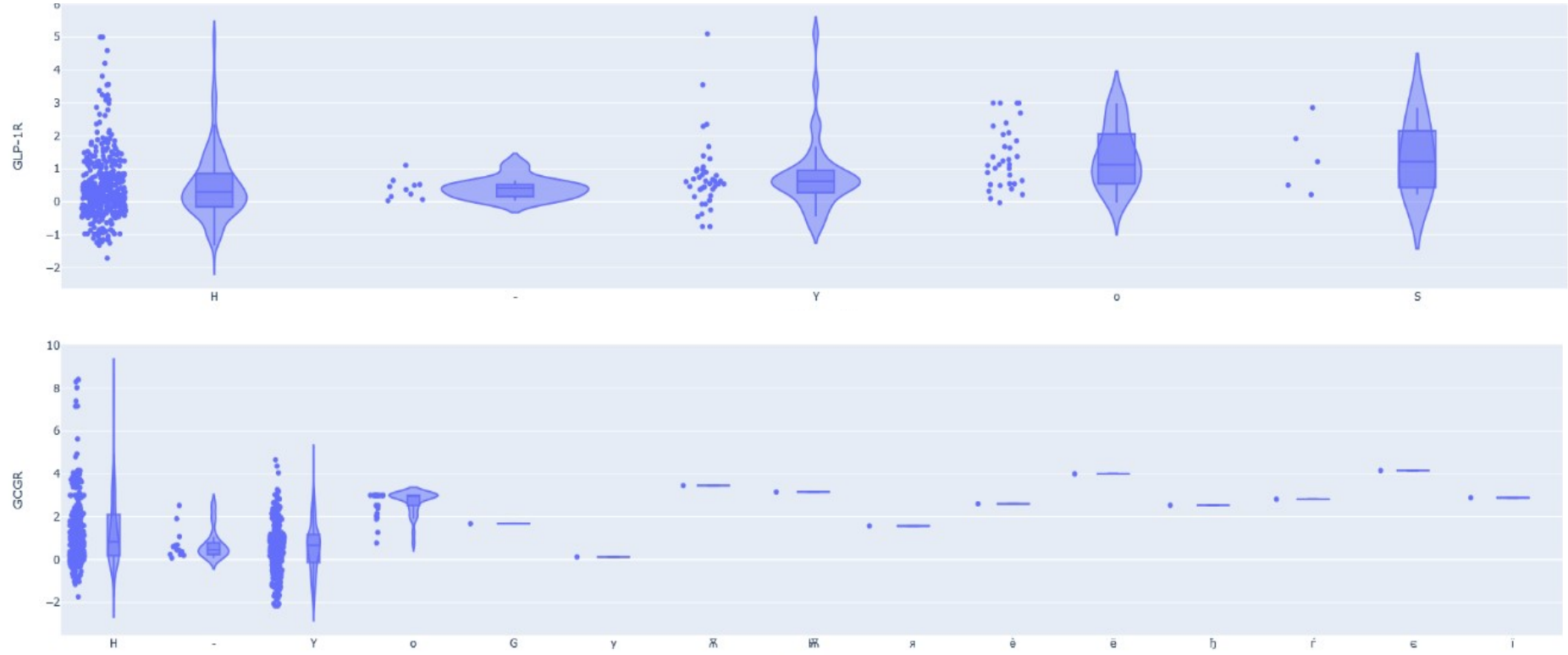

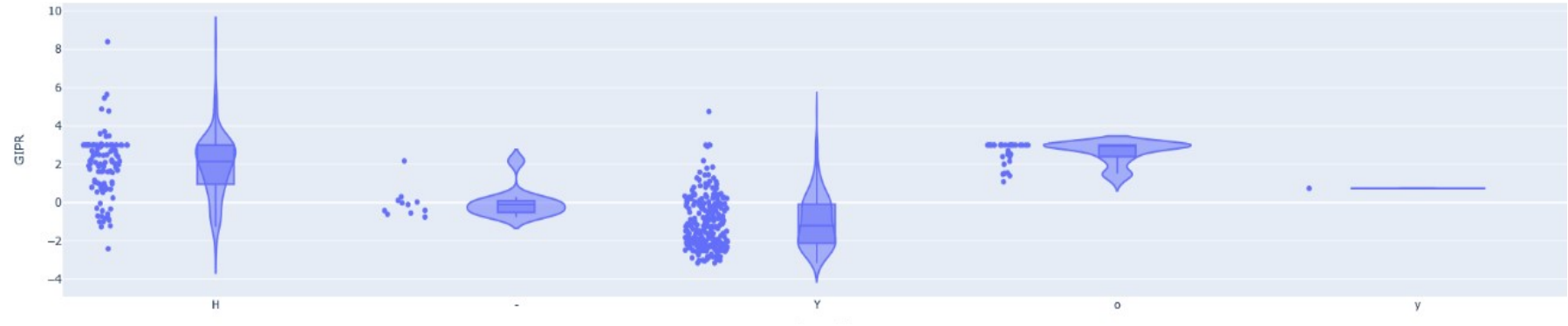
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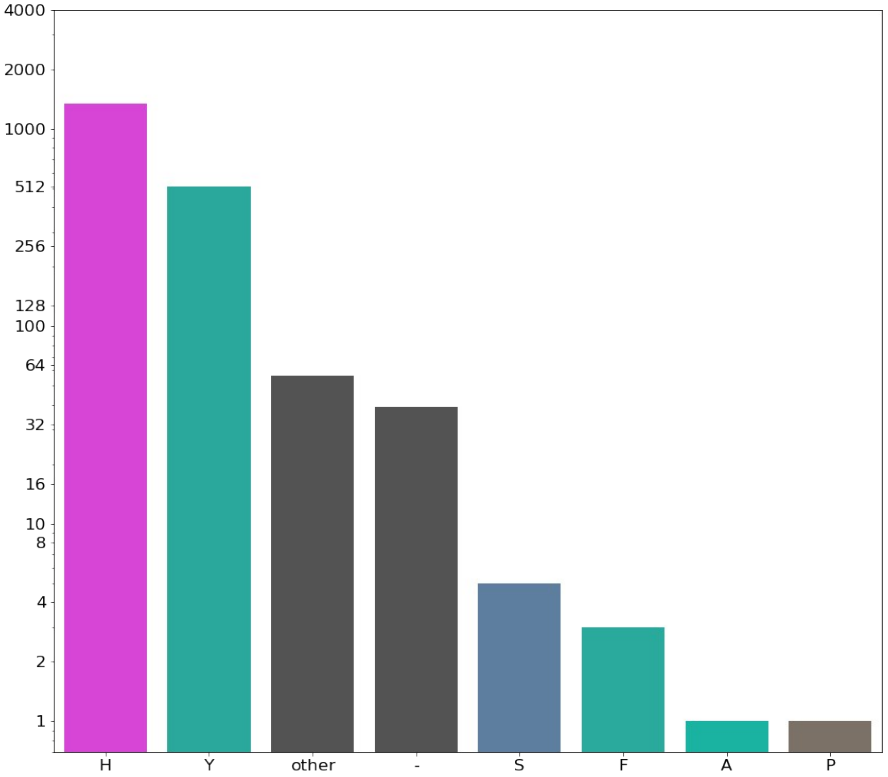
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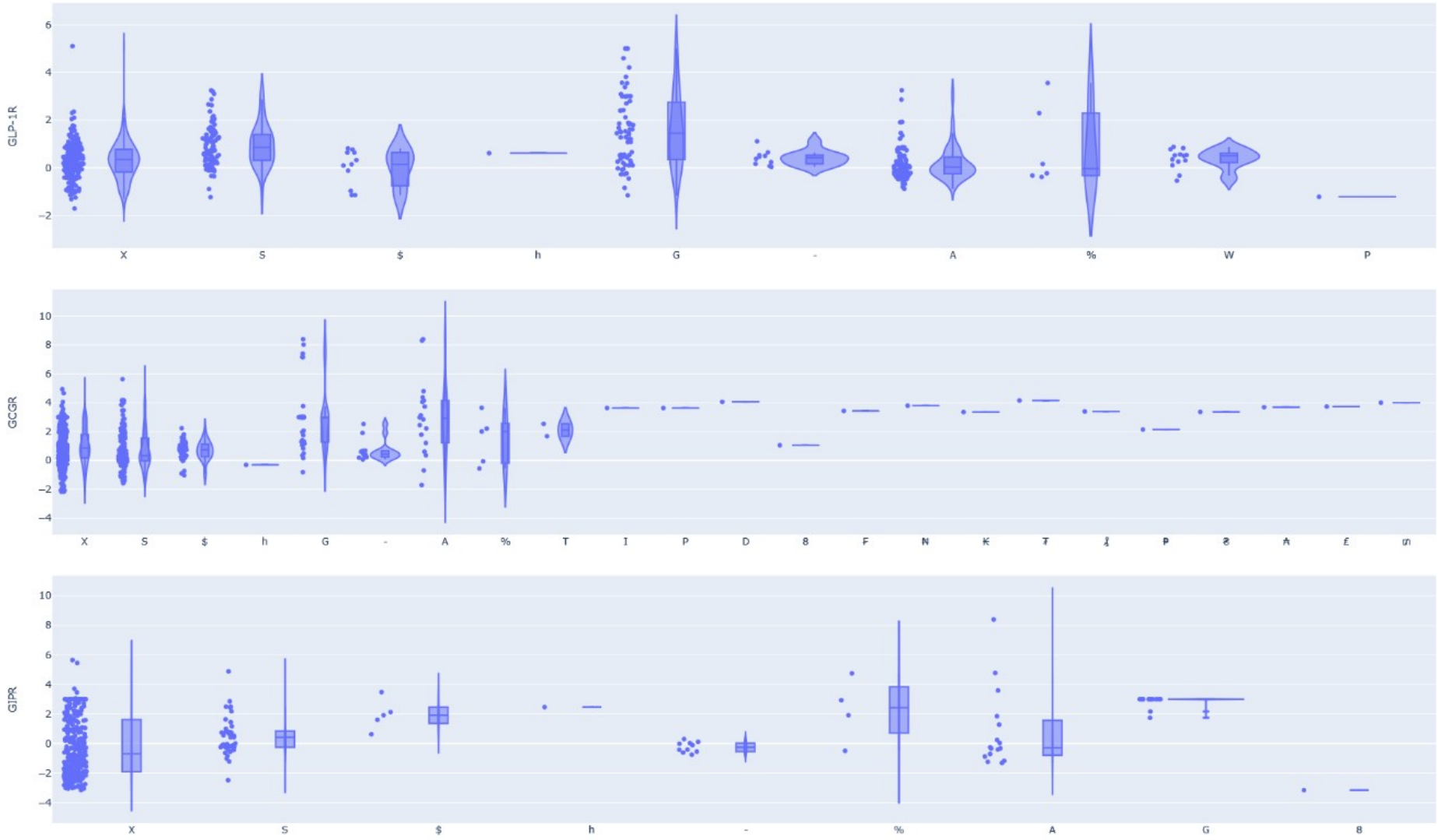

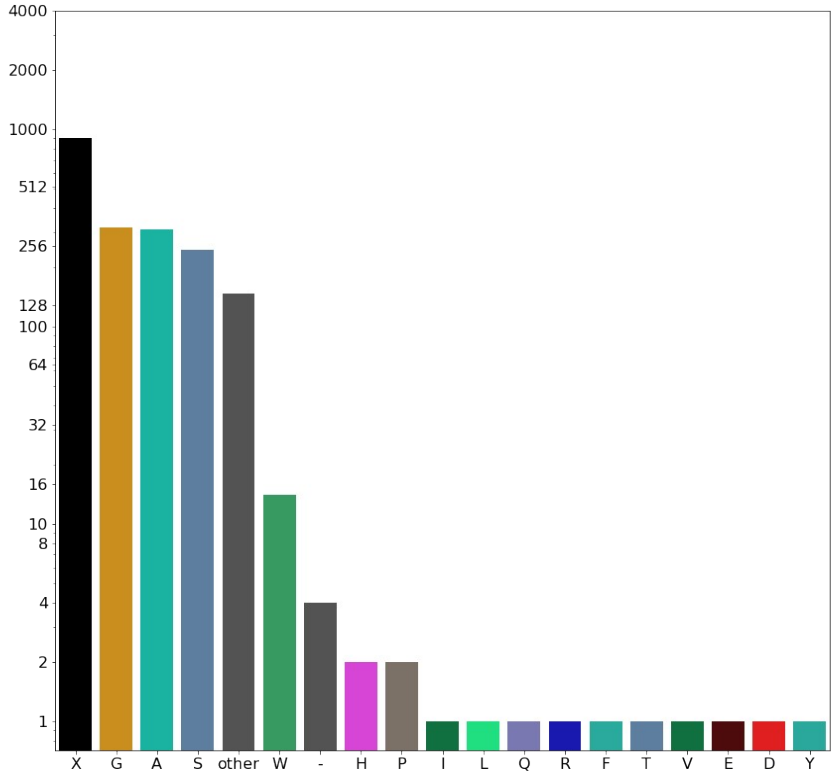
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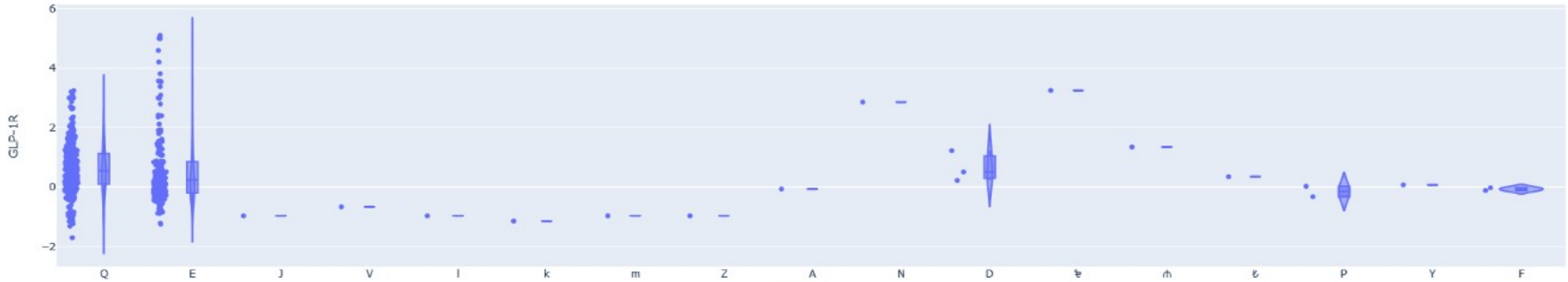
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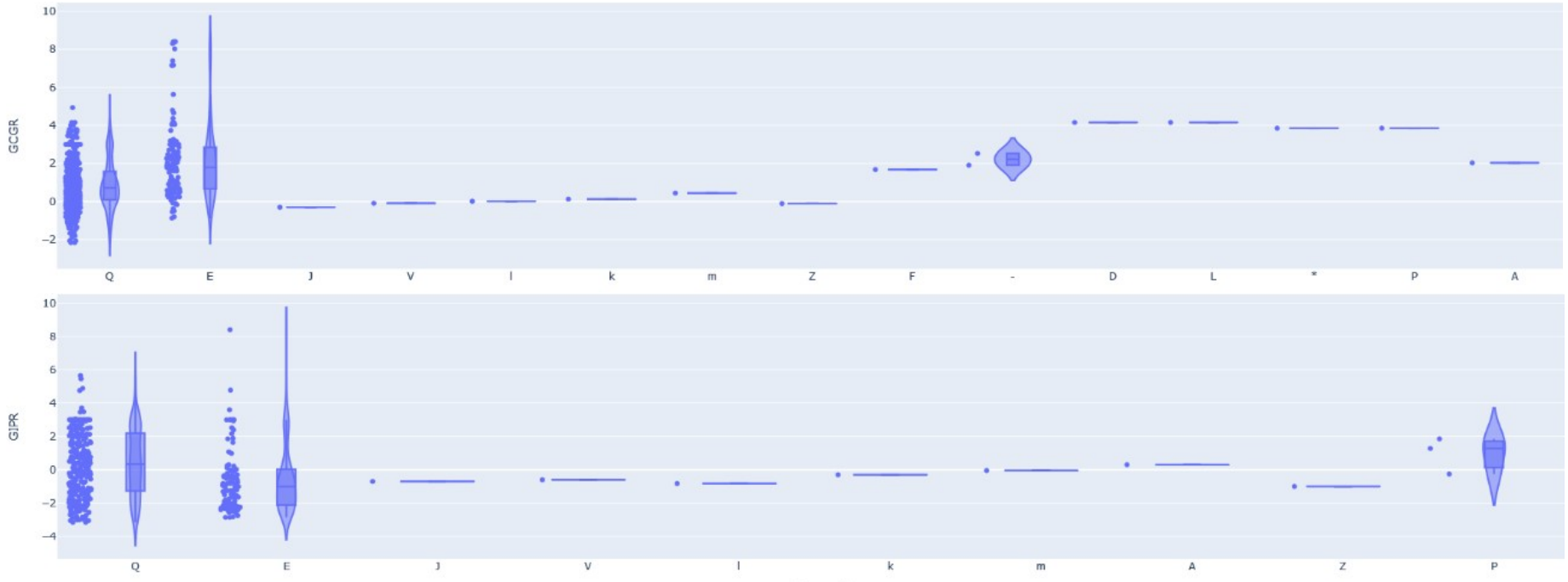
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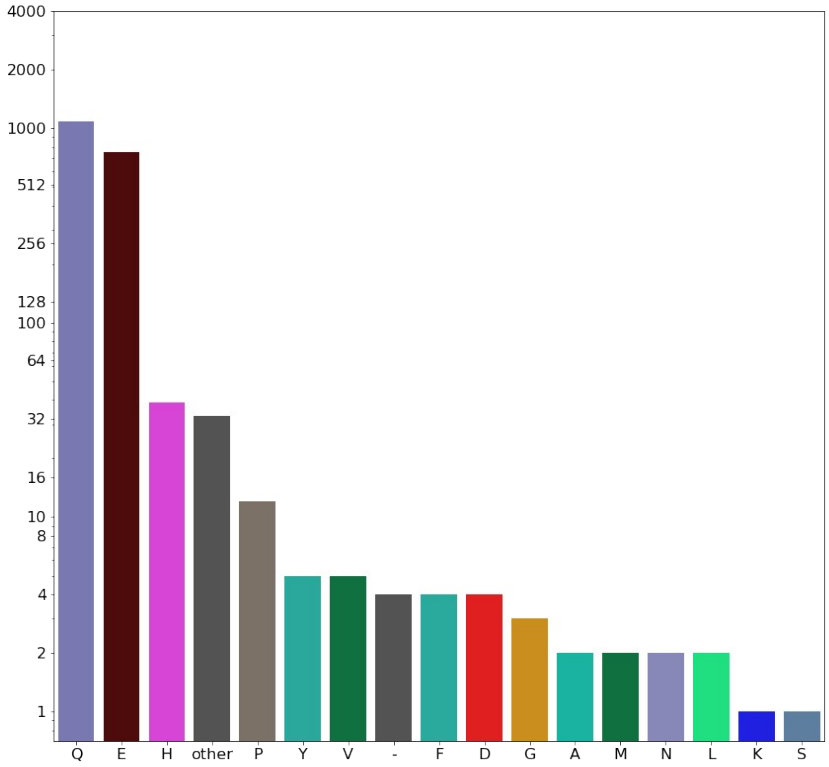
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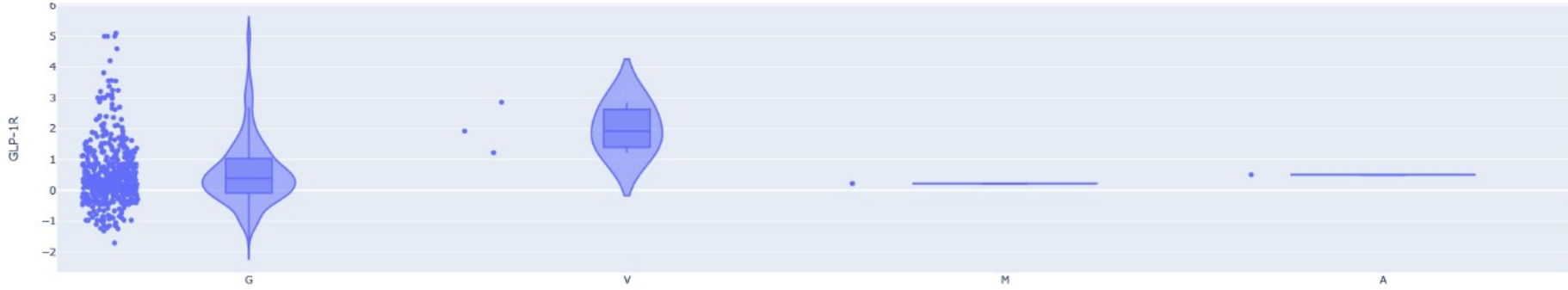
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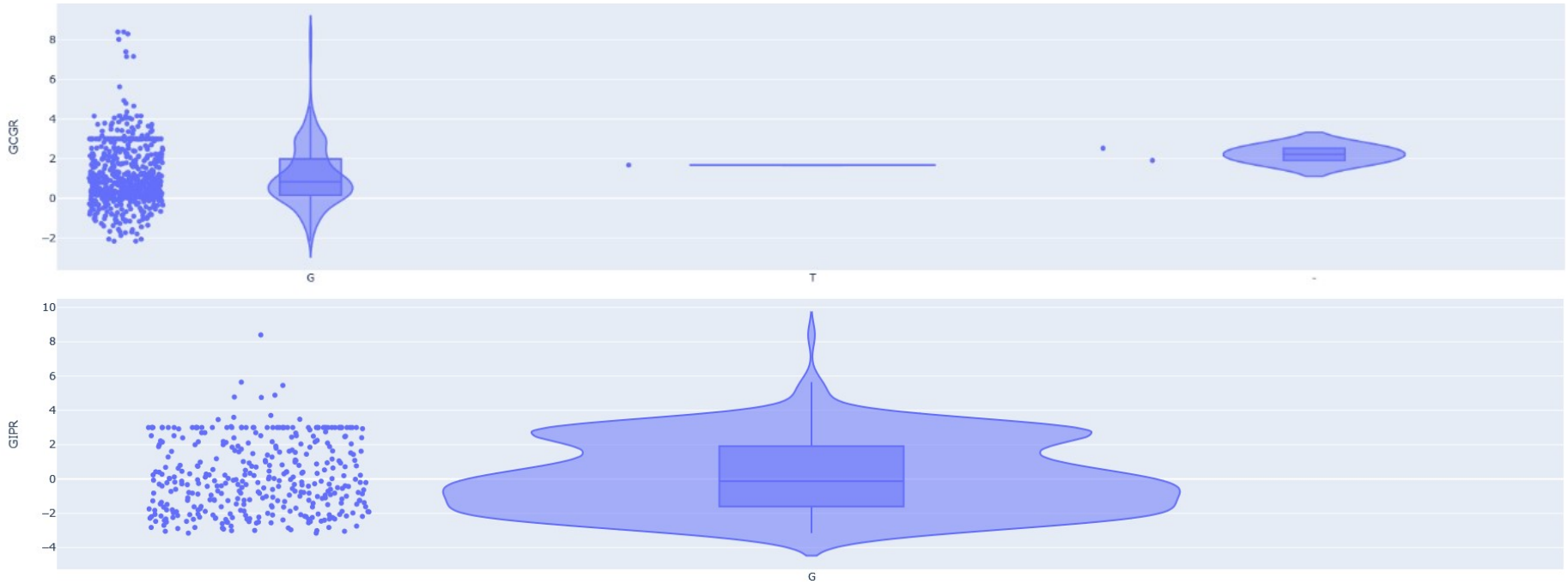
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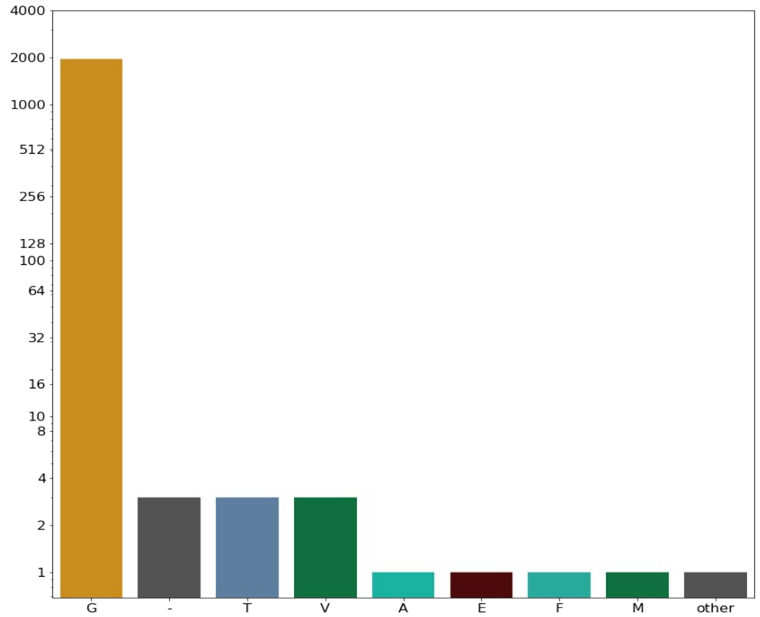
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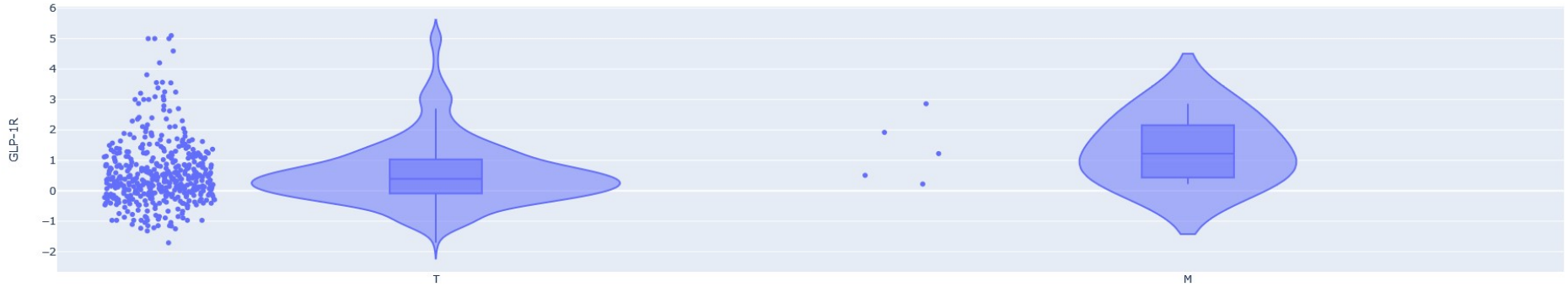
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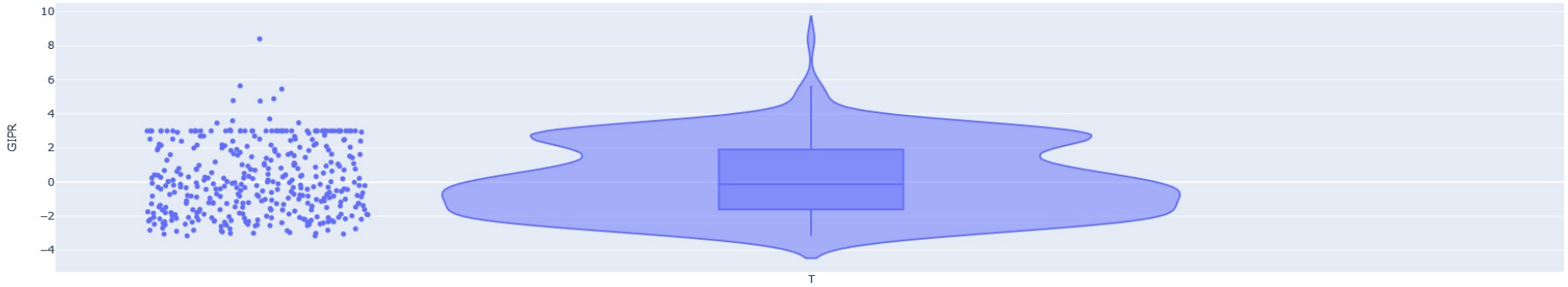
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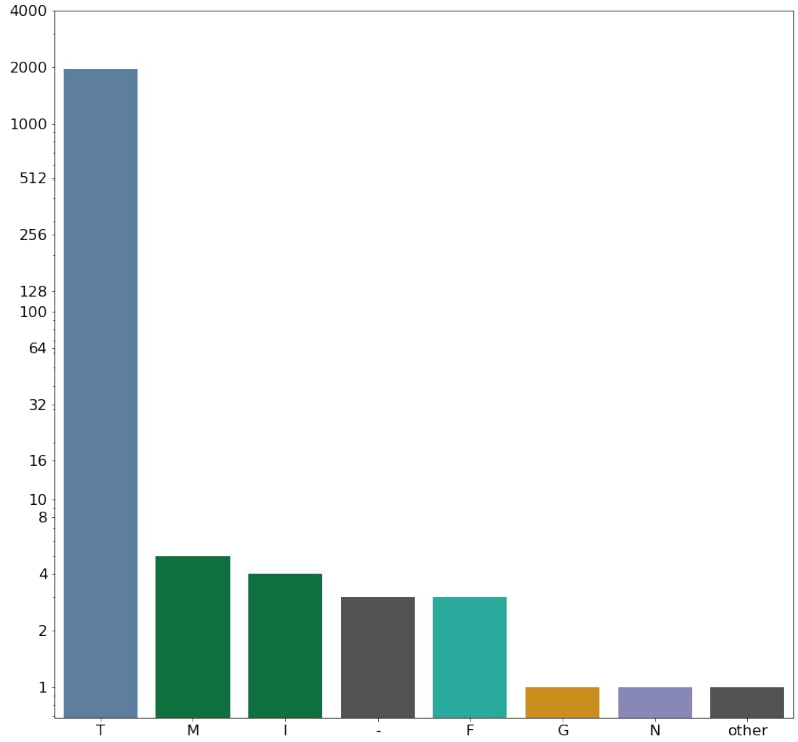
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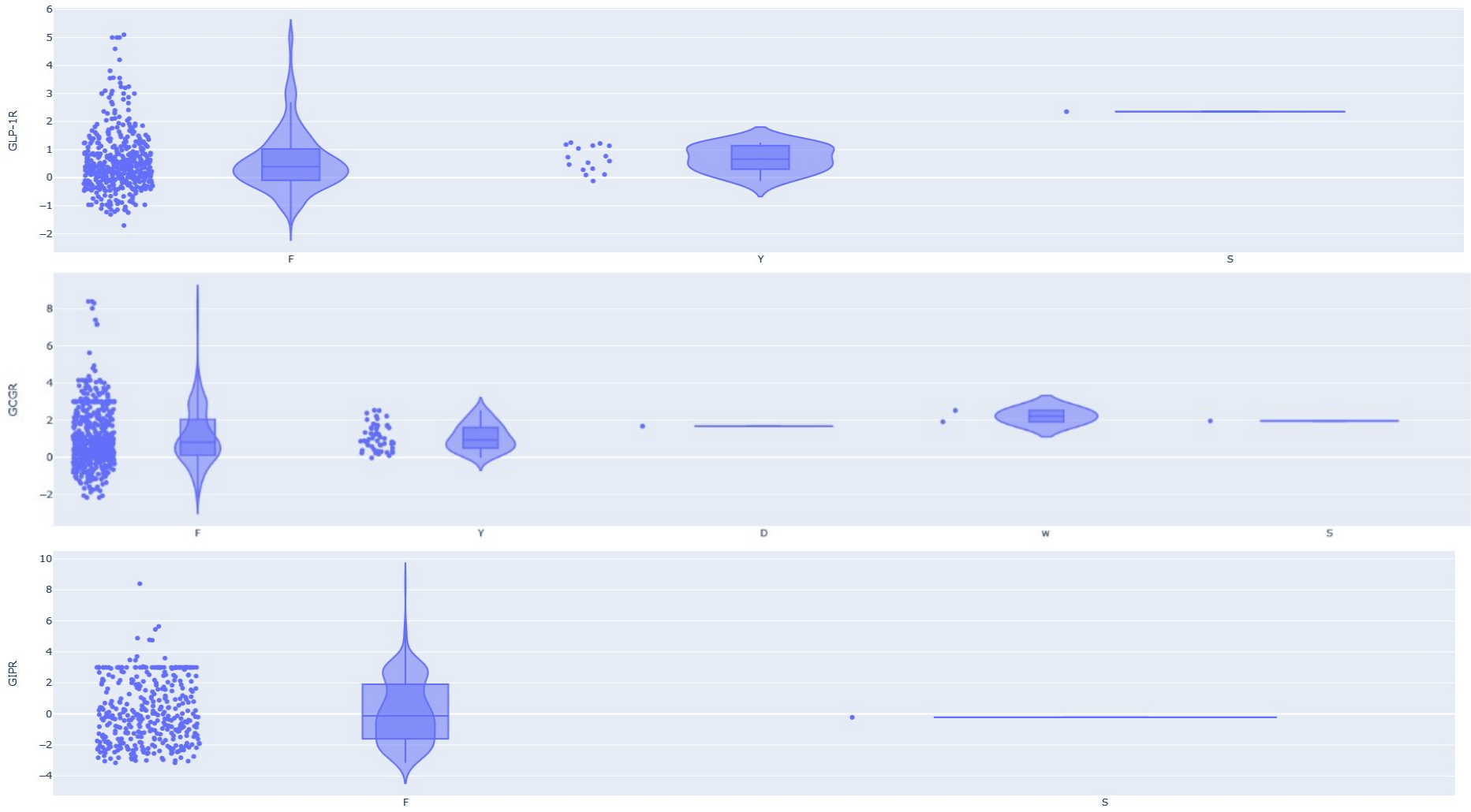
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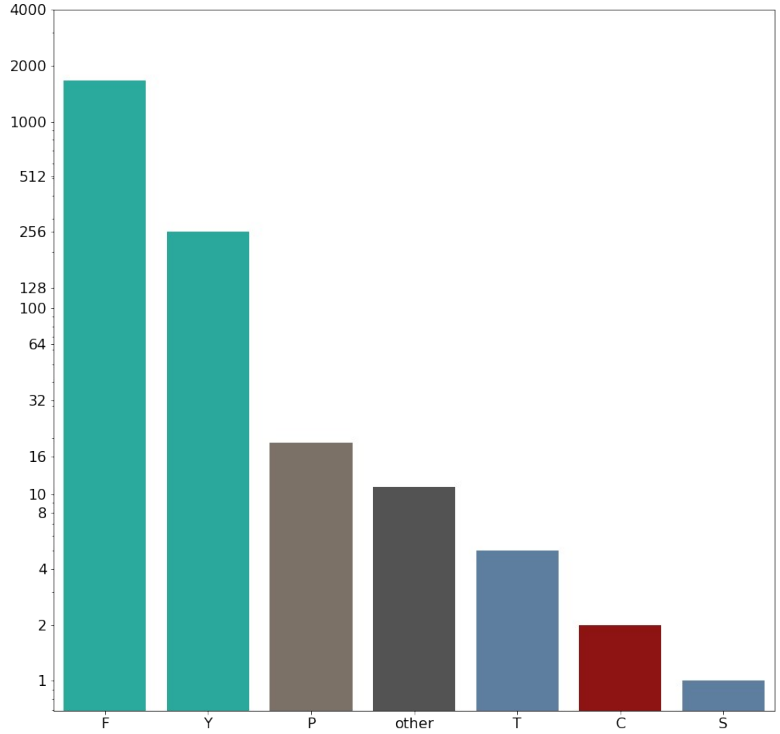
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Position 7


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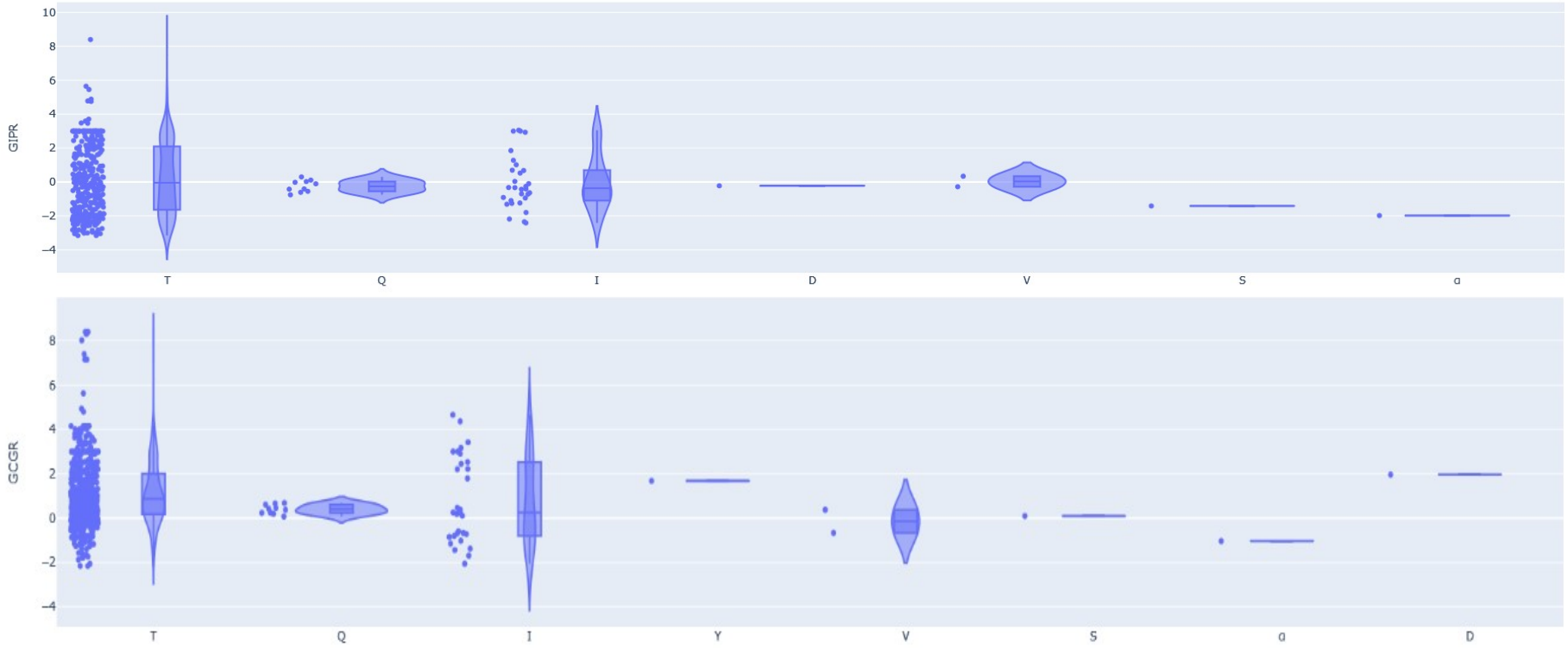
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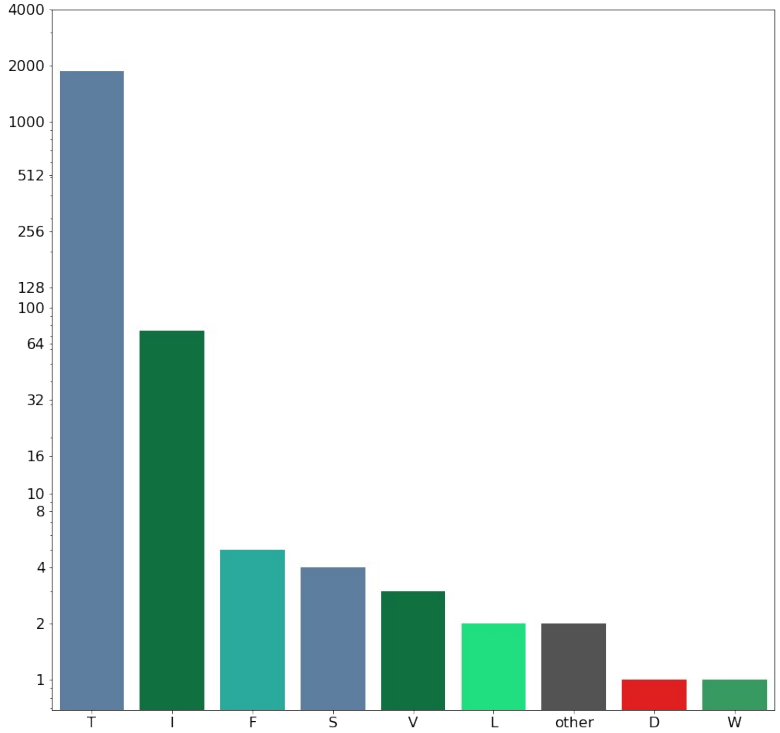
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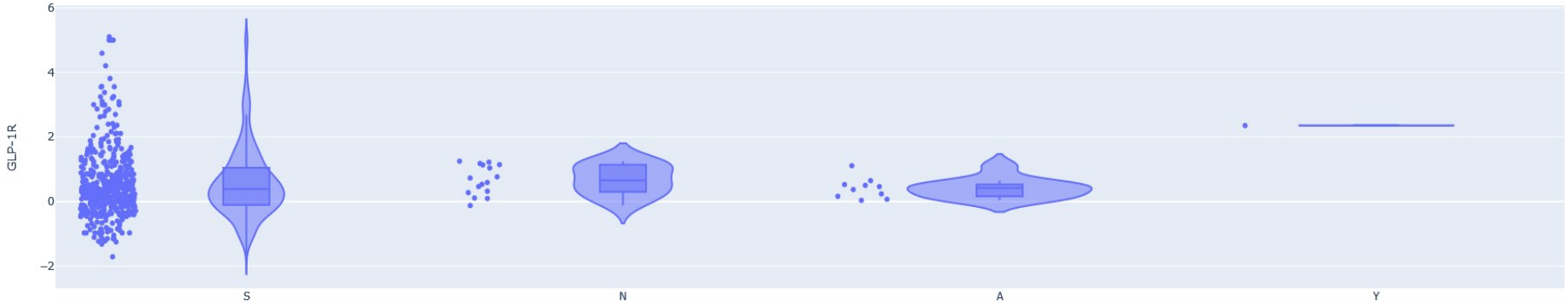
Position 8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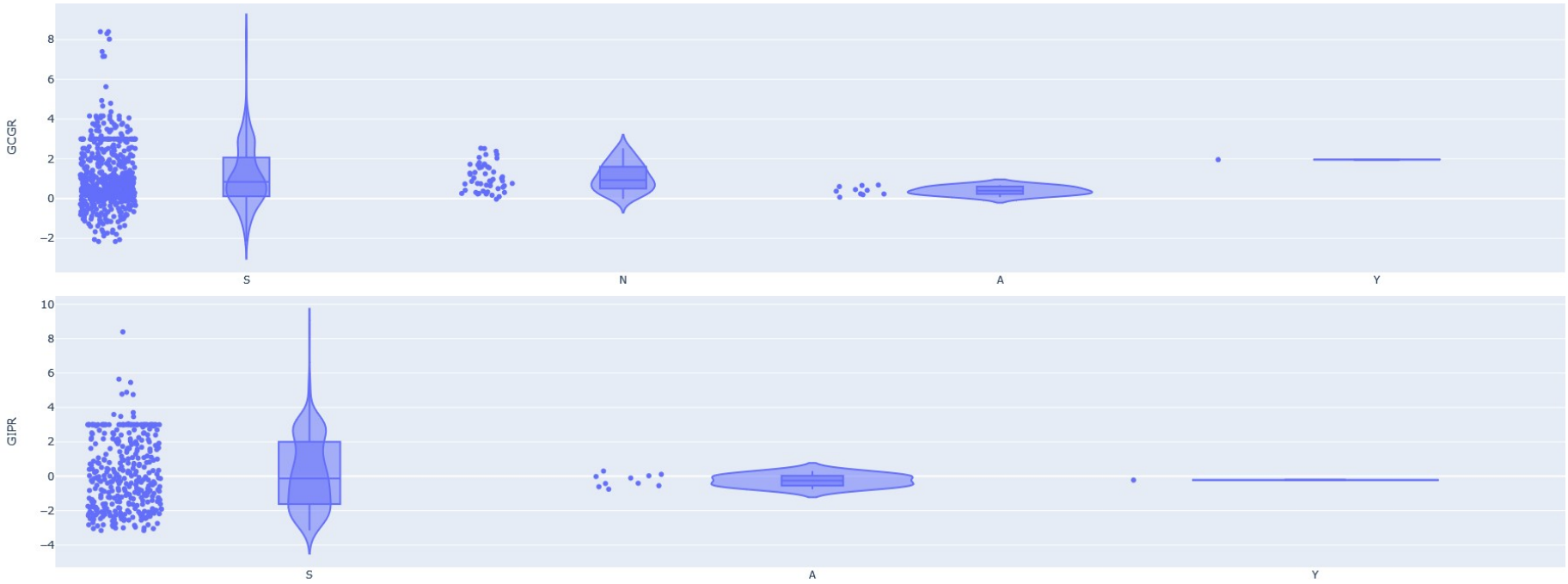
GCGR
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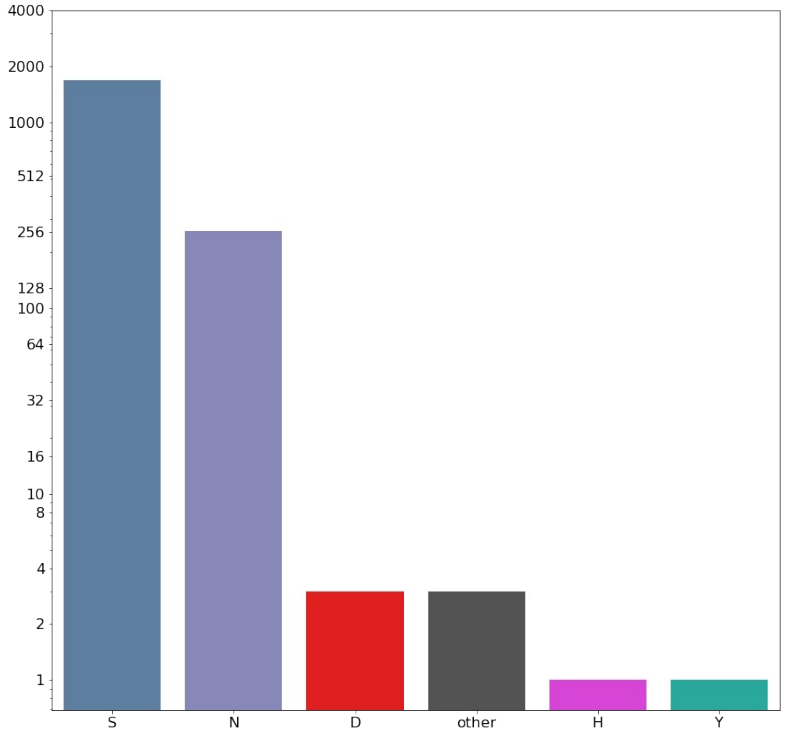
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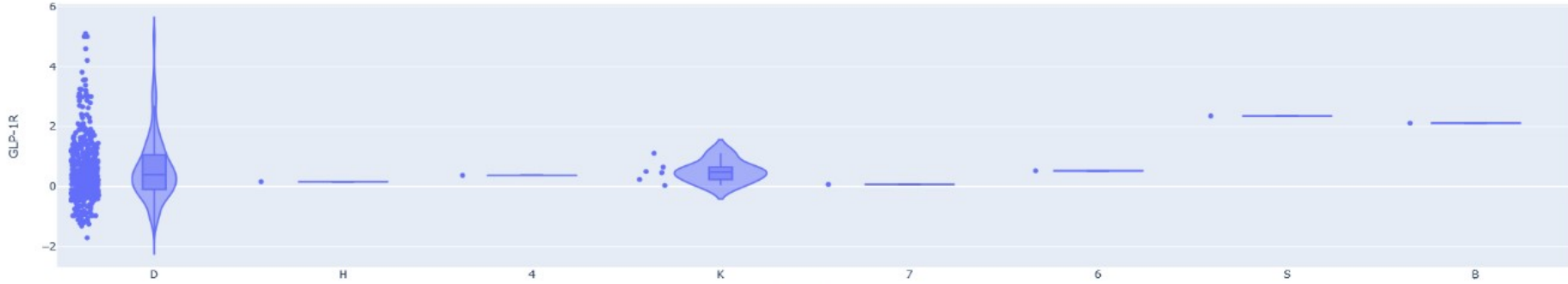
Position 9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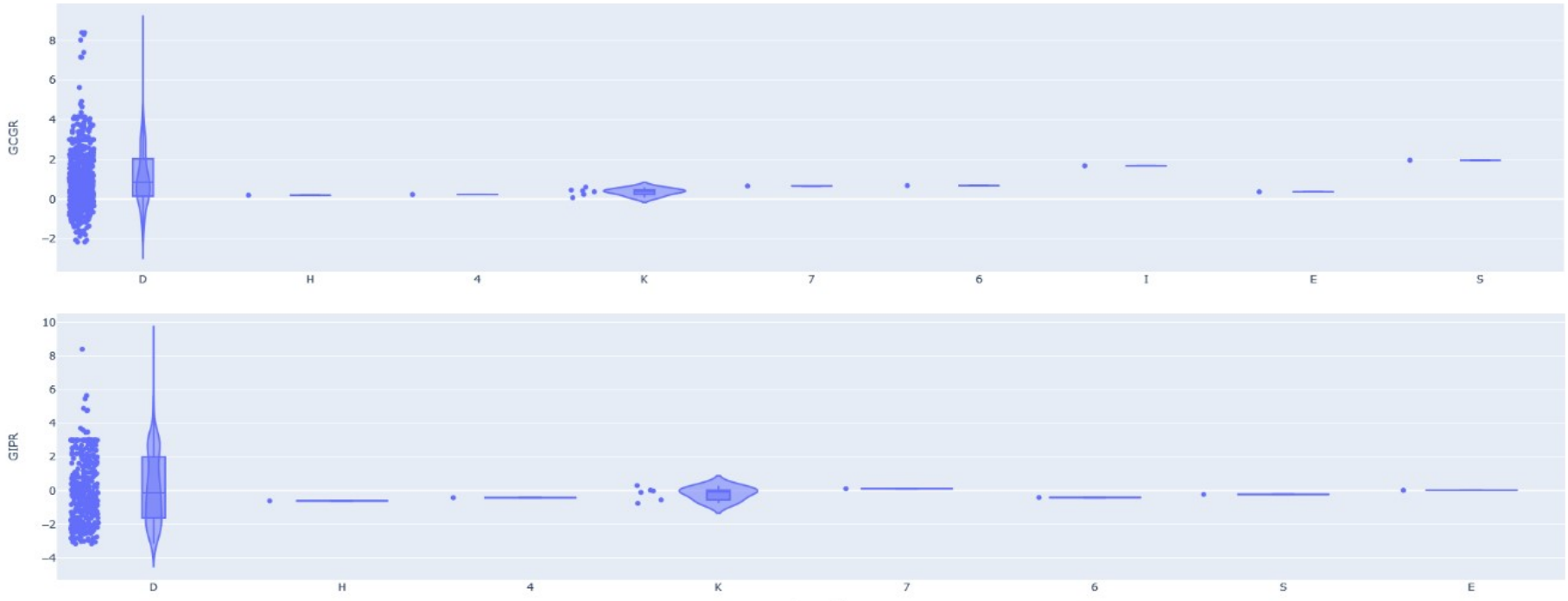
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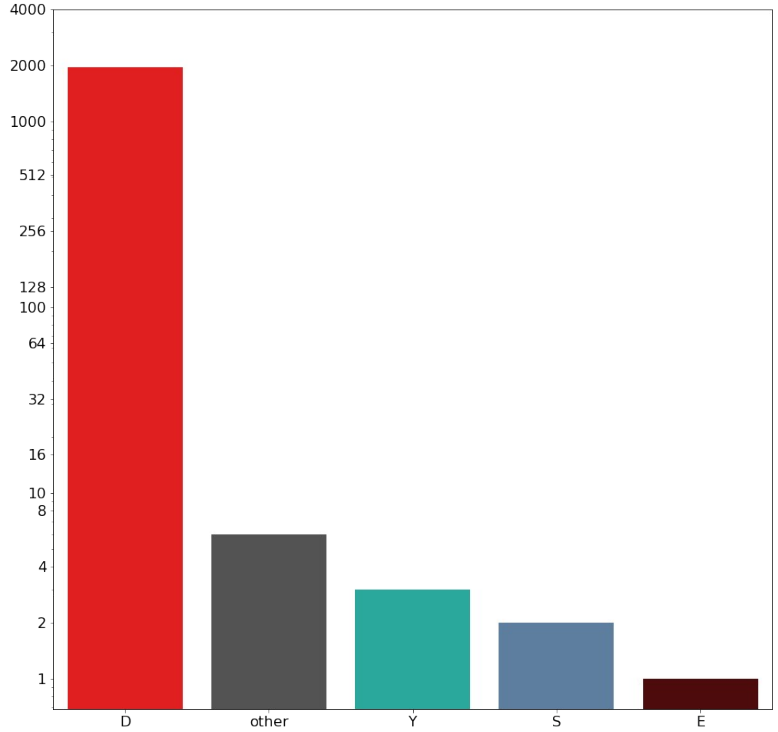
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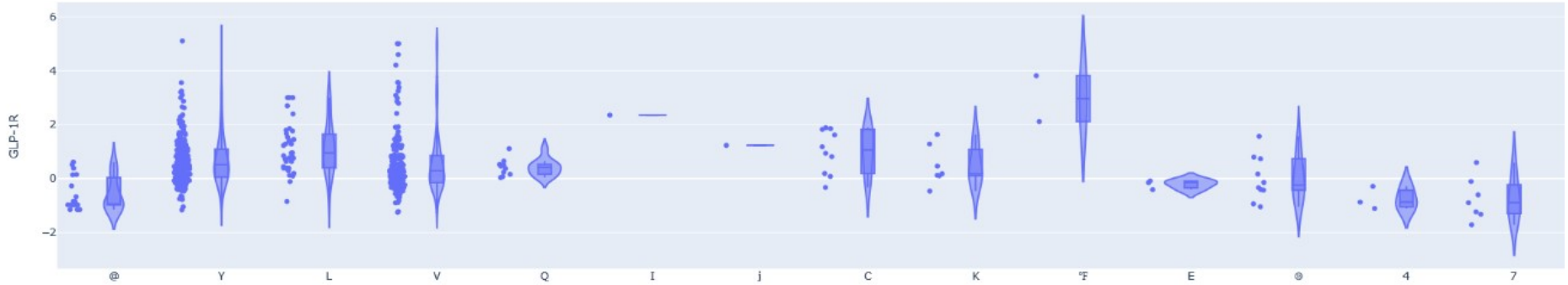
Position 10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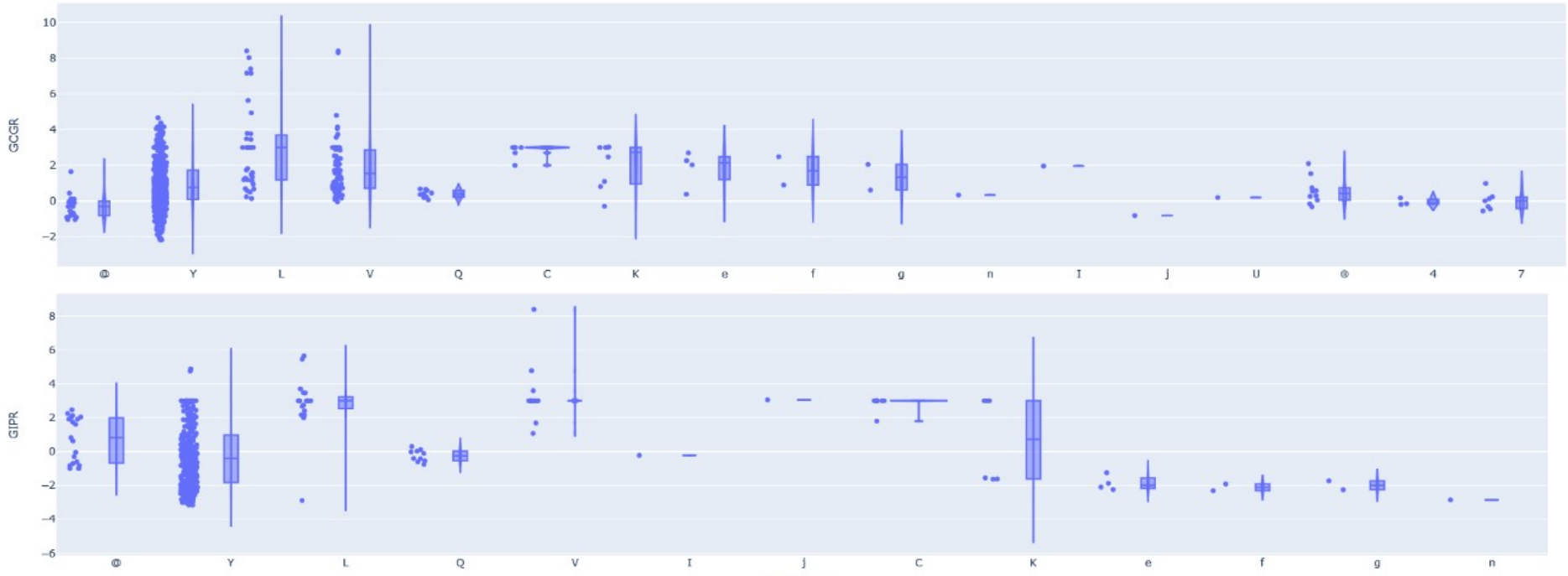
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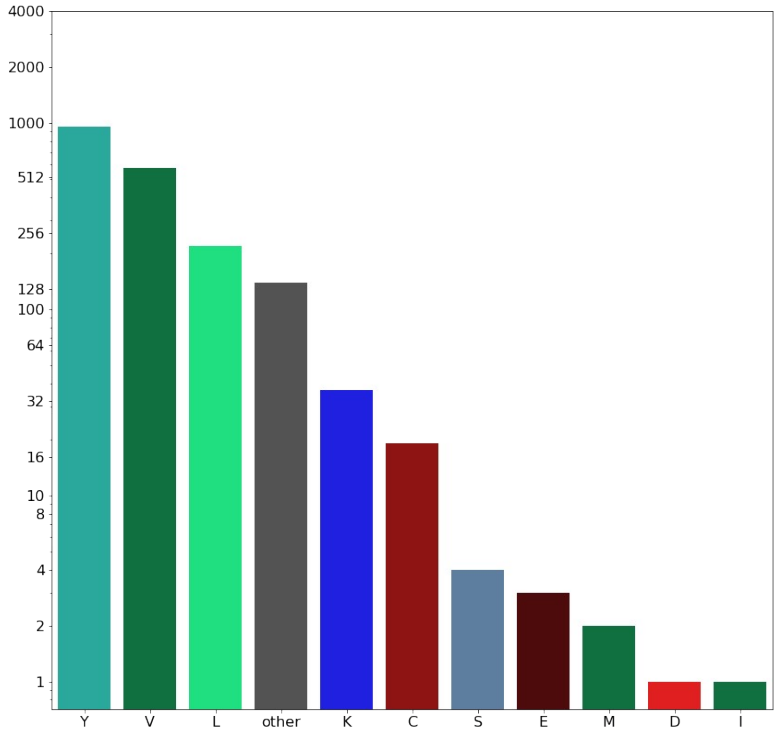
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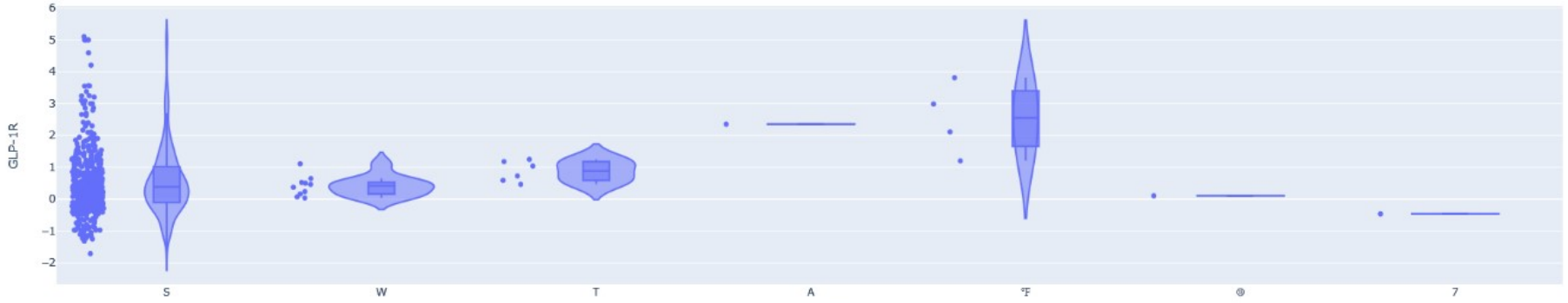
Position 11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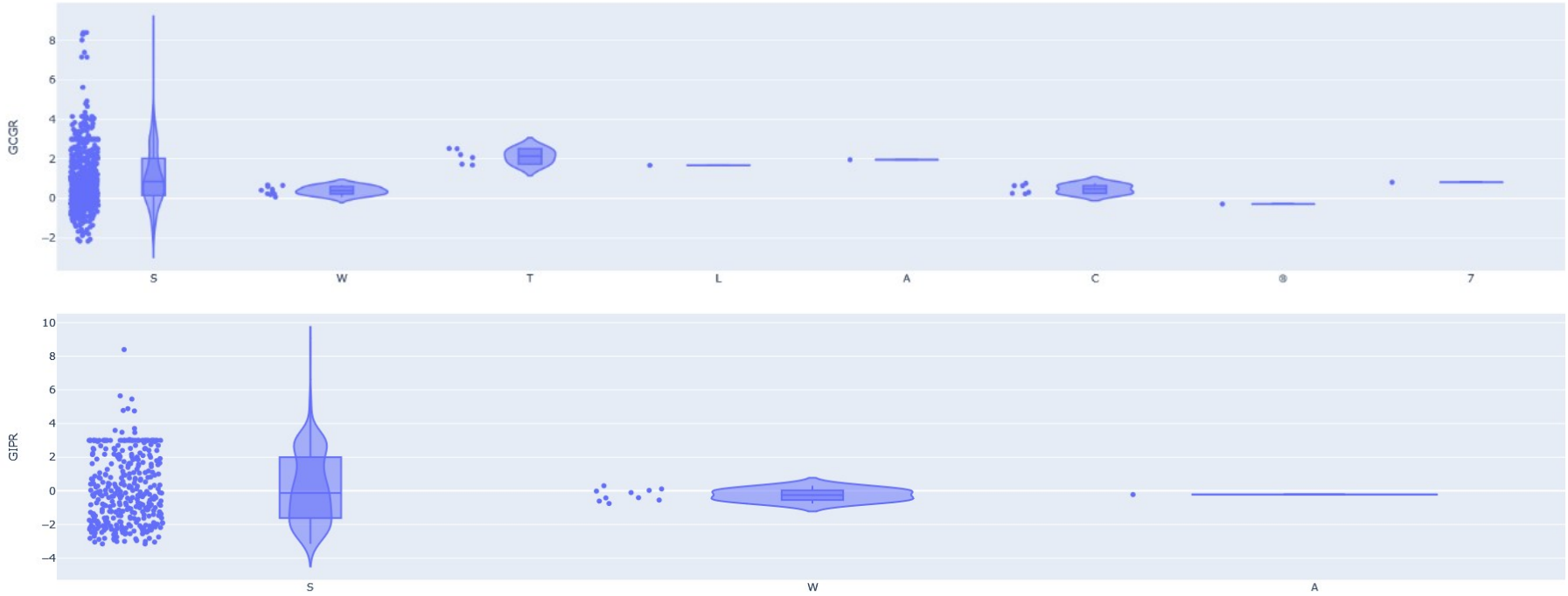
GCGR
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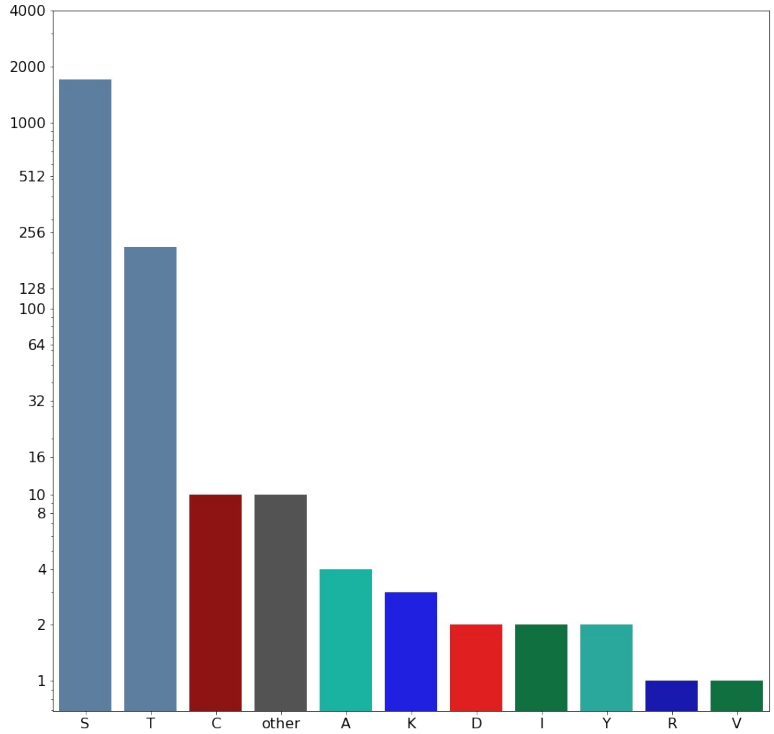
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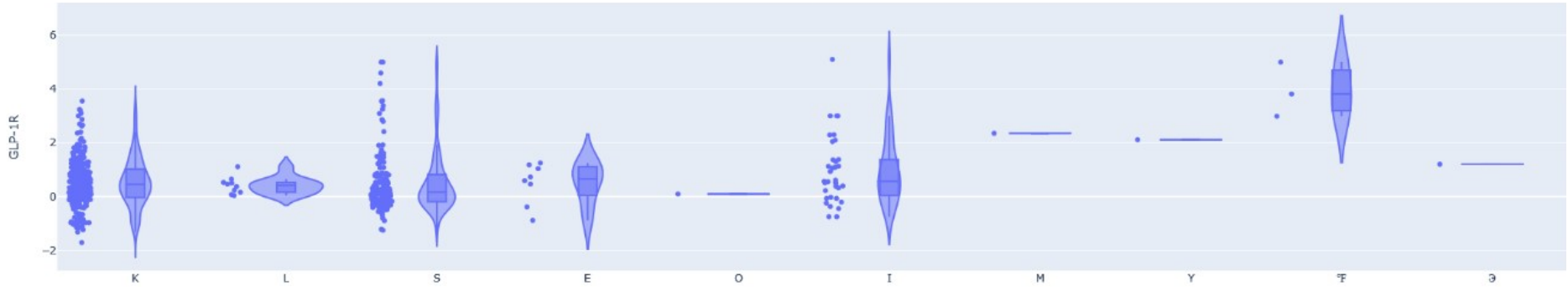
Position 12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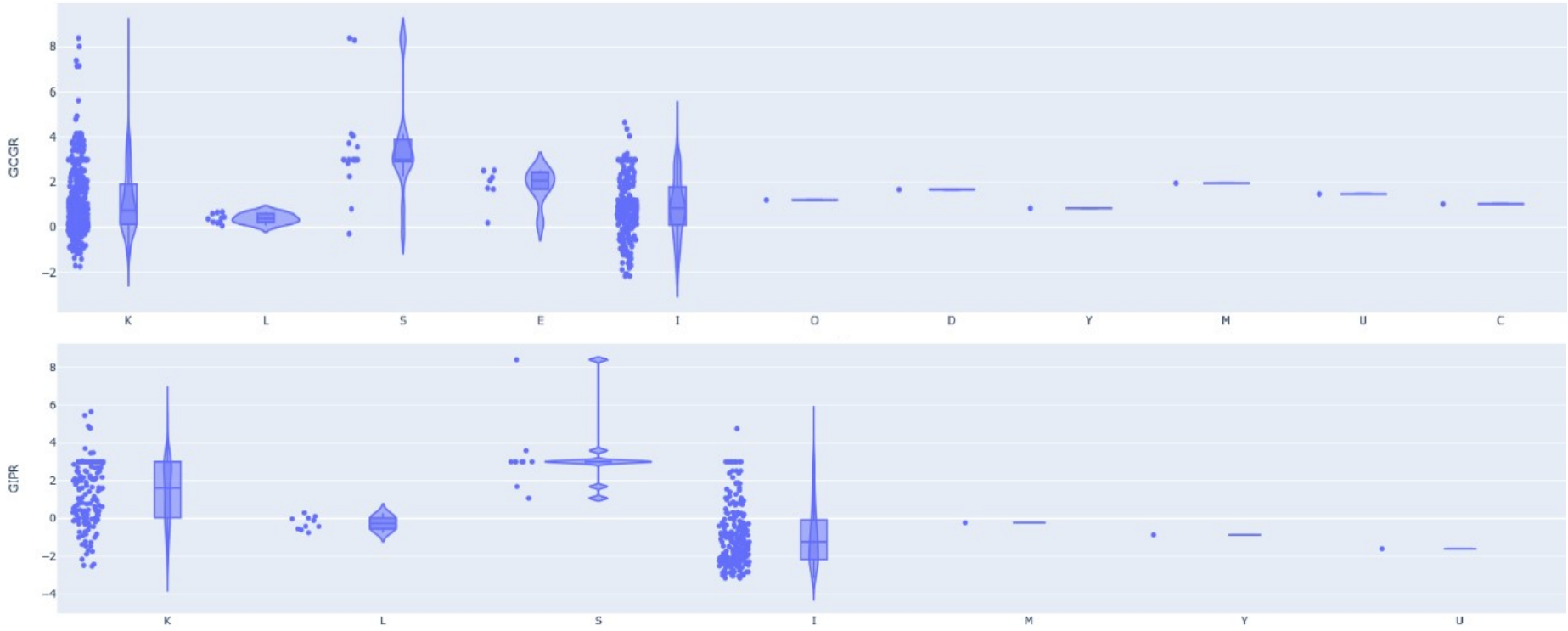
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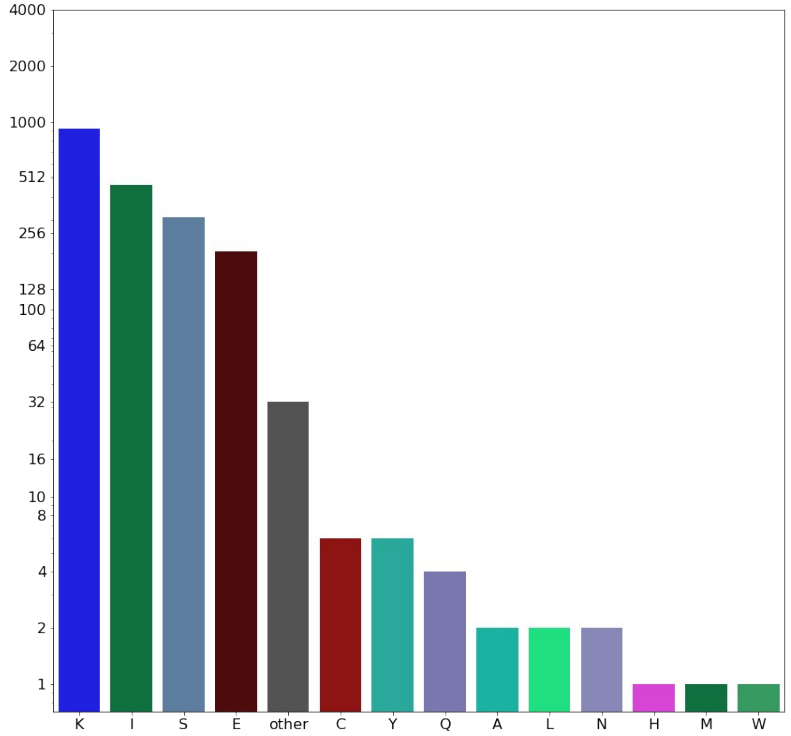
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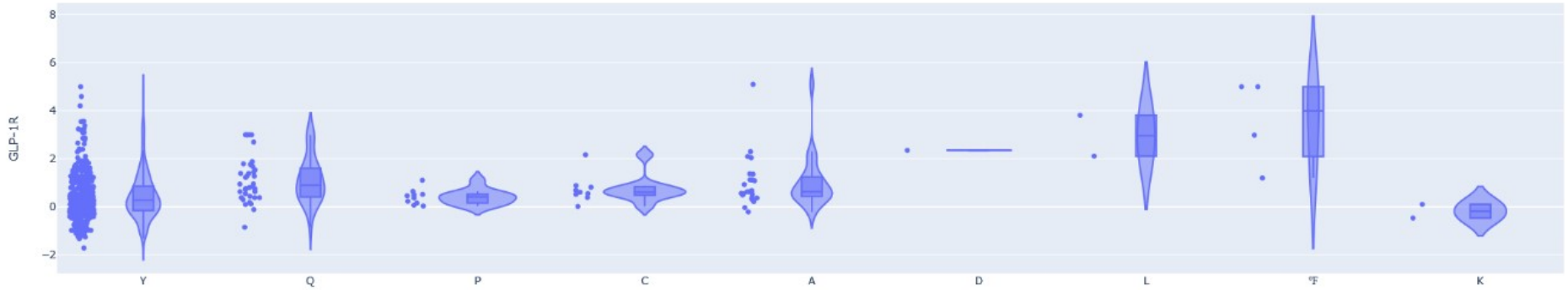
Position 13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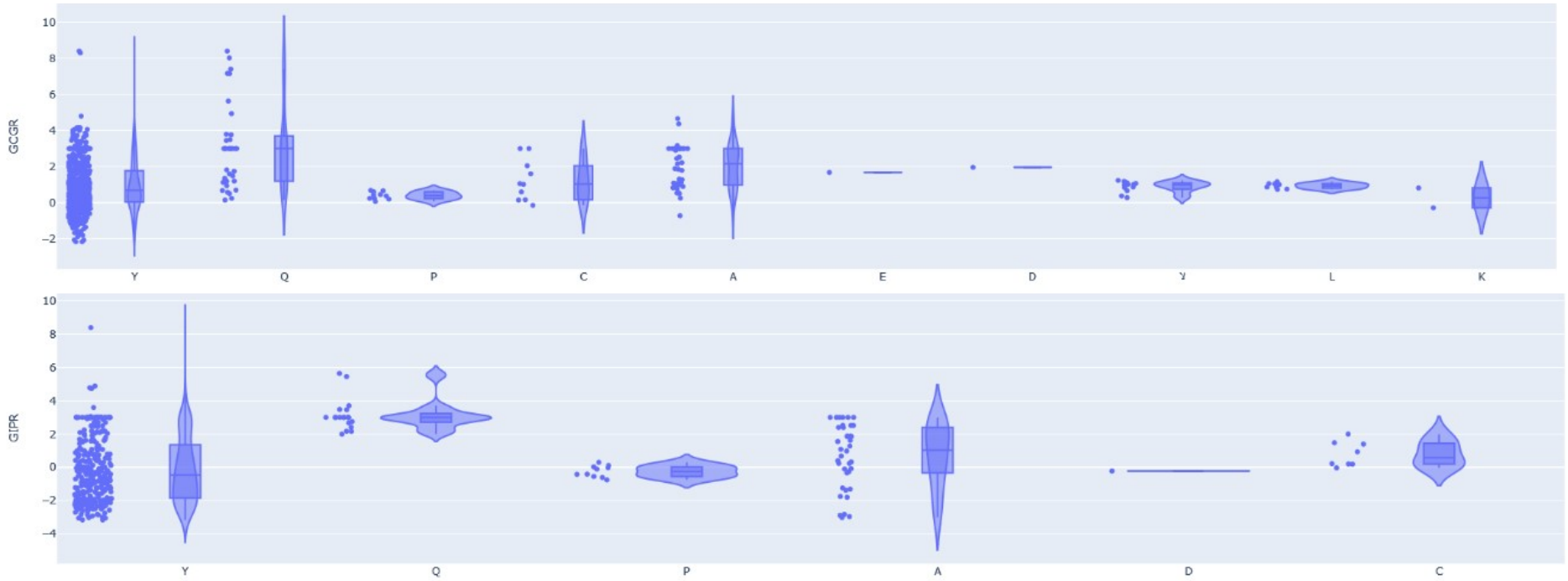
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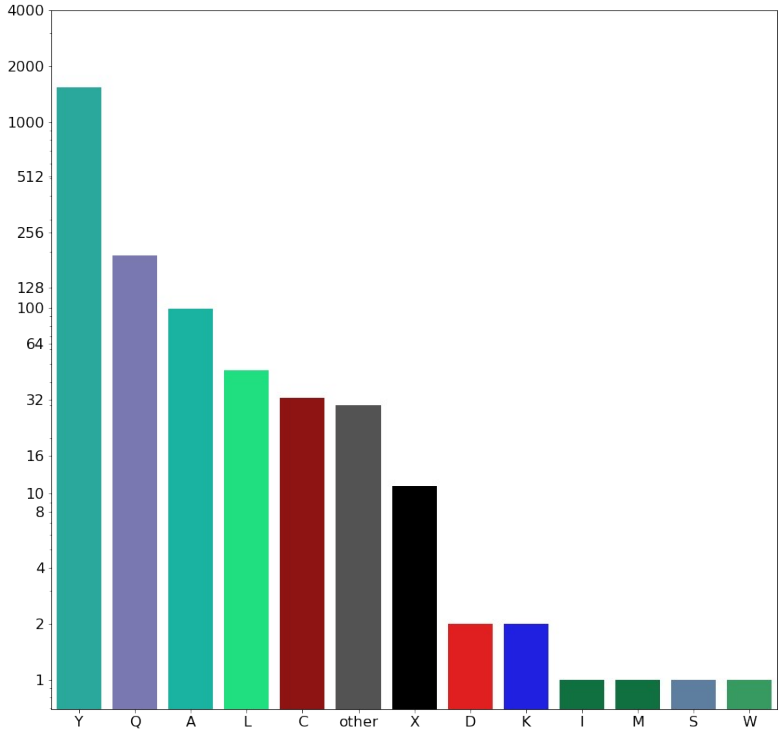
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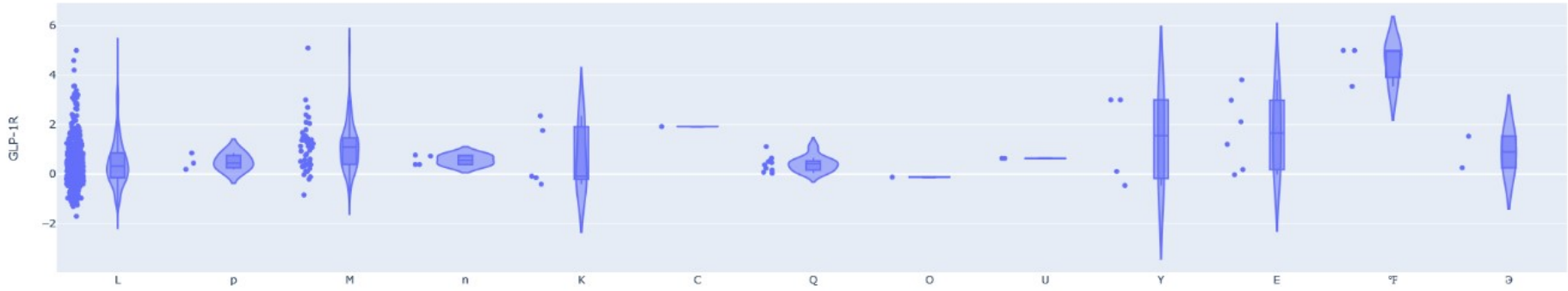
Position 14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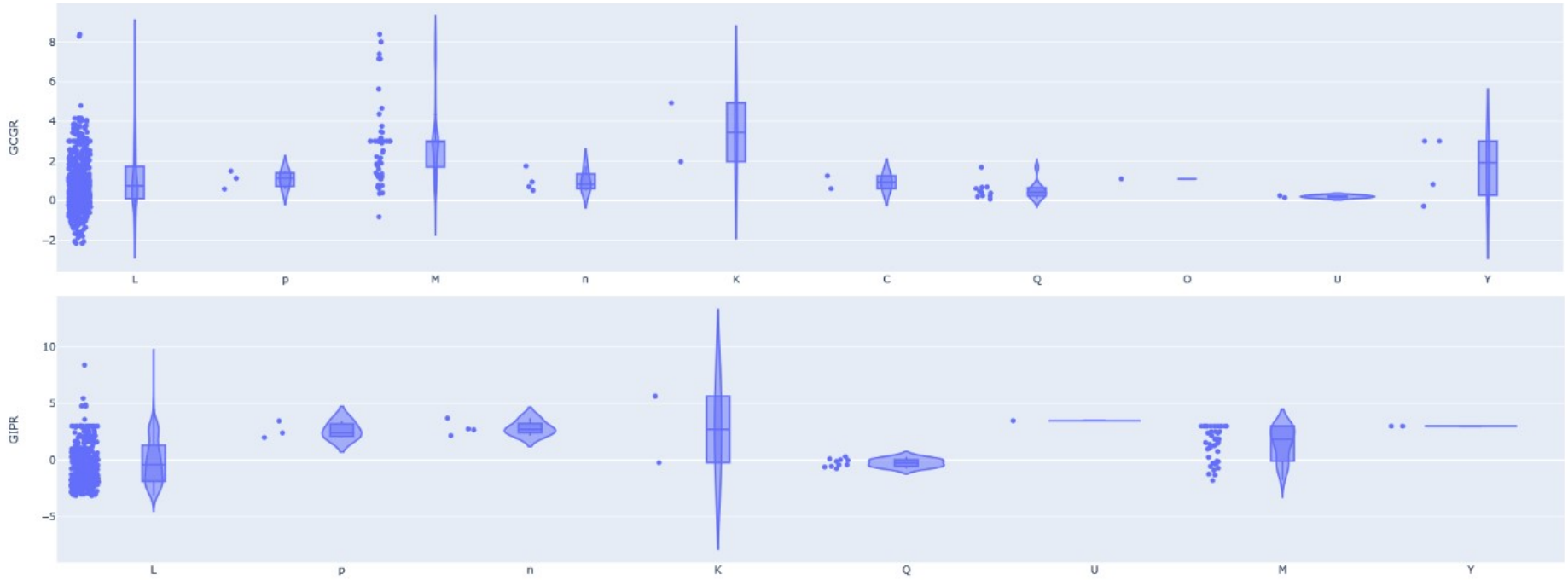
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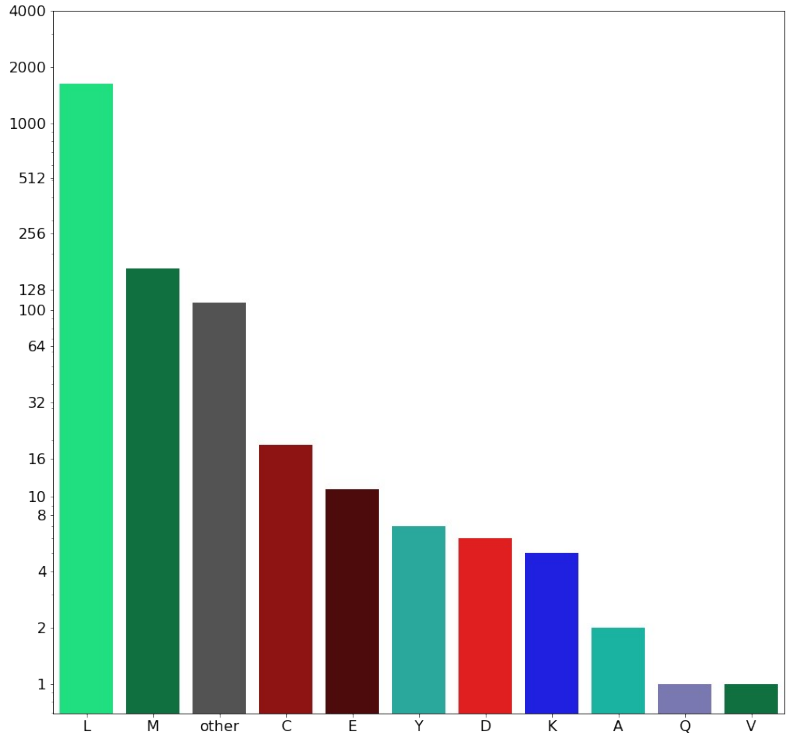
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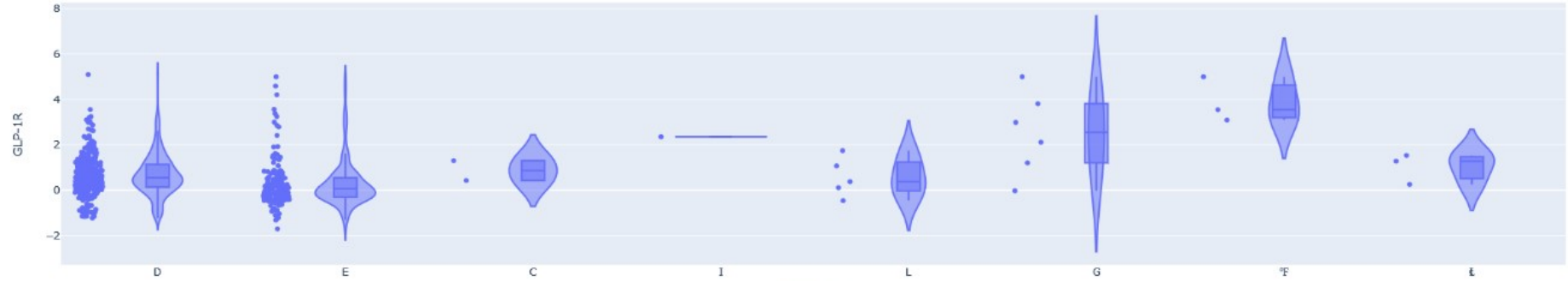
Position 15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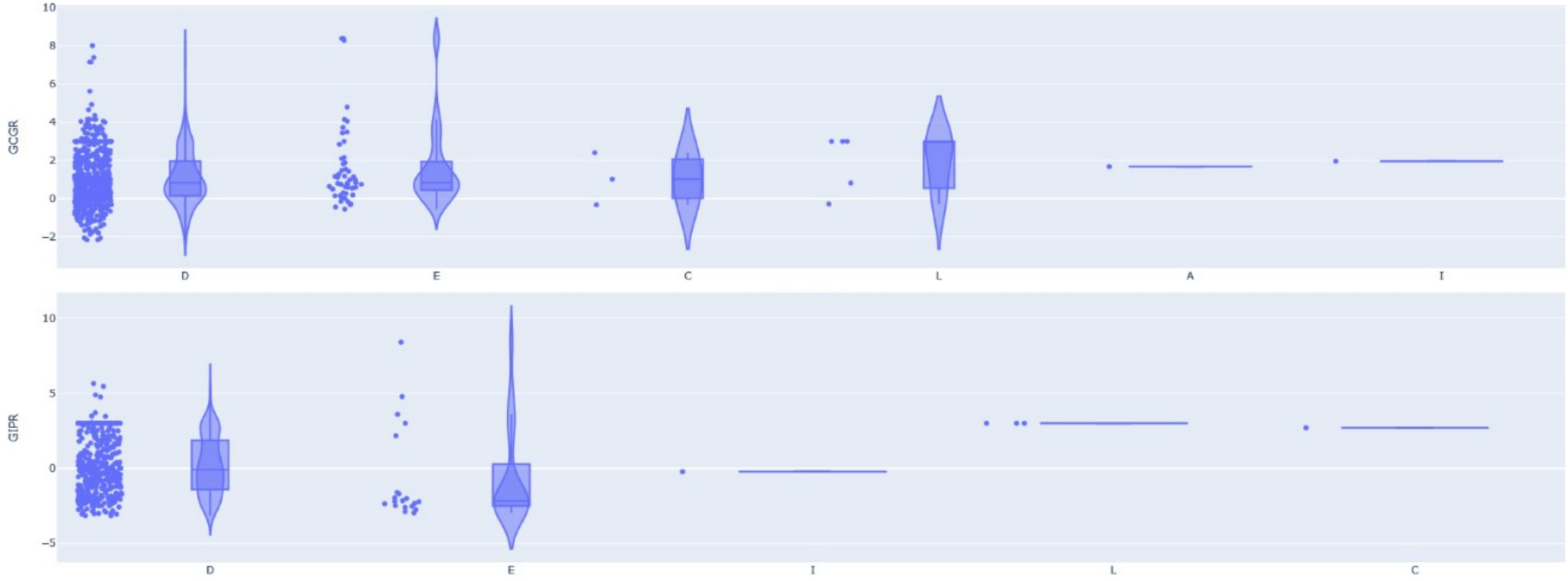
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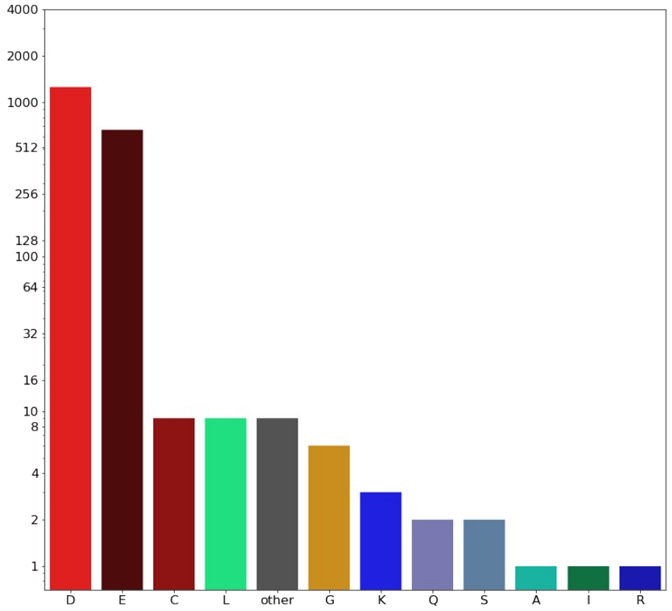
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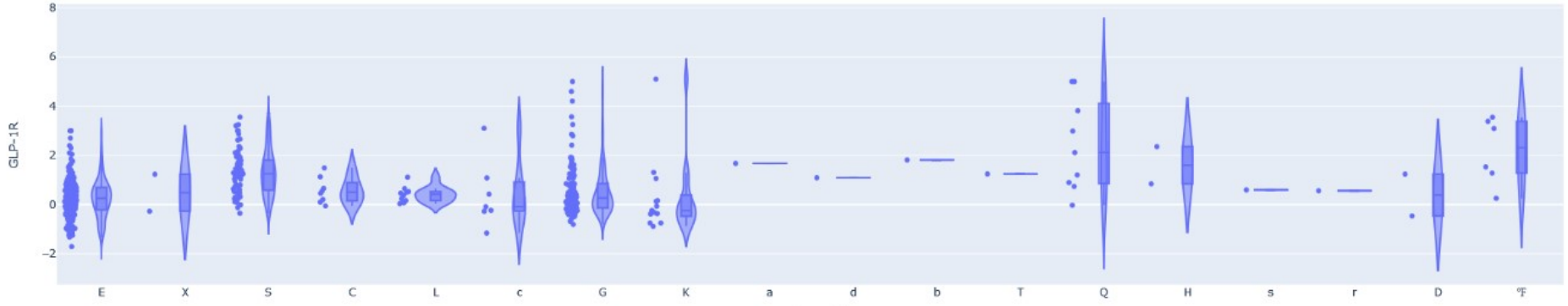
Position 16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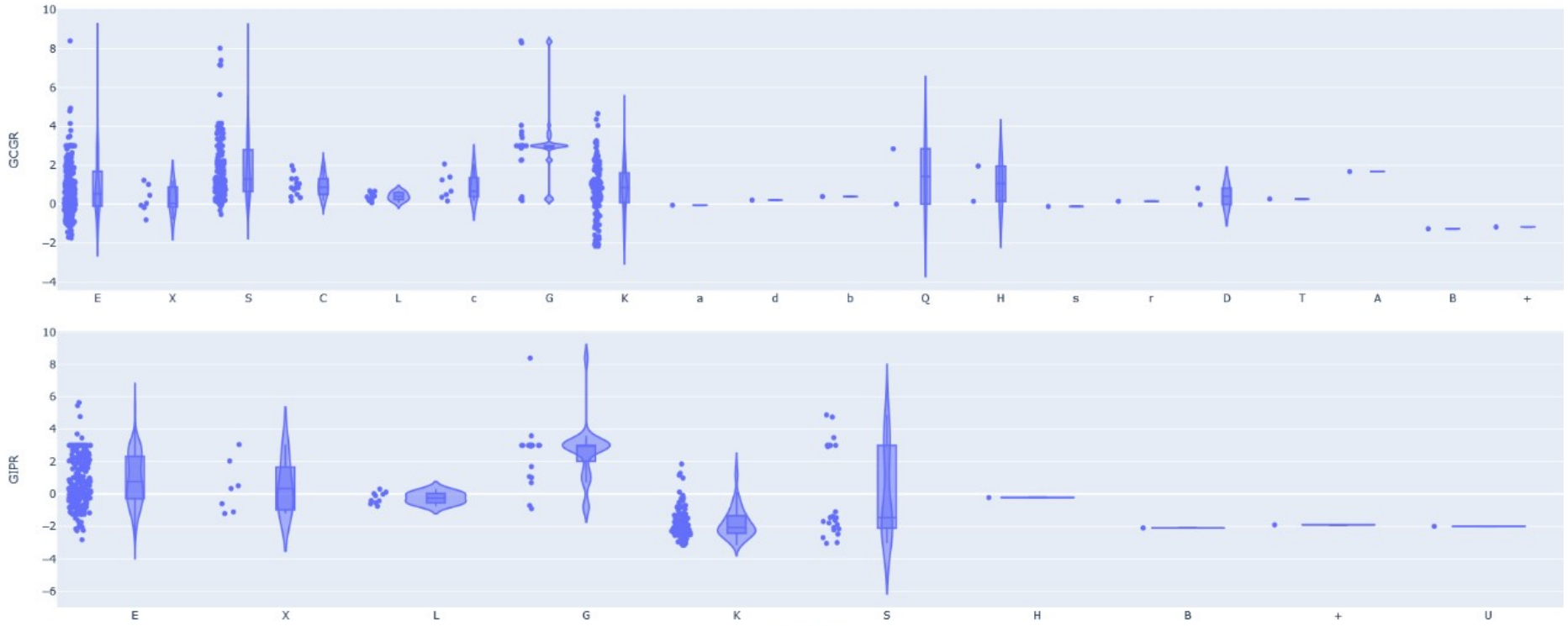
GCGR
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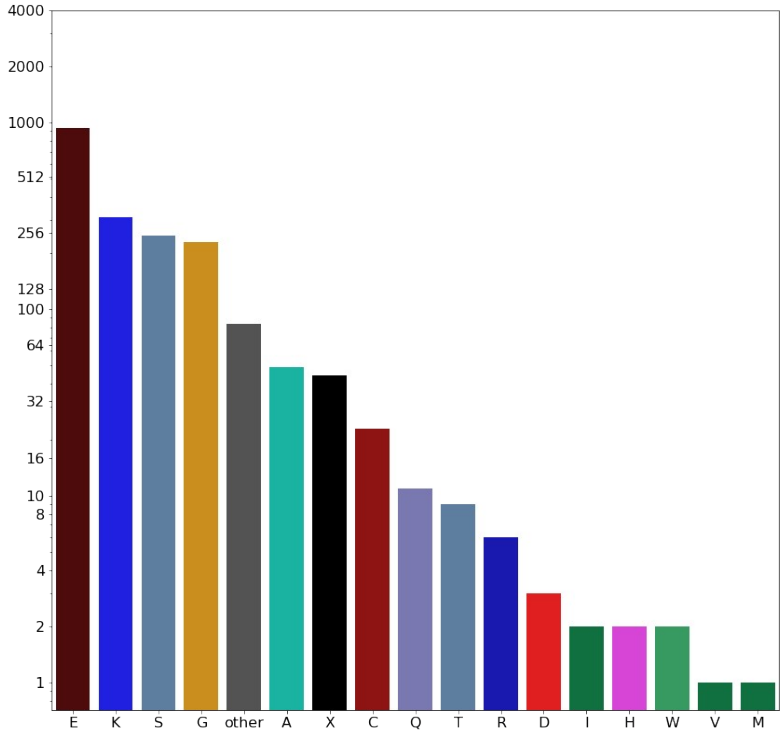
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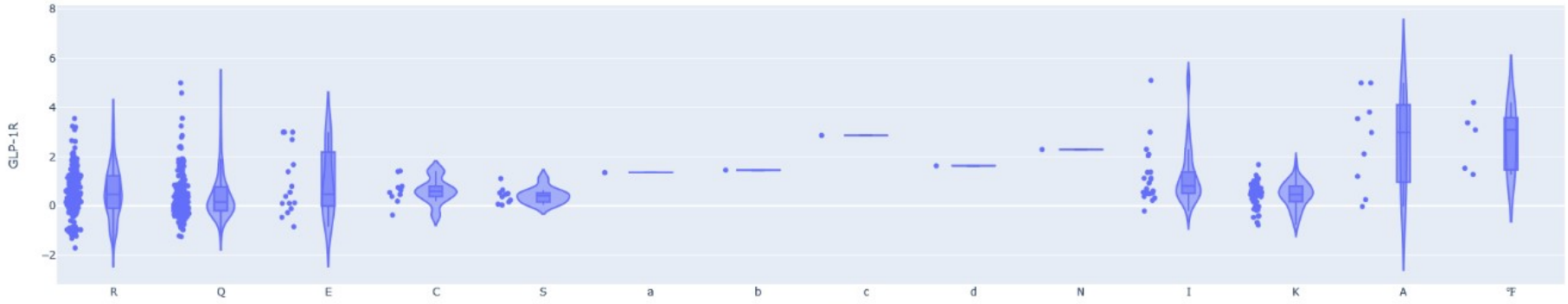
Position 17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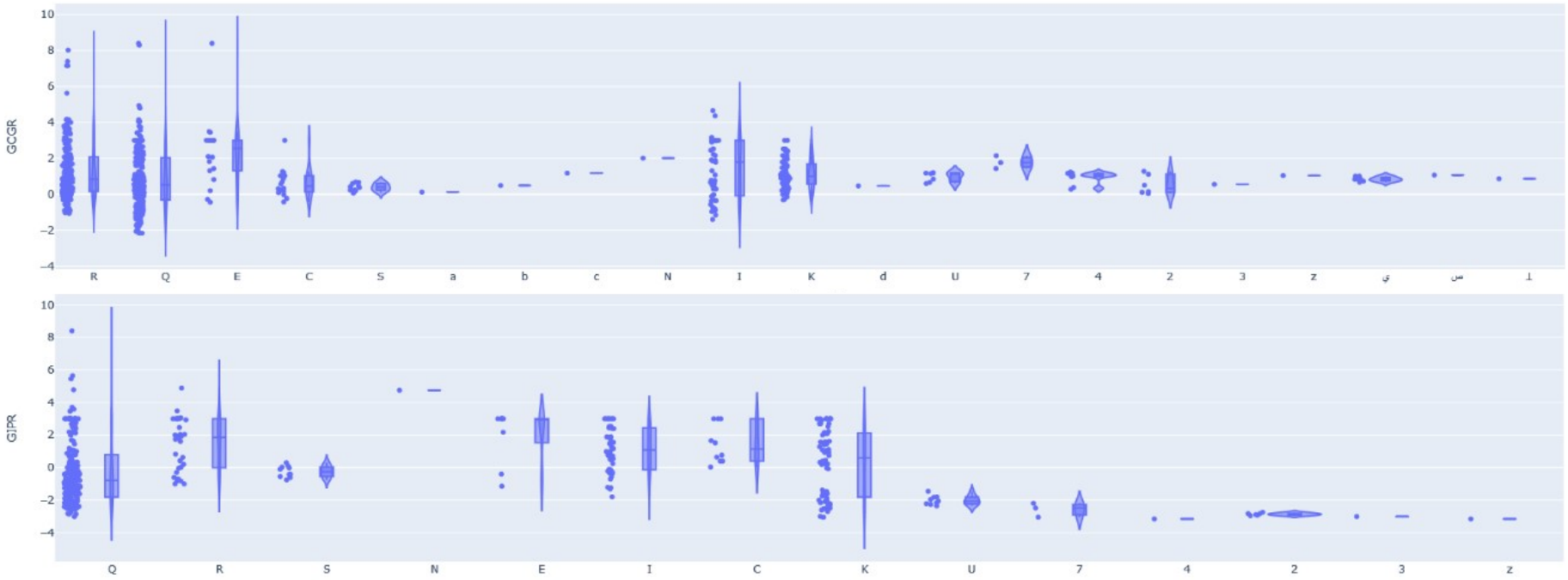
GCGR
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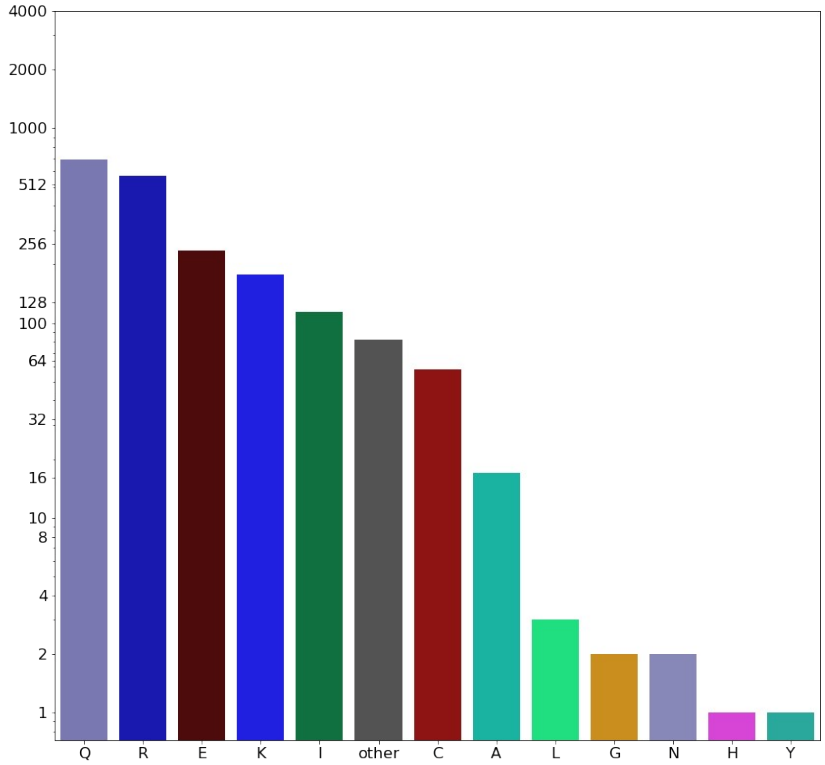
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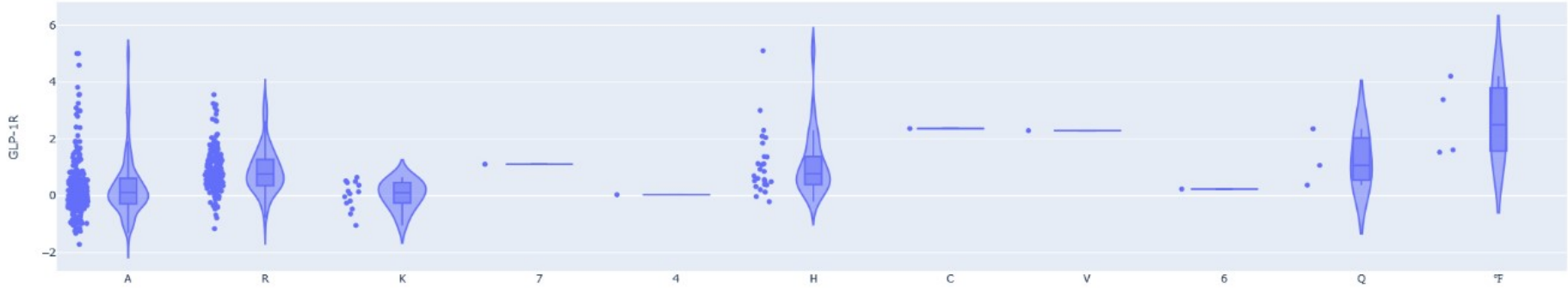
Position 18


GLP-1R
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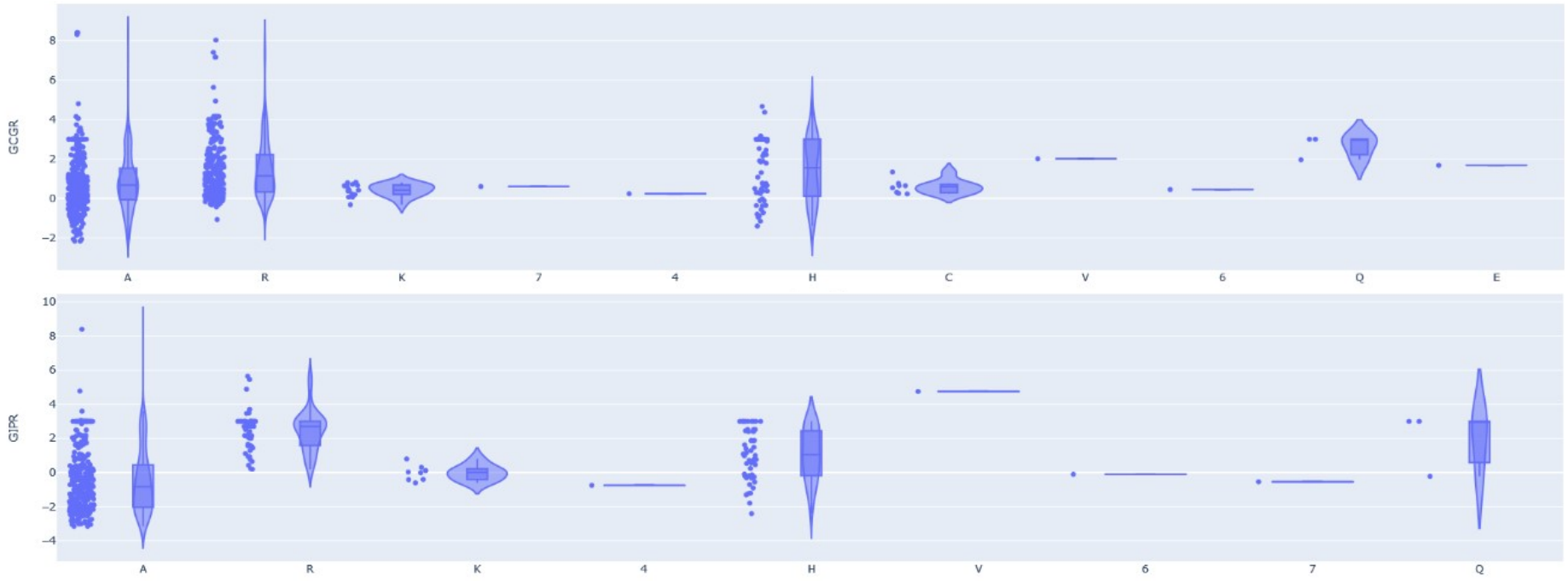
GCGR
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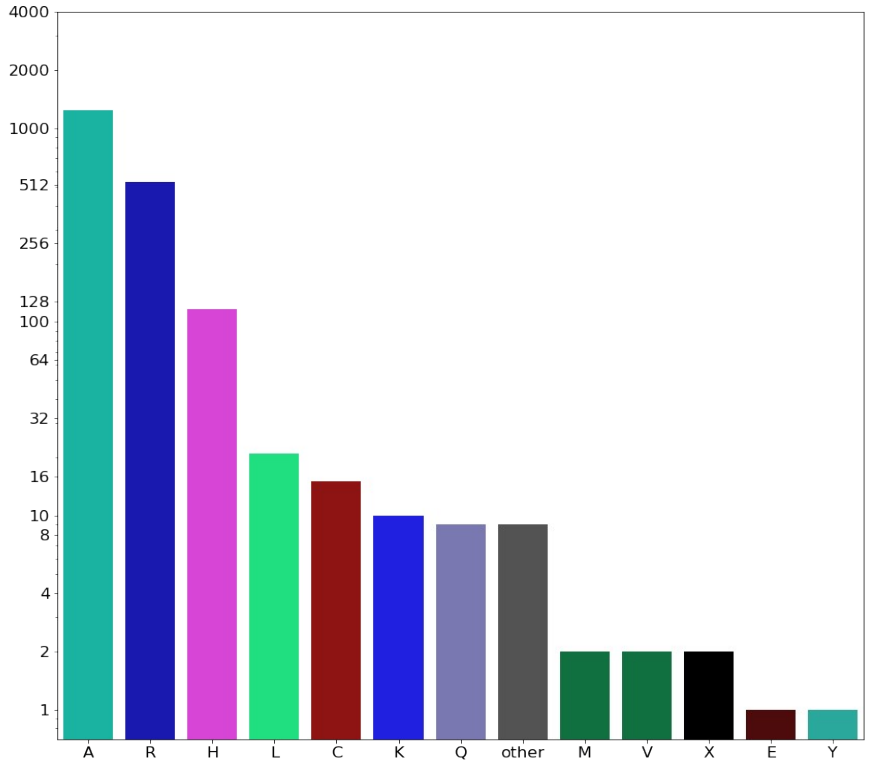
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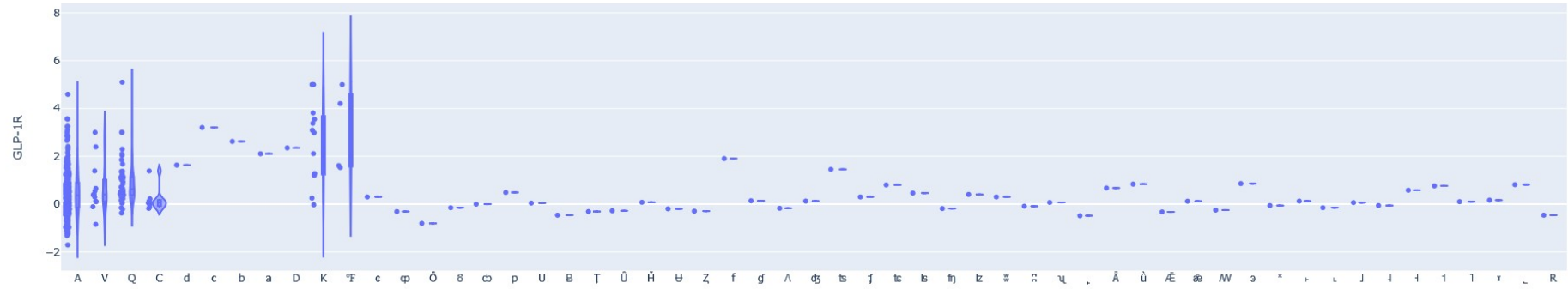
Position 19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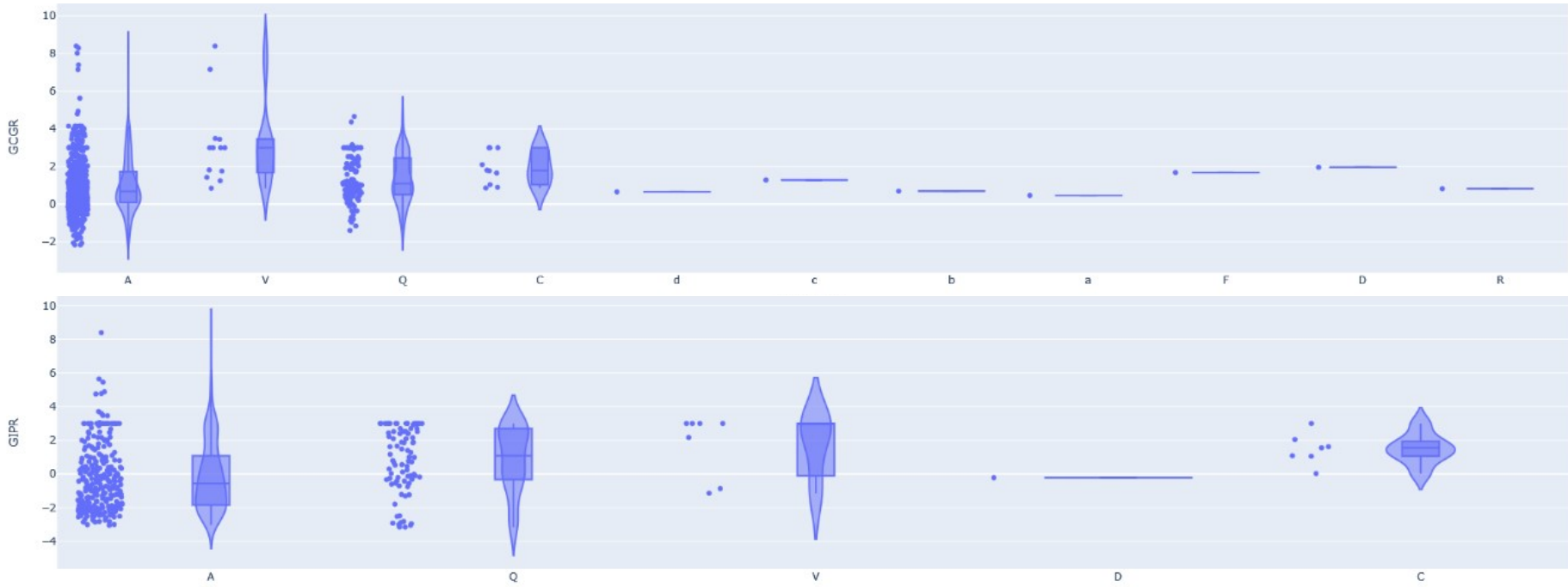
GCGR
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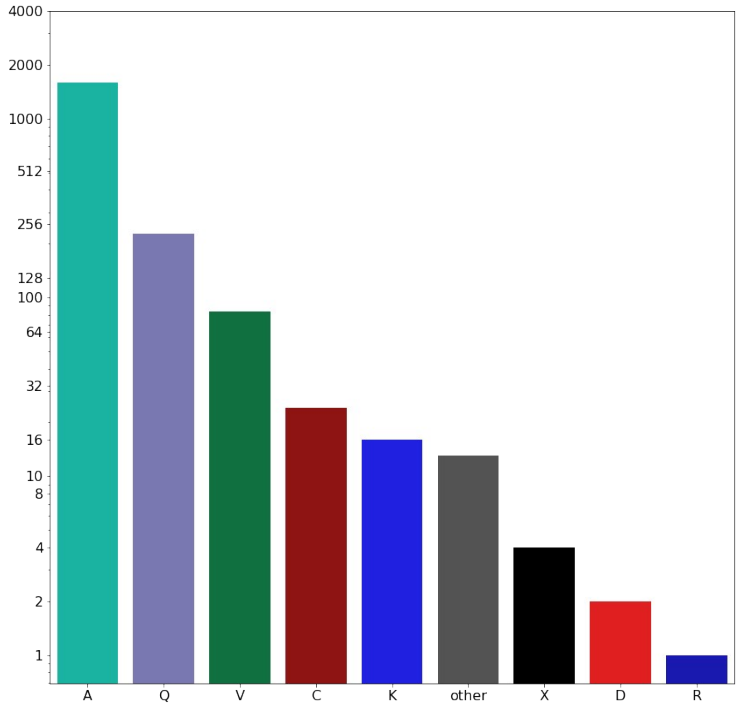
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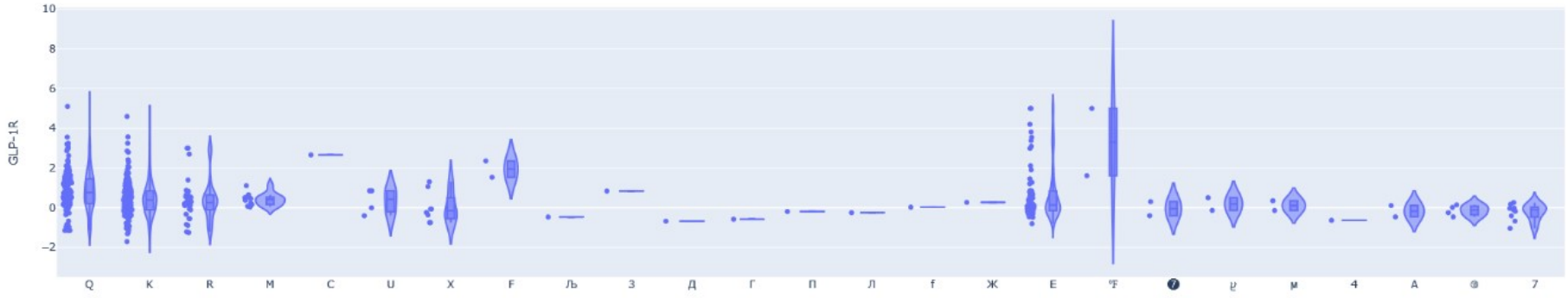
Position 20


GLP-1R
10
8
6
4
2
0
−2
Q
K
R
M
C
U
X
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Лъ
З
Д
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П
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f
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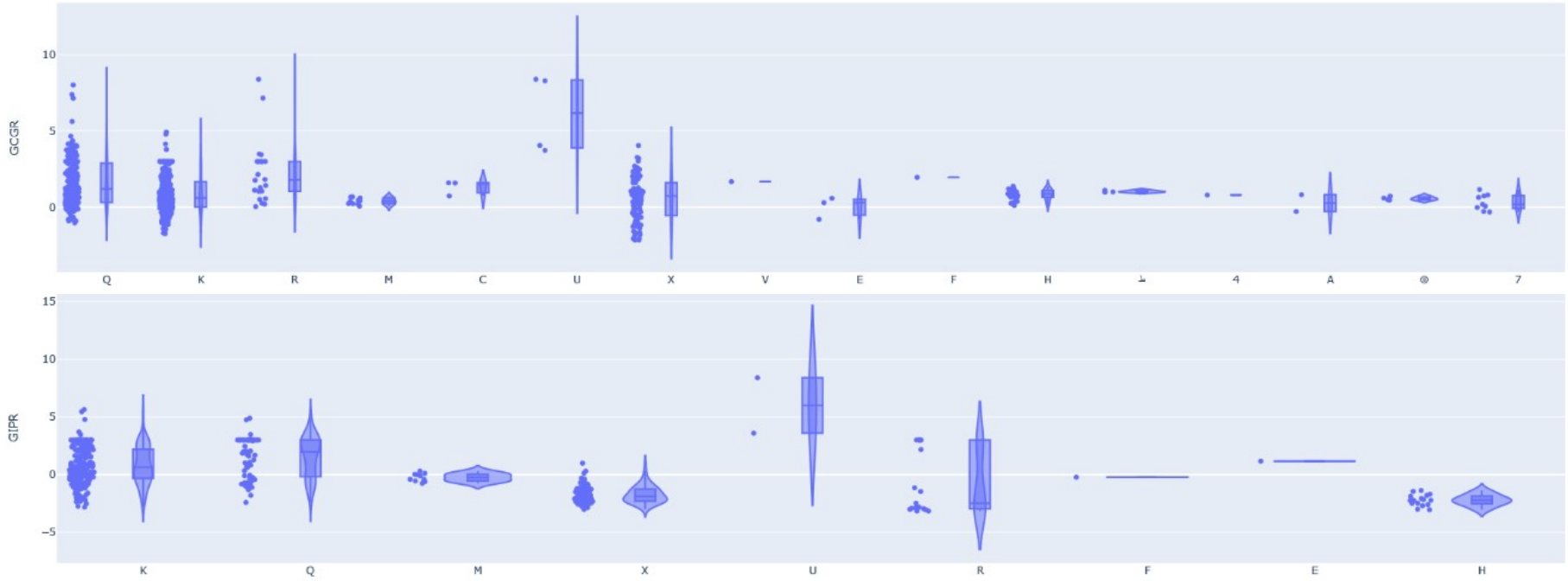
E
A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R
M
C
U
X
V
E
F
H
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GIPR
15
10
5
0
−5
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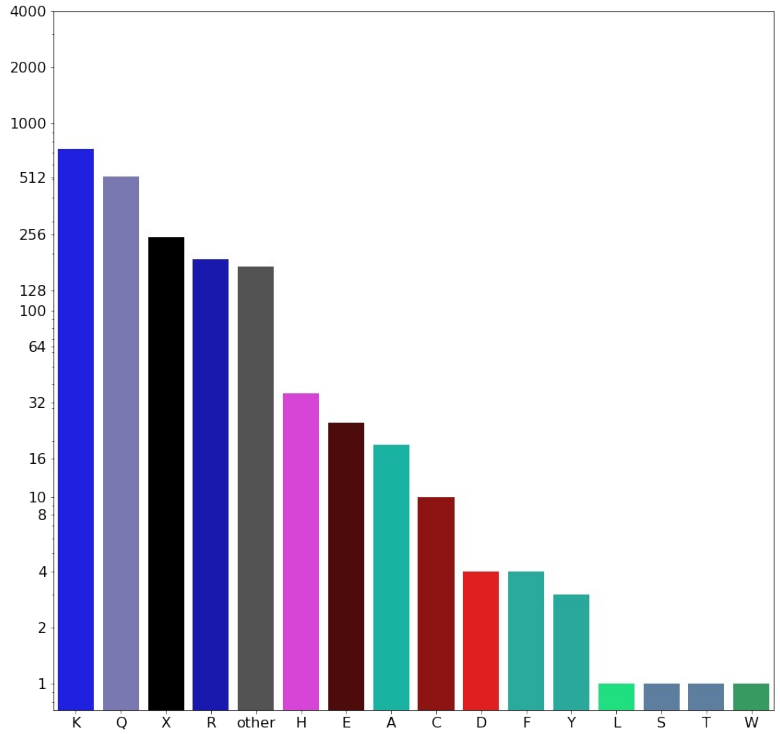
Q
M
X
U
R
F
E
H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32
16
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Q
X
R
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H
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A
C
D
F
Y
L
S
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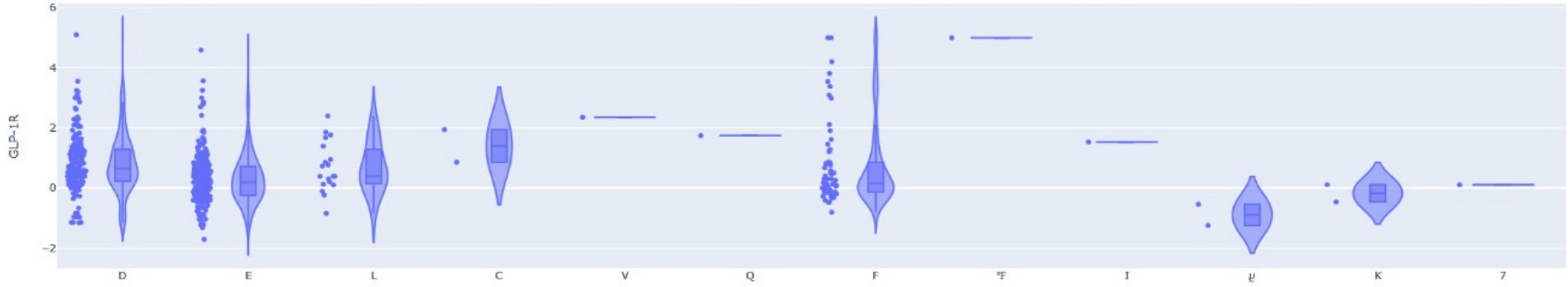
W

Position 21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GLP-1R
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L
C
V
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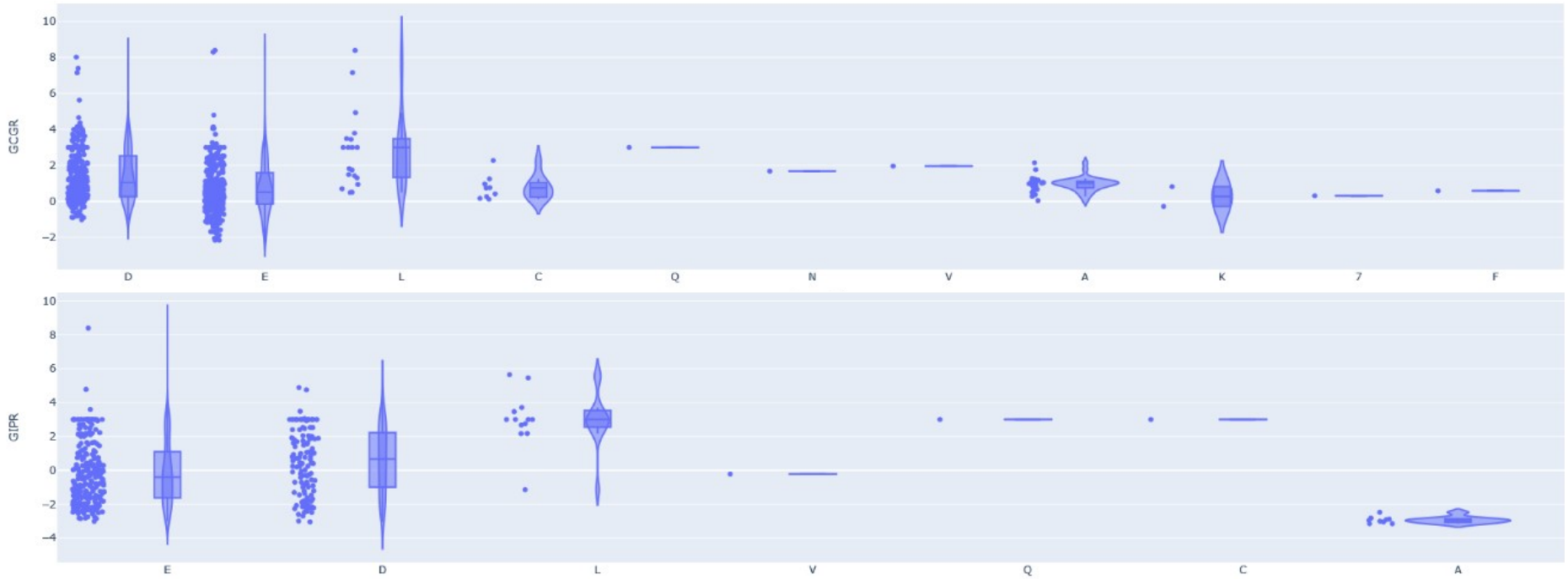
I
K


GCGR
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D
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L
C
Q
N
V
A
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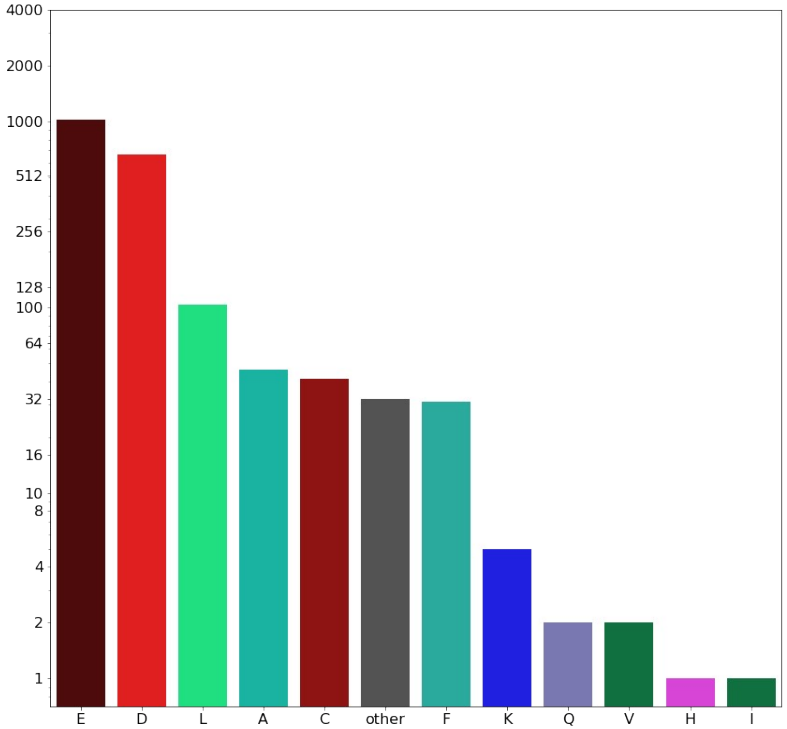
GIPR
10
8
6
4
2
0
−2
−4
E
D
L
V
Q
C
A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D
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A
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other
F
K
Q
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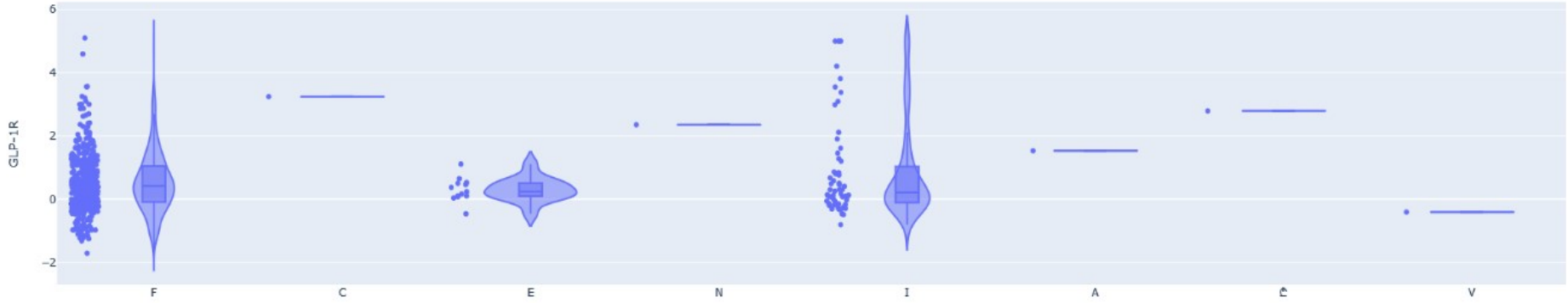
H
I

Position 22


GLP-1R
6
4
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−2
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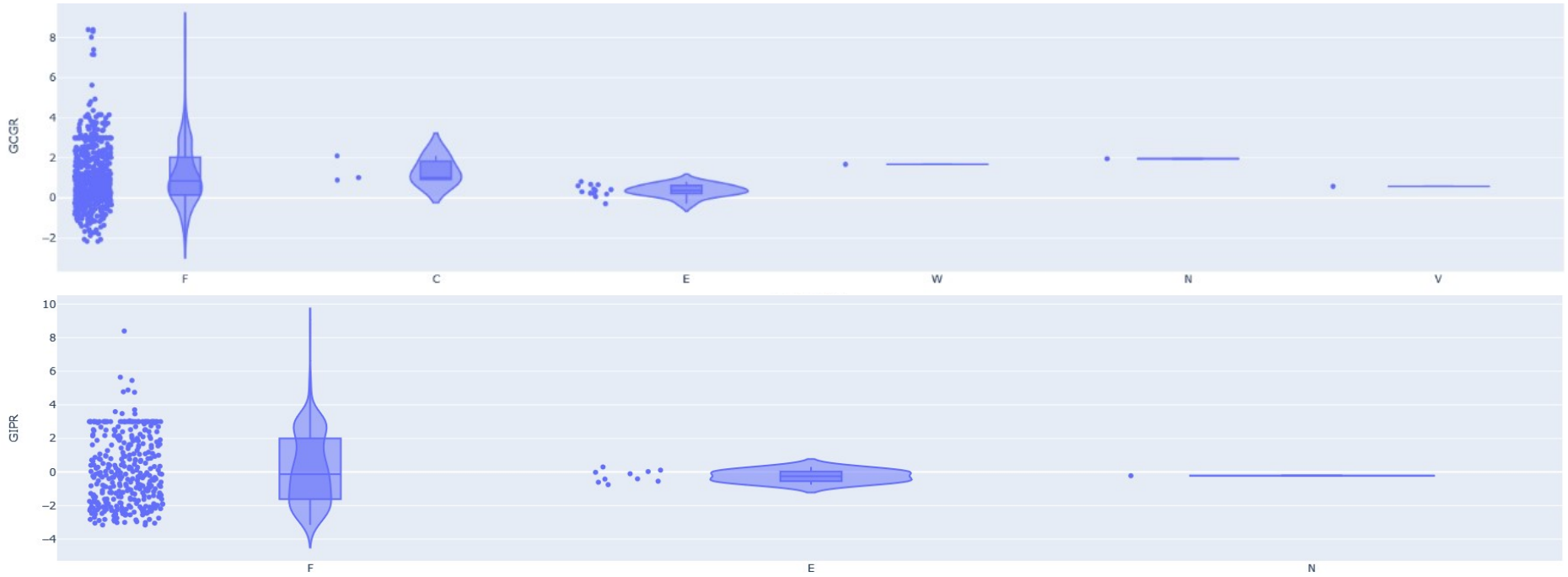
V


GCGR
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6
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−2
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W
N
V
GIPR
10
8
6
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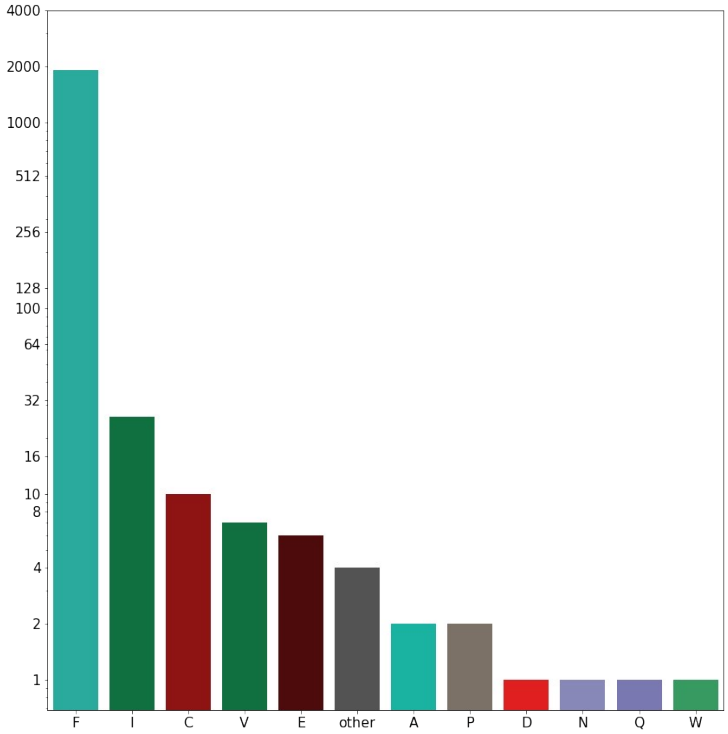
2
0
−2
−4
F
E
N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other
A
P
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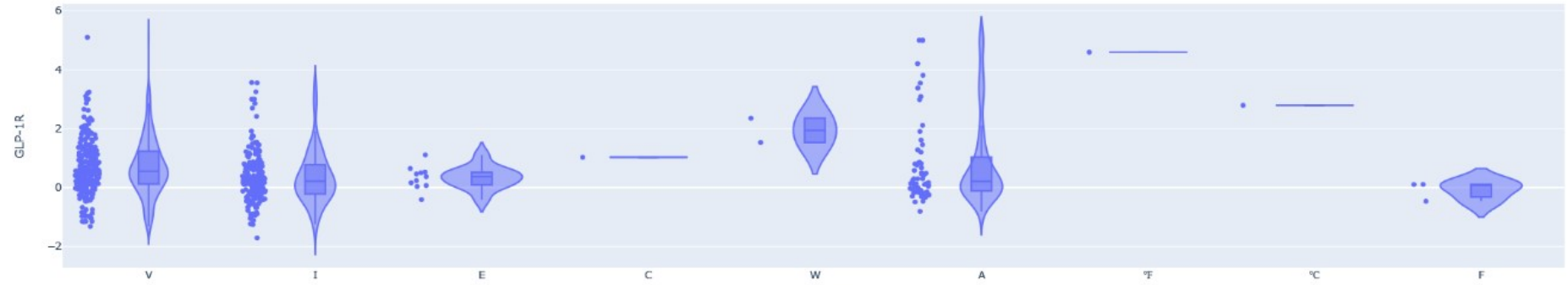
N
Q
W

Position 23


GLP-1R
6
4
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−2
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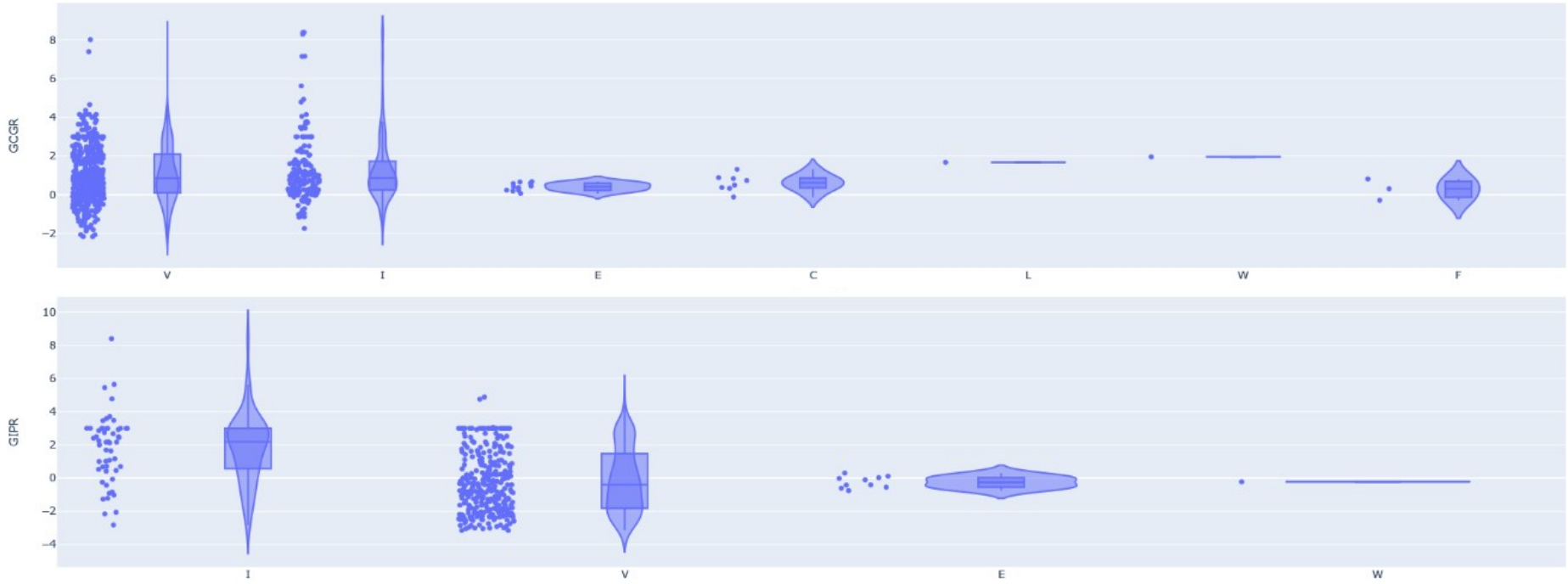
C
W
A
°F
°C
F


GCGR
8
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GIPR
10
8
6
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−4
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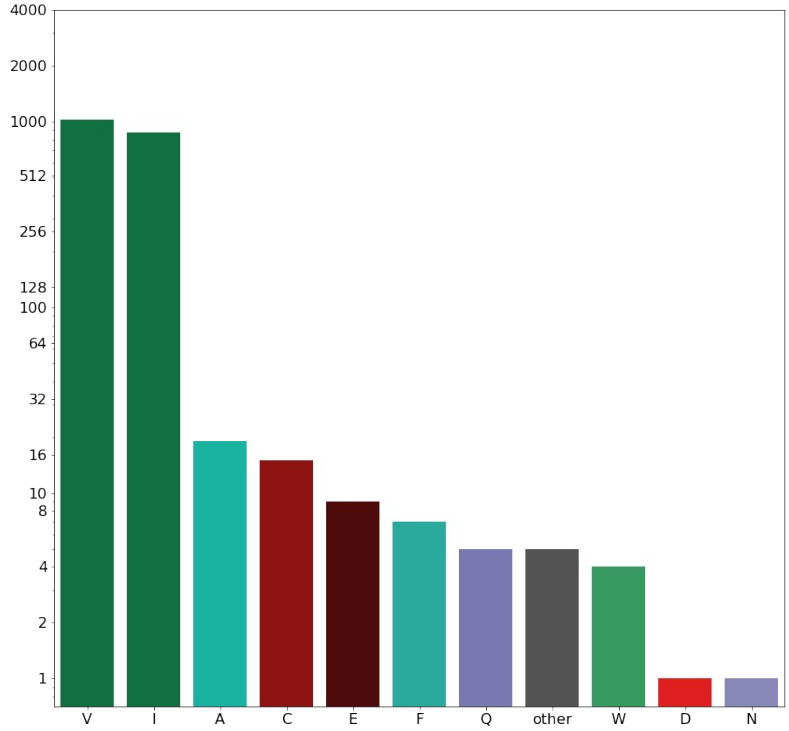
W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2000
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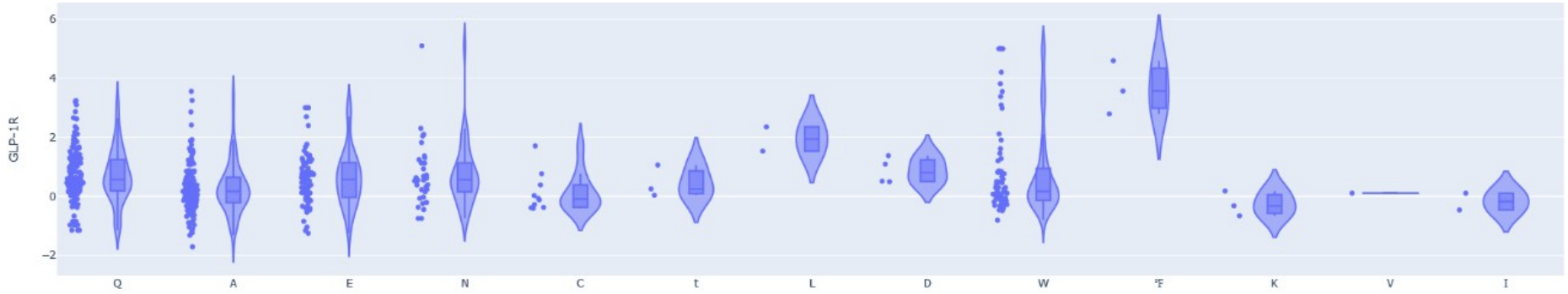
other
W
D
N

Position 24


GLP-1R
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−2
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K
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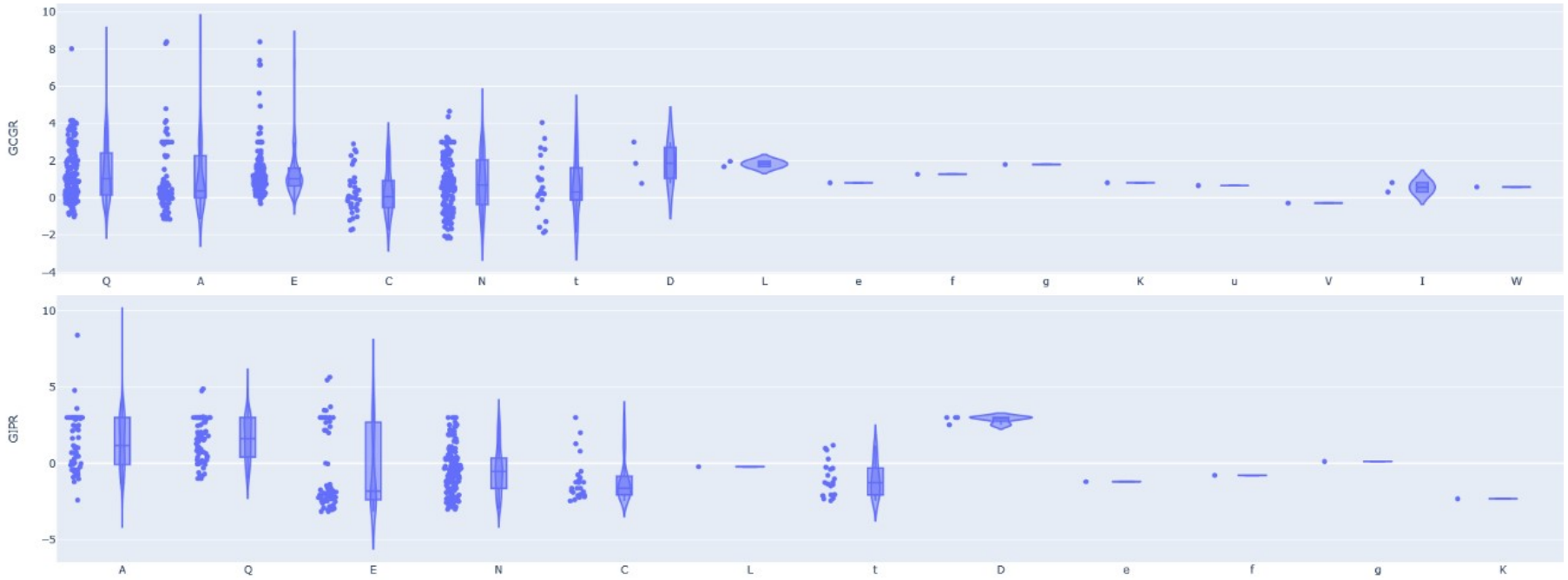
I


GCGR
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4
2
0
−2
−4
Q
A
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C
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t
D
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e
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g
K
u
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W
GIPR
10
5
0
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A
Q
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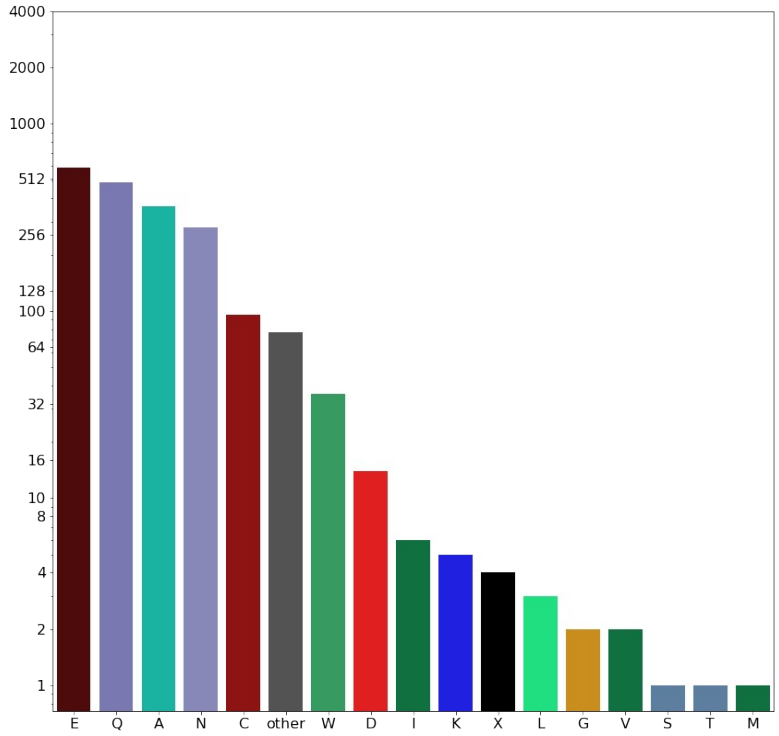
N
C
L
t
D
e
f
g
K


4000
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256
128
100
64
32
16
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E
Q
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N
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other
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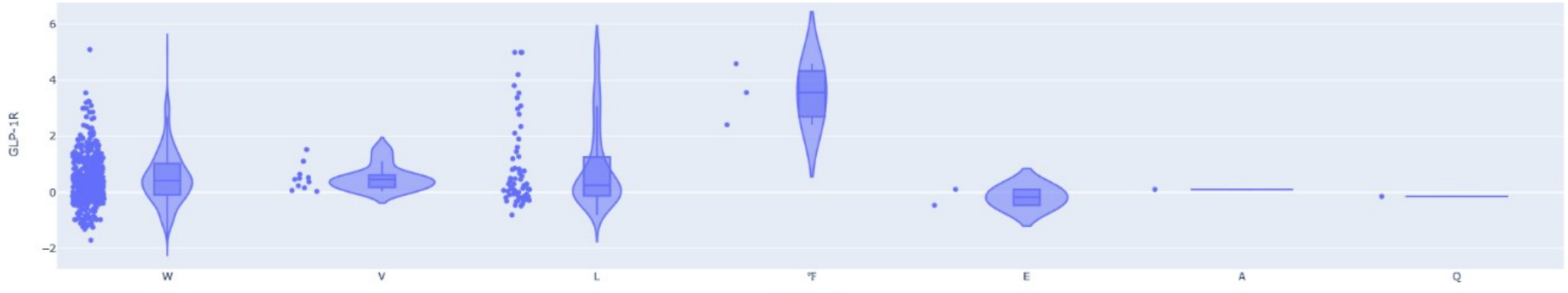
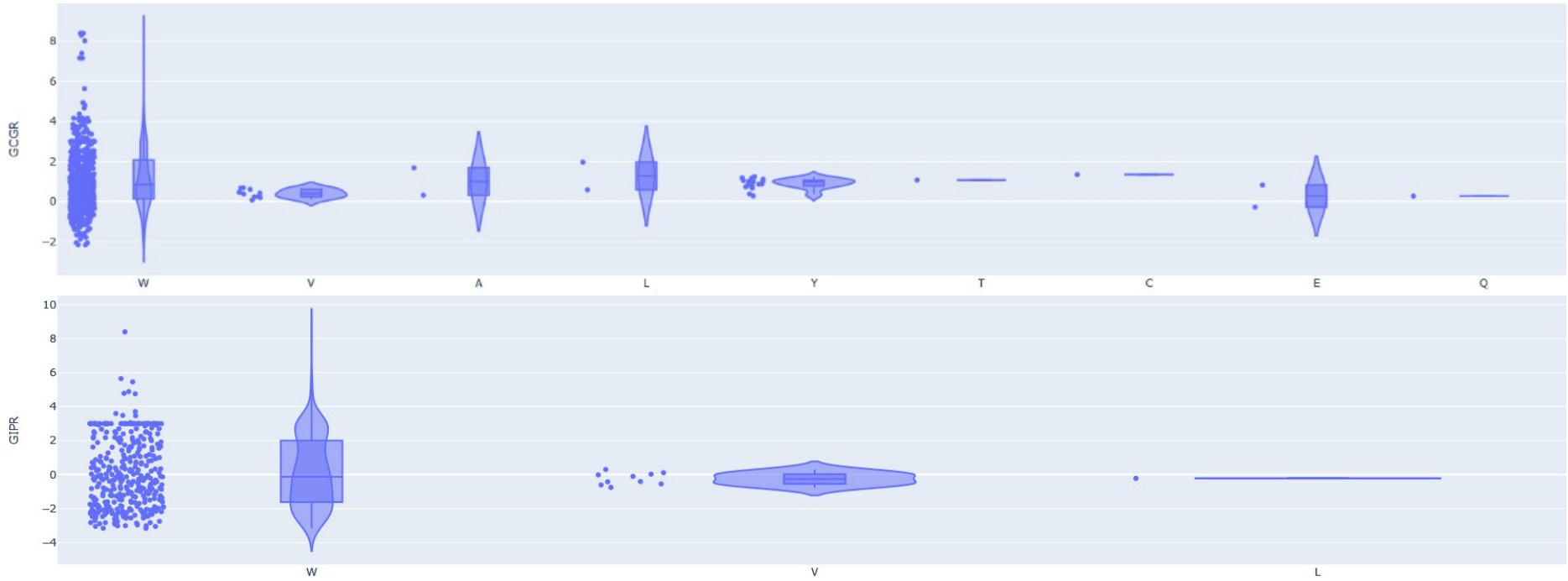
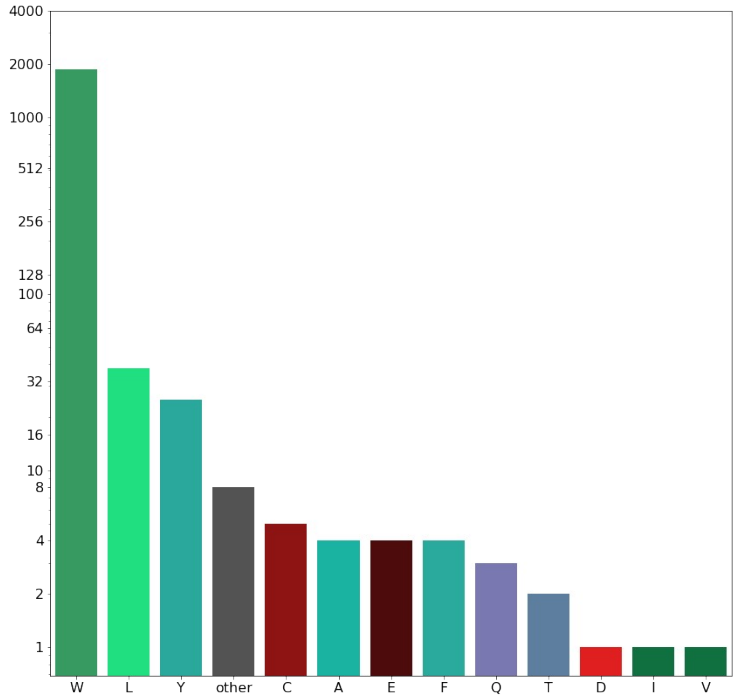
S
T
M

Position 25


GLP-1R
6
4
2
0
−2
W
V
L
F
E
A
Q


GCGR
8
6
4
2
0
−2
W
V
A
L
Y
T
C
E
Q
GIPR
10
8
6
4
2
0
−2
−4
W
V
L


4000
2000
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100
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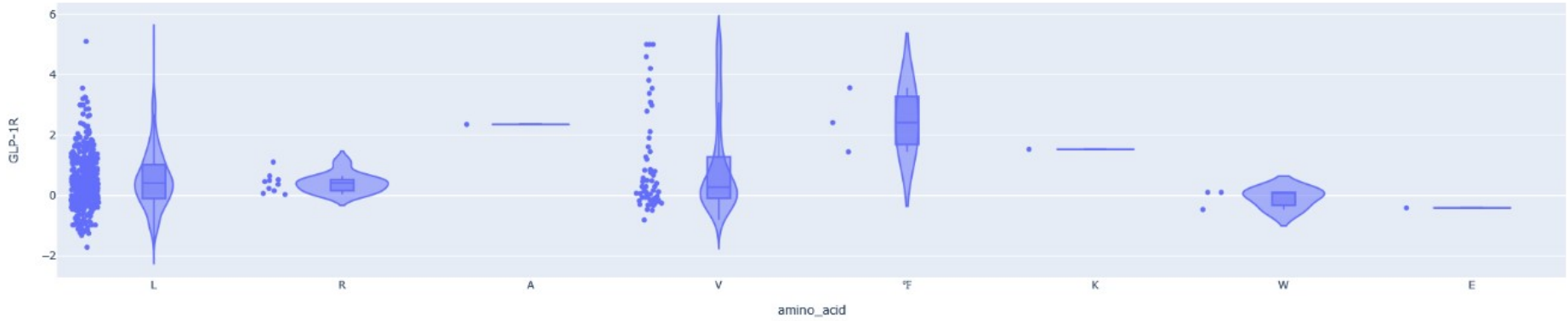
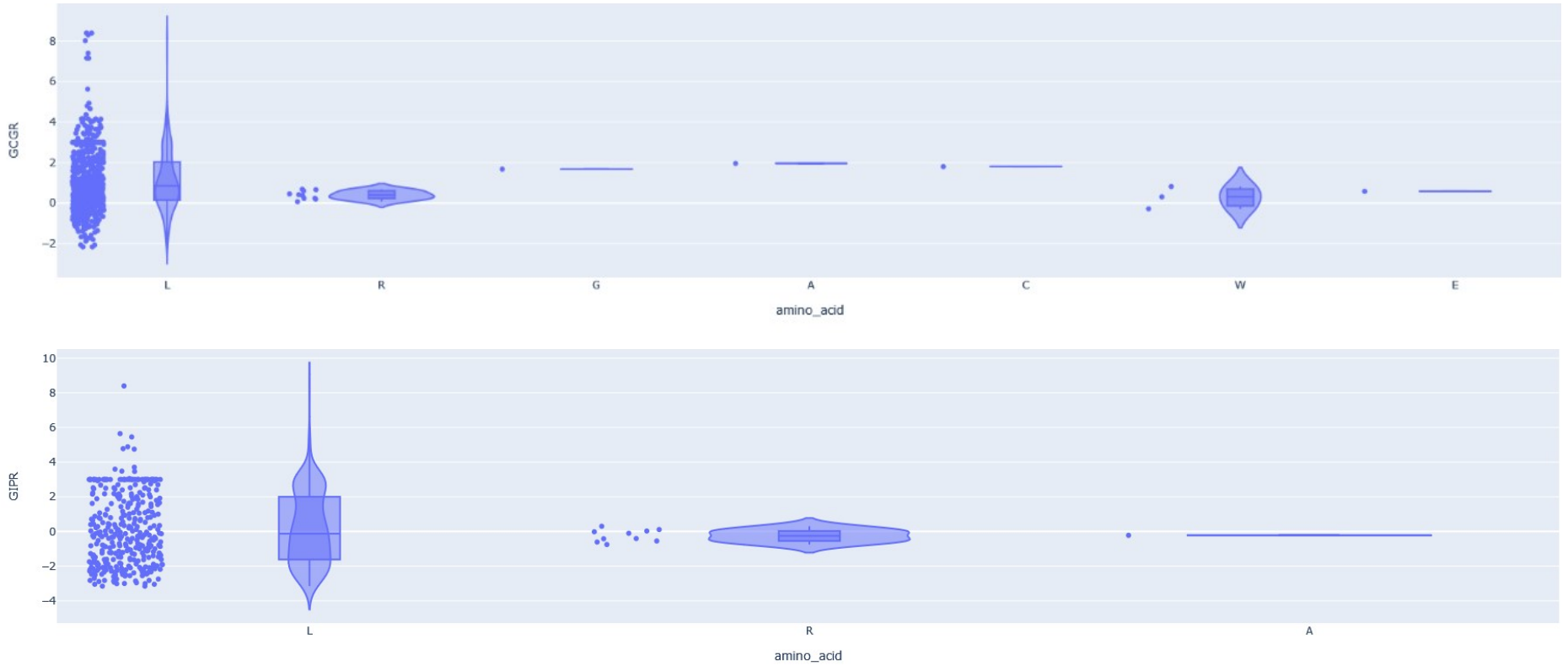
8
4
2
1
W
L
Y
other
C
A
E
F
Q
T
D
I
V

Position 26


6
4
2
0
−2
GLP-1R
L
R
A
V
F
K
W
E
amino_acid


GCGR
8
6
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−2
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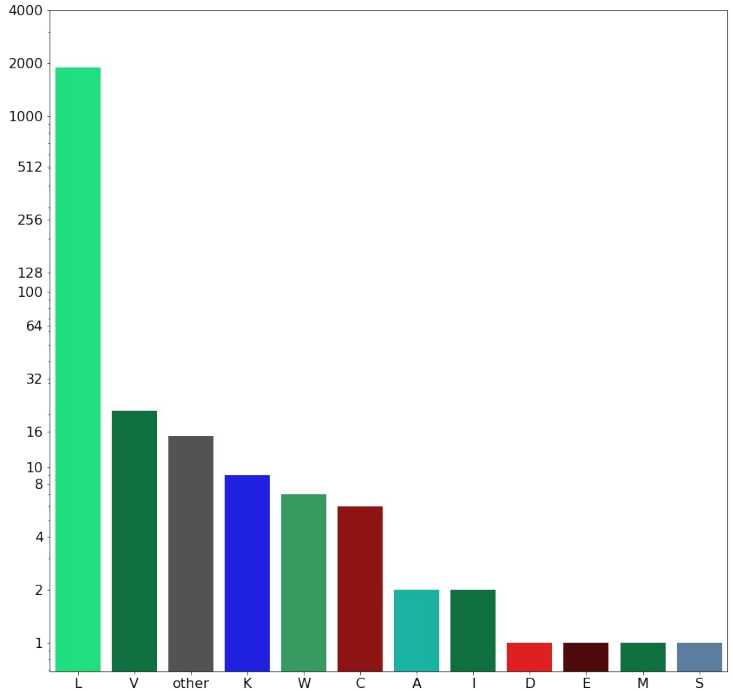
R
G
A
C
W
E
amino_acid
GIPR
10
8
6
4
2
0
−2
−4
L
R
A
amino_acid


4000
2000
1000
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256
128
100
64
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10
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4
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1
L
V
other
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W
C
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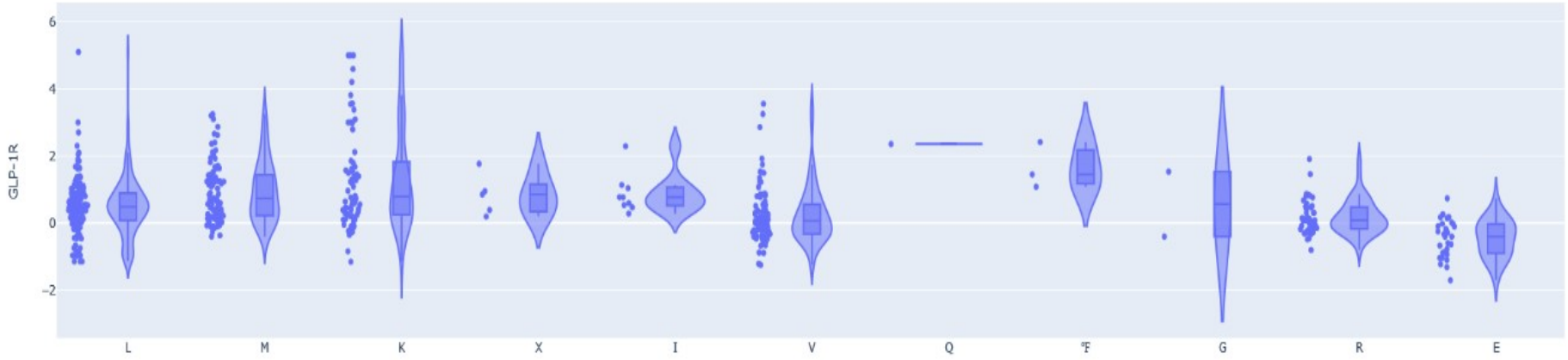
D
E
M
S

Position 27


GLP-1R
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4
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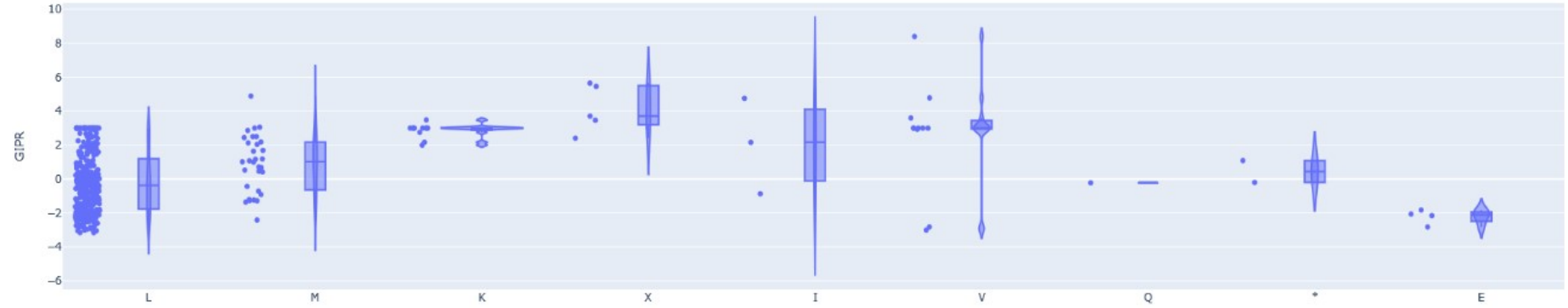
0
−2
L
M
K
X
I
V
Q
F
G
R
E


GIPR
10
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−2
−4
−6
L
M
K
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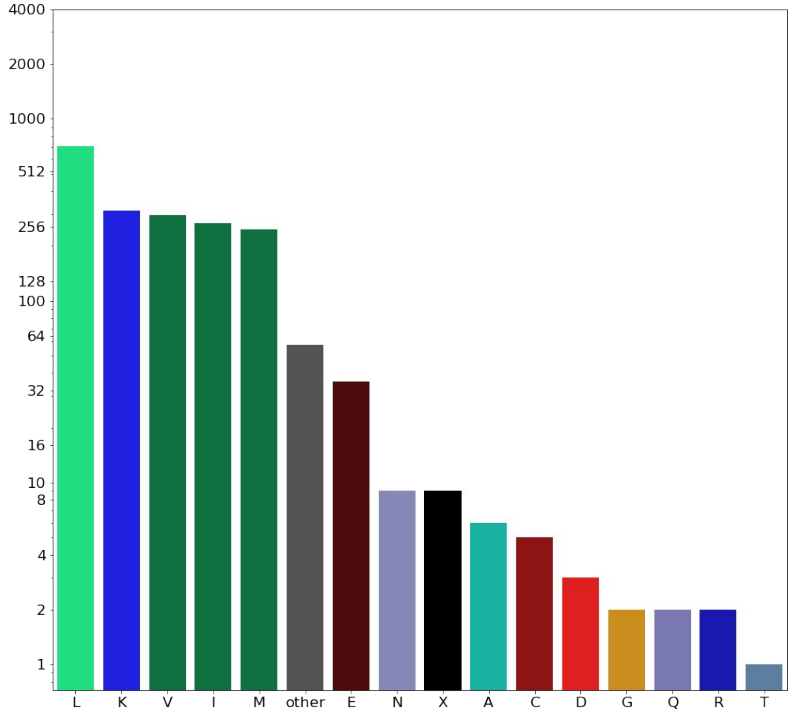
V
Q
*
E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K
V
I
M
other
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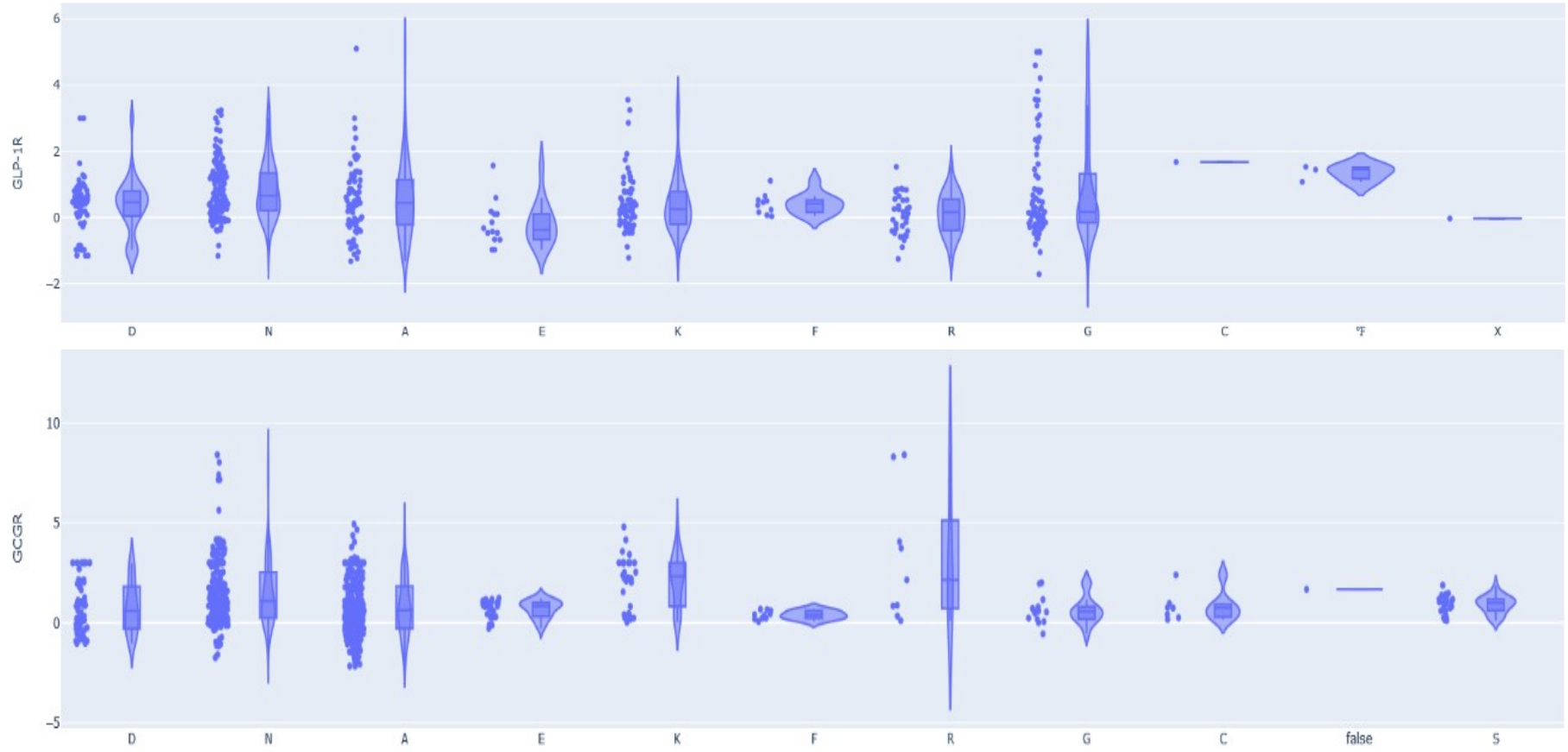
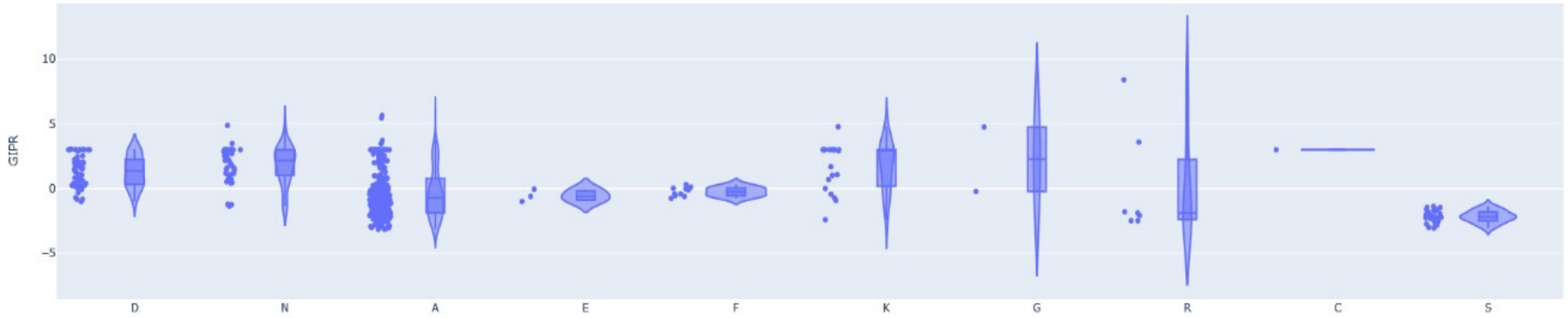
X
A
C
D
G
Q
R
T

Position 28




GIPR
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C
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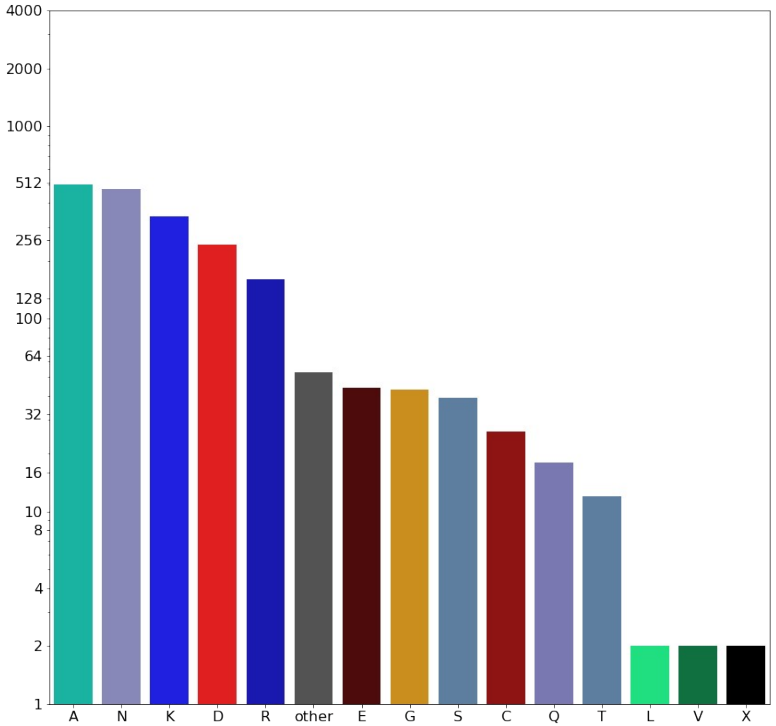
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other
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S
C
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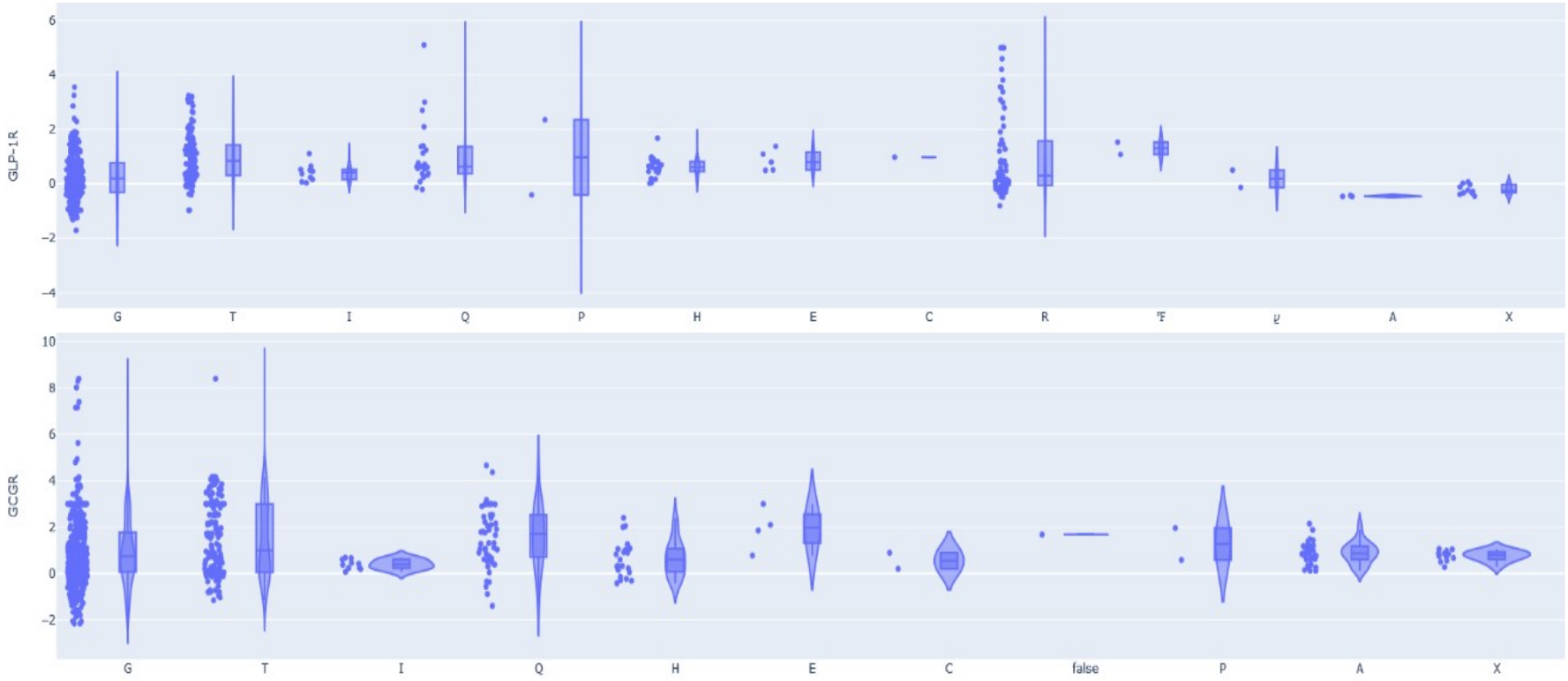
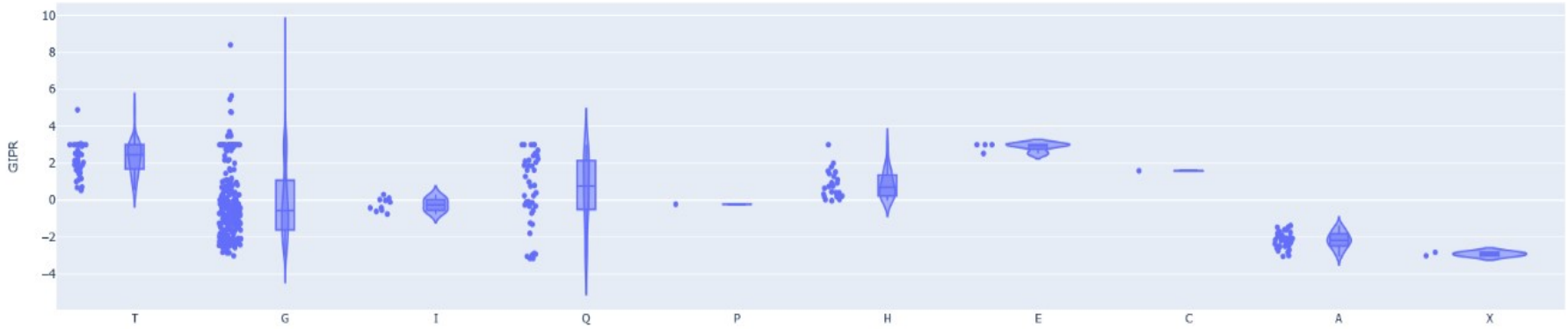
T
L
V
X

Position 29




GTPR
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6
4
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Q
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H
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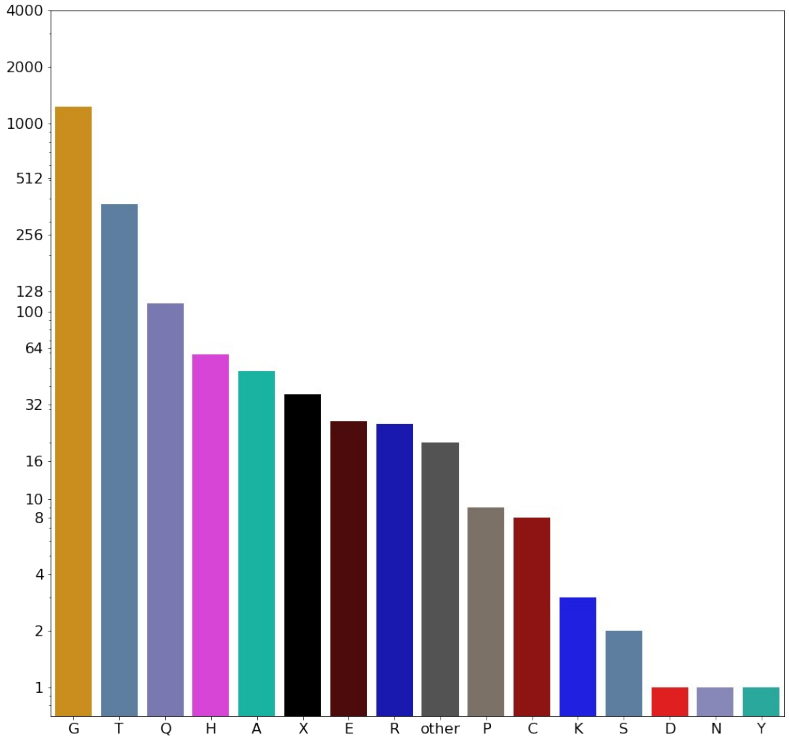
X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H
A
X
E
R
other
P
C
K
S
D
N
Y

Amino acid modifications – legend

Encoding	Modification name	Modification SMILES (additional if possible)
6	[15-carboxy-pentadecanoyl]-isoGlu-Peg3-Peg3 acylated Lysine	OC(=O)CCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCOCCOCC(=O)NCCOCCOCC(O)=O)C(O)=O
7	[17-carboxy-heptadecanoyl]-isoGlu-Peg3-Peg3 acylated Lysine	OC(=O)CCCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCOCCOCC(=O)NCCOCCOCC(O)=O)C(O)=O
4	[19-carboxy-nonadecanoyl]-isoGlu-Peg3-Peg3 acylated Lysine	OC(=O)CCCCCCCCCCCCCCCCCCC(=O)N[C@@H](CCC(=O)NCCOCCOCC(=O)NCCOCCOCC(O)=O)C(O)=O
X	Aib	CC(C)(C(=O)O)N
$	dSer	C([C@H](C(=O)O)N)O
O	N6-((S)-4-Carboxy-4-palmitamidobutanoyl)-L-lysine	CCCCCCCCCCCCCCCC(=O)N[C@@H](CCC(=O)NCCCC[C@H](N)C(O)=O)C(O)=O
U	K(Hexadecanoyl-isoGlu)	CCCCCCCCCCCCCCCC(=O)N[C@@H](CCC(*)=O)C(O)=O
@	Lys-γE-C16-acyl	
%	dAla	
Z	nVal	CCC[C@@H](C(=O)O)N
J	hSer	O=C(O)C(N)CCO
l	nLeu	CCCC[C@@H](C(=O)O)N
k	DAP	C(CC(C(=O)O)N)CC(C(=O)O)N
m	Met(O)	CS(=O)CC[C@@H](C(=O)O)N
8	Ac4c	
1	K(eicosanoyl-isoGlu-Peg3-Peg3)	[*]C(=O)COCCOCCNC(=O)COCCOCCNC(=O)CC[C@H](NC(=O)CCCCCCCCCCCCCCCCCCC)C(=O)O
a	Cys-C6	CCCCCCN1C(=O)CC(SCC(N)C=O)C1=O
b	Cys-C12	CCCCCCCCCCCCN1C(=O)CC(SCC(N)C=O)C1=O
c	Cys-C16	CCCCCCCCCCCCCCCCN1C(=O)CC(SCC(N)C=O)C1=O
d	Cys-C11-COOH	NC(CSC1CC(=O)N(CCCCCCCCCCC(O)=O)C1=O)C=O
e	K-C14	CCCCCCCCCCCCCCCC(=O)NCCCC[C@@H](N)C(O)=O
f	K-C16	CCCCCCCCCCCCCCCCCC(=O)NCCCC[C@@H](N)C(O)=O
g	K-C18	CCCCCCCCCCCCCCCCCCCC(=O)NCCCCC(N)=O
h	Sarcosine	CNCC(=O)O
i	K-C16-acyl	CC(=O)CCCCCCCCCCCCCCCCC(=O)NCCCC[C@@H](N)C(O)=O
j	K-γEγE-C16-acyl	CC(=O)CCCCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NC(CCC(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O)C(O)=O
n	K-γEγE-C16	CCCCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NC(CCC(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O)C(O)=O
2	K(Octadecanoyl-isoGlu-Peg3-Peg3)	CCCCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCOCCOCC(=O)NCCOCCOCC(=O)NCCCCC(N)C(O)=O)C(O)=O
z	[17-carboxy-heptadecanoyl]-isoGlu Lysine	NC(CCCCNC(=O)CCC(NC(=O)CCCCCCCCCCCCCCCCC(O)=O)C(O)=O)C(O)=O
3	K(Octadecanoyl-Dapa-Peg3-Peg3)	CCCCCCCCCCCCCCCCCC(=O)NC(CN)C(=O)NCCOCCOCC(=O)NCCOCCOCC(=O)NCCCCC(N)C(O)=O
o	CA - 4-imidazoacetyl	CC(=O)C1=CN=CN1
p	K(ioctadecanoyl-isoGlu)	CCCCCCCCCCCCCCCCCC(=O)N[C@@H](CCC(*)=O)C(O)=O
t	Cys-40kDa PEG	C(C(C(=O)O)N)S
r	hCys	NC(CCS(O)(=O)=O)C(O)=O
s	hE	NC(CCCC(O)=O)C(O)=O
L10	L10-staple	BrCC(=O)NCCCCCCCCCCCCNC(=O)CBr

L9	L9-staple	BrCC(=O)NCCCCCCCCCCCNC(=O)CBr
L8	L8-staple	BrCC(=O)NCCCCCCCCCCNC(=O)CBr
L7	L7-staple	BrCC(=O)NCCCCCCCCCNC(=O)CBr
L6	L6-staple	BrCC(=O)NCCCCCCCCNC(=O)CBr
L5	L5-staple	BrCC(=O)NCCCCCCCNC(=O)CBr
L4	L4-staple	BrCC(=O)NCCCCCCNC(=O)CBr
L3	L3-staple	BrCC(=O)NCCCCCNC(=O)CBr
L2	L2-staple	BrCC(=O)NCCCCNC(=O)CBr
L1	L1-staple	BrCC(=O)NCCCNC(=O)CBr
L11	L11-staple	BrCC(=O)NCCOCCOCCNC(=O)CBr
L12	L12-staple	BrCC(=O)NCCOCCOCCOCCNC(=O)CBr
L15	L15-staple	OC(=O)CCCCCCCCCCCCCCCCCC(=O)NC(CCCCNC(=O)CCOCCOCCNC(=O)CCOCCOCCNC(=O)C(CCCNC(=O)CBr)NC(=O)CBr)C(O)=O
L14	L14-staple	OC(=O)CCCCCCCCCCCCCCCCCC(=O)NCCOCCOCCOCCNC(=O)C(CCCNC(=O)CBr)NC(=O)CBr
L13	L13-staple	CCCCCCCCCCCCCC(=O)NCCOCCOCCNC(=O)C(CCCNC(=O)CBr)NC(=O)CBr
u	Cys-20kDa PEG	C(C(C(=O)O)N)S-PEG
w	3-phenyllactic acid Phe-OH	C1=CC=C(C=C1)CC(C(=O)O)O
y	hippuric acid	C1=CC=C(C=C1)C(=O)NCC(=O)O
*****	Nle	CCCCC(N)C(O)=O
+	ornithine	NCCC[C@H](N)C(O)=O
B	2,4-diaminobutyric acid	C(CN)C(C(=O)O)N
x	K-3-SH-propionic acyl group	CC(=O)C(S)CC(=O)NCCCC[C@@H](N)C(O)=O
!	K-C8-acyl	CC(=O)CCCCCCCCCC(=O)NCCCC[C@@H](N)C(O)=O
α	hA	CCC(C(=O)O)N
5	dLys	NCCCC[C@H](N)C(O)=O
9	dLys-C14-acyl	CC(=O)CCCCCCCCCCCCCCCC(=O)NCCCC[C@H](N)C(O)=O
^	Cys-5kDa PEG	C(C(C(=O)O)N)S
#	Epsilon amine of Lys is attached to Ala-Ac-Cys(PEG), wherein the Ac-Cys(PEG) is a Cys residue comprising an alpha amino group capped with an acetyl group (CH3CO) and comprising a 40 kDa PEG covalently attached to its side chain	
γ	Cys-PEG covalently linked via thioether linkage	
δ	Substituted amino acid covalently linked to a hydrophilic group	
ε	can be any naturally occurring amino acid	
η	Substitute amino acid covalently linked to an acyl or alkyl group	
μ	various modifications - described in metadata	
κ	Glutamine analog	
λ	Acetamidomethyl-cysteine	CC(=O)NCSCC(N)C(O)=O

χ	Acetyldiaminobutanoic acid	CC(C(=O)C)C(C(=O)O)(N)N
ψ	carbamoyldiaminopropanoic acid	NC(=O)C(N)(N)CC(O)=O
ω	Methylglutamine	
τ	Acetylornithine	CC(=O)NC(CCC[NH3+])C(=O)[O-]
ç	Methionine sulfoxide	O=C(O)C(N)CCS(=O)C
ф	Epsilon amine is peptide bonded to Ala residue which is attached to an acetylated Cys which is attached to a 40kDa PEG	
ш	Epsilon amine is peptide bonded to Glu residue which is attached to an acetylated Cys which is attached to a 40kDa PEG	
ч	Epsilon amine is peptide bonded to Arg residue which is attached to an acetylated Cys which is attached to a 40kDa PEG	
ь	Epsilon amine is peptide bonded to Phe residue which is attached to an acetylated Cys which is attached to a 40kDa PEG	
и	K-Acylated with C16 fatty acyl group via epsilon amine	
м	K-γE-C16	CCCCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O
Б	DMIA - 2,3-Dimethyl-2H-indazol-6-amine	CC1=C2C=CC(=CC2=NN1C)N
Ь	Covalently bound to 40kDa PEG via thioether made by reaction of peptide with haloacetyl-activated PEG	
ϑ	1-aminocyclopropane-1-carboxylate	[NH3+]C1(CC1)C([O-])=O
ƨ	K-γE-C14	CCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O
Ƶ	Cys - covalently bound to a 40kDa PEG via thioether made by reaction of peptide with maleimide-activated PEG	
Б	Acylated with a C16 fatty acyl group via acid amino acid spacer	
ϙ	Acylated with a C16 fatty acyl group via dipeptide spacer	
ϟ	Acylated with C16 fatty acyl group via acidic amino acid spacer	
Ҽ	Acylated with a C14 acyl group via acidic amino acid spacer	
Љ	C16-γGlu-Muc-K	CCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCNC(=O)[C@@H](O)[C@H](O)[C@H](O)[C@@H](O)C(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O
З	C18-γGlu-Muc-K	CCCCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCNC(=O)[C@@H](O)[C@H](O)[C@H](O)[C@@H](O)C(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O
Ж	C20-γGlu-Muc-K	CCCCCCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCNC(=O)[C@@H](O)[C@H](O)[C@H](O)[C@@H](O)C(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O
Д	C16-γGlu-Muc-Muc-K	CCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCNC(=O)[C@@H](O)[C@H](O)[C@H](O)[C@@H](O)C(=O)NCCNC(=O)[C@@H](O)[C@H](O)[C@H](O)[C@@H](O)C(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O
Г	C18-γGlu-Muc-Muc-K	CCCCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCNC(=O)[C@@H](O)[C@H](O)[C@H](O)[C@@H](O)C(=O)NCCNC(=O)[C@@H](O)[C@H](O)[C@H](O)[C@@H](O)C(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O
П	C20-γGlu-Muc-Muc-K	CCCCCCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCNC(=O)[C@@H](O)[C@H](O)[C@H](O)[C@@H](O)C(=O)NCCNC(=O)[C@@H](O)[C@H](O)[C@H](O)[C@@H](O)C(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O
Л	C16-γGlu-O2Oc-K	CCCCCCCCCCCCCCCC(=O)NC(CCC(=O)NCCOCCOCC(=O)NCCCC[C@@H](N)C(O)=O)C(O)=O
Ж	Ac-H	CC(=O)N[C@H](CC1=CN=CN1)C(O)=O

Ж	Pyr-H	OC(=O)[C@@H](CC1=CN=CN1)NC(=O)C1CCC(=O)N1
я	Bzl-H	OC(=O)[C@@H](CC1=CN=CN1)NCC1=CC=CC=C1
è	Bz-H	OC(=O)[C@@H](CC1=CN=CN1)NC(=O)C1=CC=CC=C1
ë	Peg4-H	COCCOCCOCCOCCC(=O)N[C@H](CC1=CN=CN1)C(O)=O
ђ	Imi-H	O[C@H](CC1=CN=CN1)C(O)=O
ѓ	Me-H	CN[C@H](CC1=CN=CN1)C(O)=O
є	Me2-H	CN(C)[C@H](CC1=CN=CN1)C(O)=O
ї	desNH2-H	OC(=O)CCC1=CN=CN1
£	L-Abu	CC[C@H](N)C(O)=O
₣	D-Abu	CC[C@@H](N)C(O)=O
₡	Cpa	N[C@@H](CC1CC1)C(O)=O
₥	Nva	CCC[C@H](N)C(O)=O
₦	Prg	N[C@@H](CC#C)C(O)=O
₩	Alg	N[C@@H](CC=C)C(O)=O
₭	Vlg	N[C@@H](C=C)C(O)=O
₮	D-tbg	CC(C)(C)[C@@H](N)C(O)=O
₯	2-Cha	C[C@](N)(C1CCCCC1)C(O)=O
₰	Acp	NC1(CC1)C(O)=O
₱	Acb	NC1(CCC1)C(O)=O
₴	Acpe	NC1(CCCC1)C(O)=O
₳	Acx	NC1(CCCCC1)C(O)=O
₼	PS	NC(COP([O-])([O-])=O)C(O)=O
₺	NPA	NC(CC1=CC=C(C=C1)[N+]([O-])=O)C(O)=O
₺	CYA	NC(CS([O-])(=O)=O)C(O)=O
₾	F-ualfa	NCC(CC1=CC=CC=C1)NC(N)=O
₿	D-ualfa	NCC(CC(O)=O)NC(N)=O
₵	E-u	NC(CCC(O)=O)CNC(N)=O
ɘ	Y-u	NC(CNC(N)=O)CC1=CC=C(O)C=C1
℉	A-u	CC(N)CNC(N)=O
℃	I-u	CC[C@H](C)C(N)CNC(N)=O
Bph	4,4’-bis(bromomethyl)biphenyl staple	BrCC1=CC=C(C=C1)C1=CC=C(CBr)C=C1
∞	dCys	C([C@H](C(=O)O)N)S
Bpy	Bpy (CL-2) staple	BrCC1=CC=C(C=N1)C1=CN=C(CBr)C=C1
mBph	mBph (CL-2)	BrCC1=CC(=CC=C1)C1=CC(CBr)=CC=C1
Phe	Phe (CL-4)	BrCC1=CC2=NC3=CC(CBr)=CC=C3N=C2C=C1
Alk	Alk (CL-3)	BrC\C=C\C1=CC=C(\C=C\CBr)C=C1
S1	S1	O.OC(=C)CCCCCCCCCCCCCCCCCC(=O)N[C@@H](CCCCNC(=O)CCOCCOCCNC(=O)CCOCCOCCNC(=O)[C@H](CCCNC(=O)CS)NC(=O)CS)C(O)=O
S2	S2	O.OC(=C)CCCCCCCCCCCCCCCCCC(=O)N[C@@H](CCCCNC(=O)CCOCCOCCNC(=O)CCOCCOCCNC(=O)CCC(=O)

		N(CCNC(=O)CS)CCNC(=O)CS)C(O)=O
⓪	K-(D-glucoside)-C16	
①	K-(D-glucoside)-C18	
②	K-(D-glucoside)-C8	
③	K-(D-glucoside)-C10	
④	K-(D-glucoside)-C12	
⑤	K-(D-glucoside)-C14	
⑥	K-(D-maltoside)-C12	
⑦	K-(D-melibioside)-C12	
⑧	K-(D-melibioside)-C14	
⑨	K-(D-melibioside)-C16	
⑩	K-(D-melibioside)-C18	
⑫	K-S2-(D-glucoside)-C14	
⑬	K-(D-glucoside)-C16(carboxylate at the end)	
⑭	K-(D-glucoside)-C18(carboxylate at the end)	
⑮	Cys-maleimidelinker-40kDaPEG	
⑪	K-S1-(D-glucoside)-C14	
Ţ	K-γGlu-CO-(CH2)12-CH3	CCCCCCCCCCCCCC(=O)N[C@@H](CCC(*)=O)C(O)=O
Û	K-γGlu-CO-(CH2)10-CH3	CCCCCCCCCCCC(=O)N[C@@H](CCC(*)=O)C(O)=O
Ĥ	K-γGlu-CO-(CH2)9-CH3	CCCCCCCCCCC(=O)N[C@@H](CCC(*)=O)C(O)=O
४	K-γGlu-CO-(CH2)8-CH3	CCCCCCCCCC(=O)N[C@@H](CCC(*)=O)C(O)=O
Ʉ	K-Gaba-CO-(CH2)12-CH3	CCCCCCCCCCCCCC(=O)NCCCC(*)=O
Ƀ	K-Gaba-CO-(CH2)10-CH3	CCCCCCCCCCCC(=O)NCCCC(*)=O
Ő	K-Gaba-CO-(CH2)9-CH3	CCCCCCCCCCC(=O)NCCCC(*)=O
Ƶ	K-Gaba-CO-(CH2)8-CH3	CCCCCCCCCC(=O)NCCCC(*)=O
Λ	K-βAla-CO-(CH2)9-CH3	CCCCCCCCCCC(=O)NCCC(*)=O
ɸ	K-βAla-CO-(CH2)10-CH3	CCCCCCCCCCCC(=O)NCCC(*)=O
ȸ	K-βAla-CO-(CH2)12-CH3	CCCCCCCCCCCCCC(=O)NCCC(*)=O

9. HPLC and MS analysis for final compounds – raw data

P1-L1

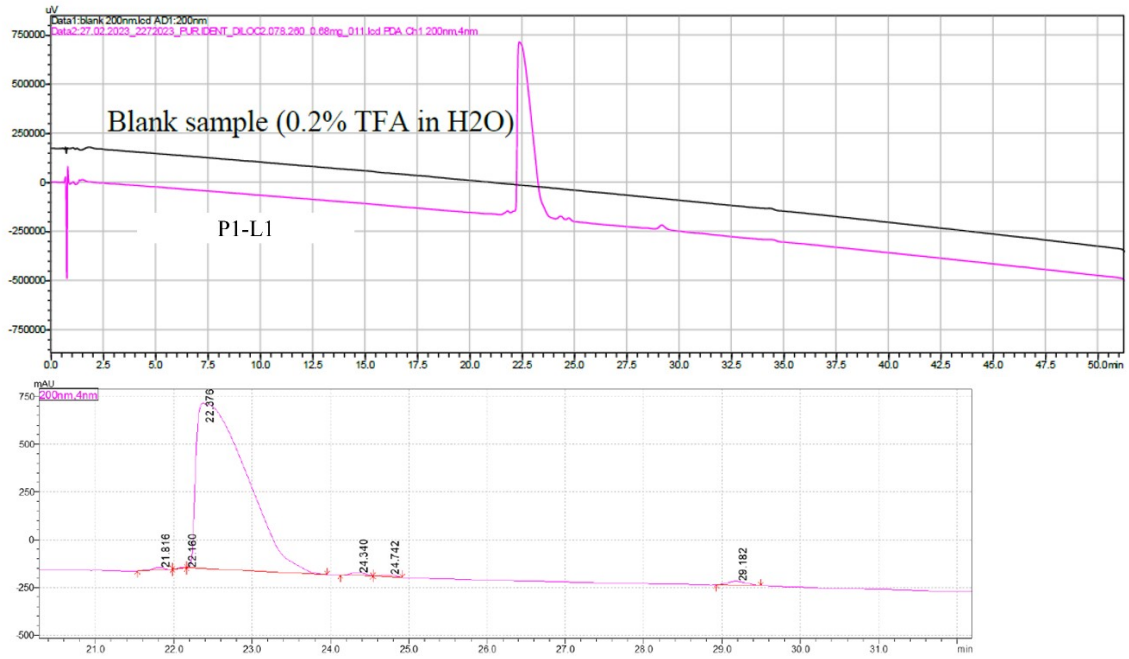
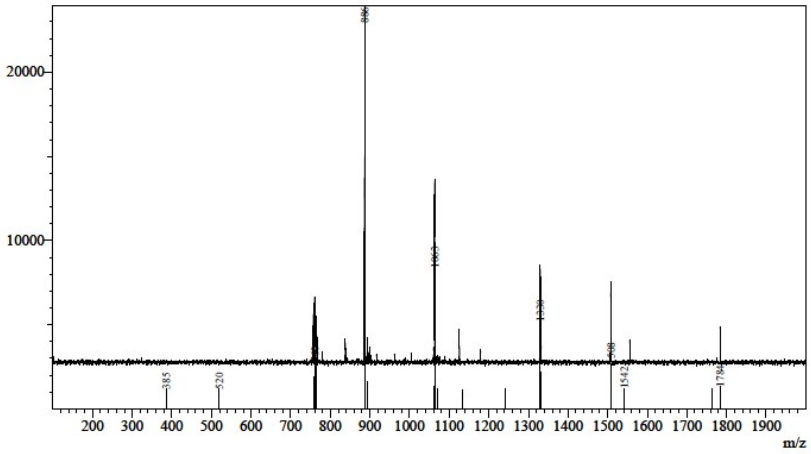
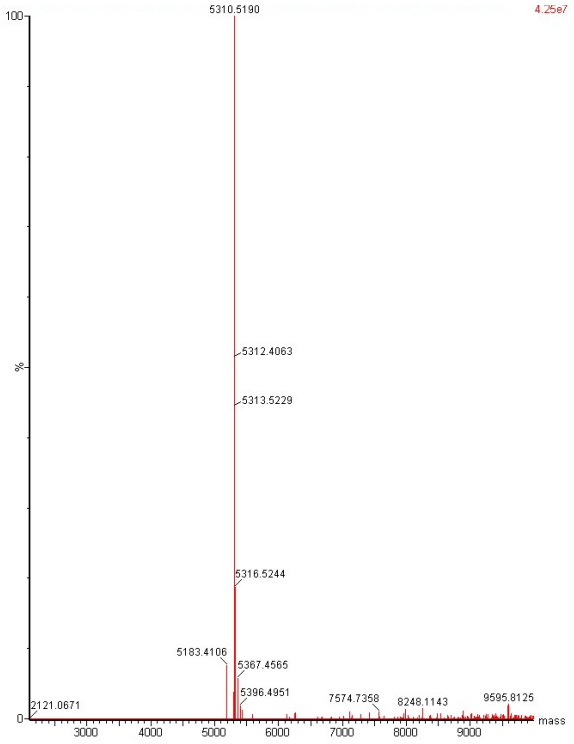


TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	21.816	142008	0.69
2	22.160	14875	0.07
3	23.301	7895760	38.57
4	23.387	11790720	57.60
5	24.340	188849	0.92
6	24.742	108597	0.53
7	29.182	330047	1.61





P2-L2

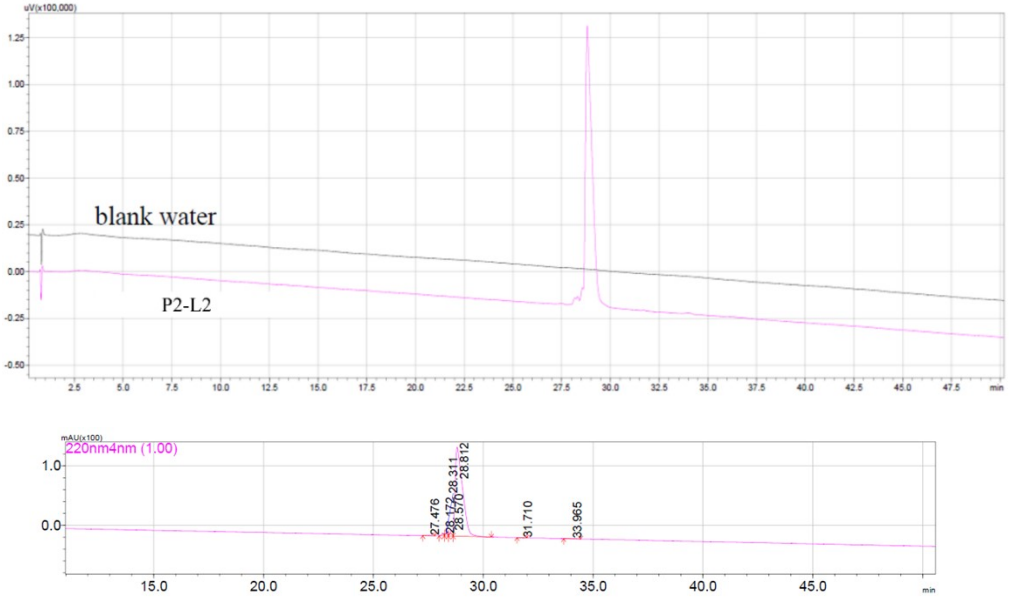
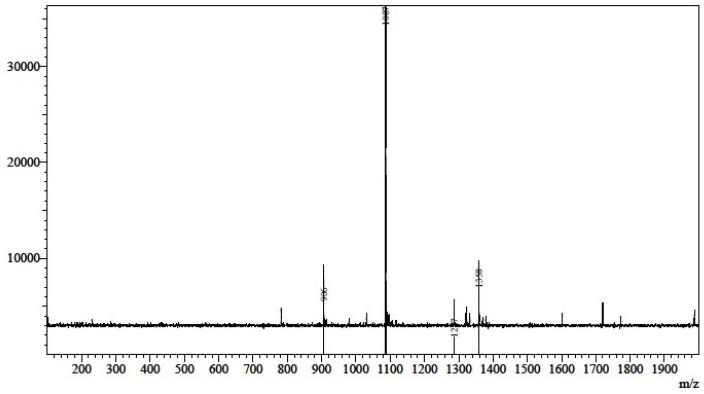


TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	27.476	7054	0.19
2	28.172	27490	0.76
3	28.311	39333	1.08
4	28.570	88521	2.43
5	28.812	3455724	95.05
6	31.710	3416	0.09
7	33.965	13983	0.38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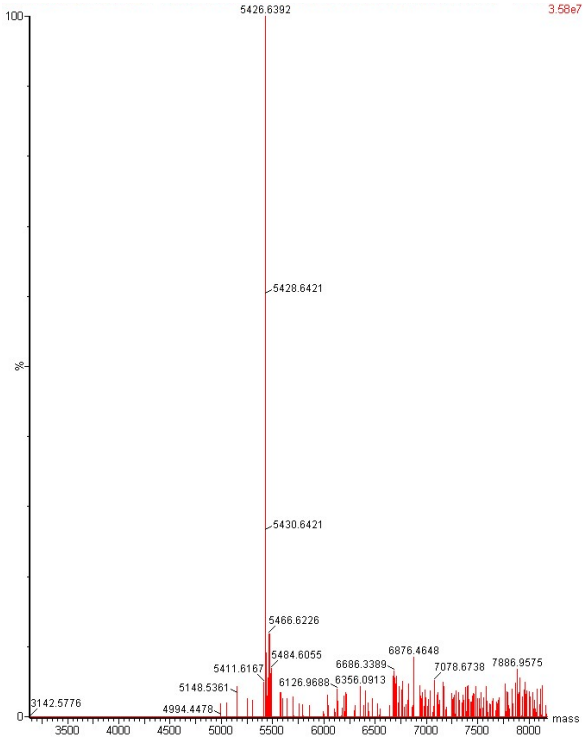
20000
10000
200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900
m/z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%
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3142.5776
4994.4478
6126.9688
6356.0913
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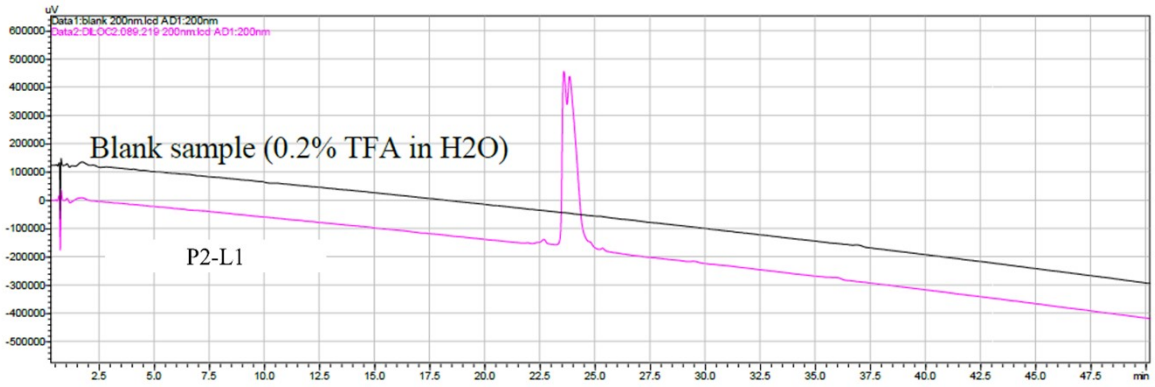
mass

P2-L1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500000
400000
300000
200000
100000
0
-100000
-200000
-300000
-400000
-500000
Blank sample (0.2% TFA in H2O)
P2-L1
2.5 5.0 7.5 10.0 12.5 15.0 17.5 20.0 22.5 25.0 27.5 30.0 32.5 35.0 37.5 40.0 42.5 45.0 47.5
min

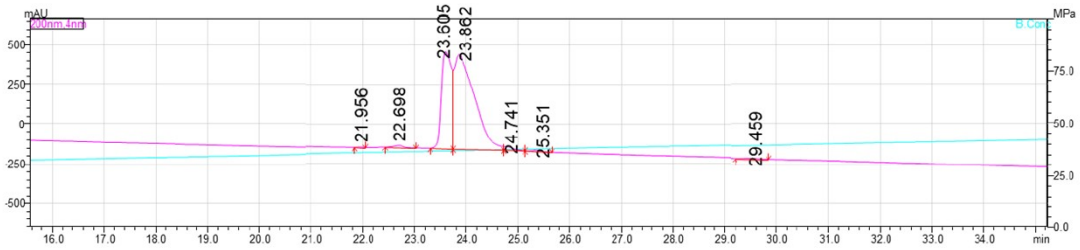
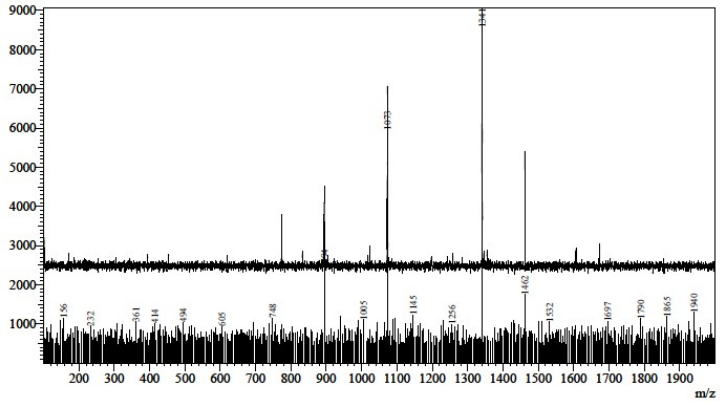
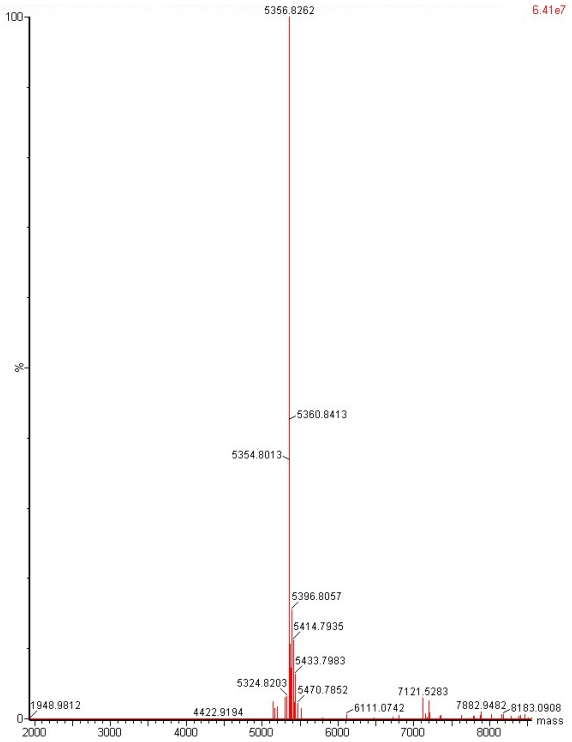


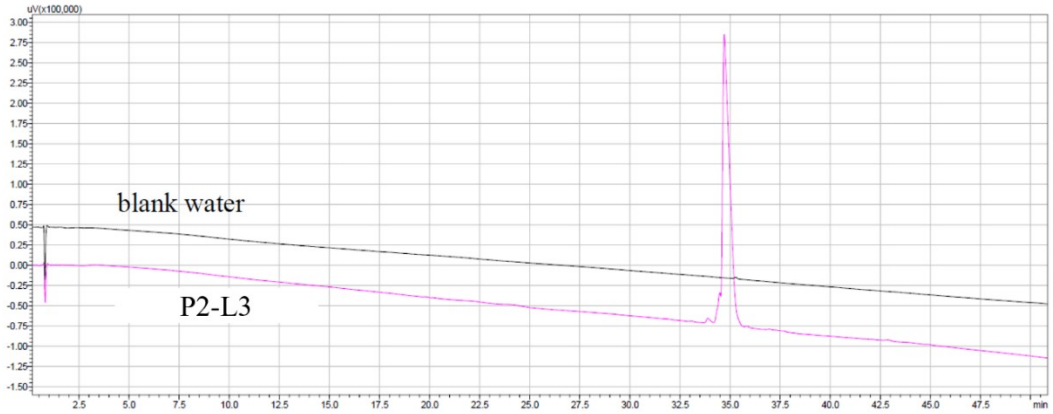
TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	21.956	10545	0.04
2	22.698	172361	0.68
3	23.605	8244453	32.60
4	23.862	16458483	65.09
5	24.741	271801	1.08
6	25.351	65888	0.26
7	29.459	63546	0.25





P2-L3



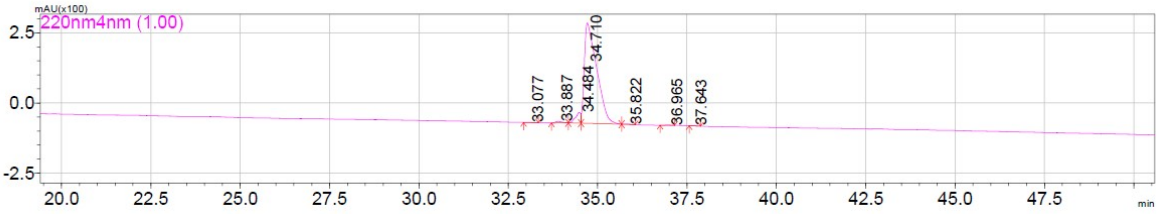
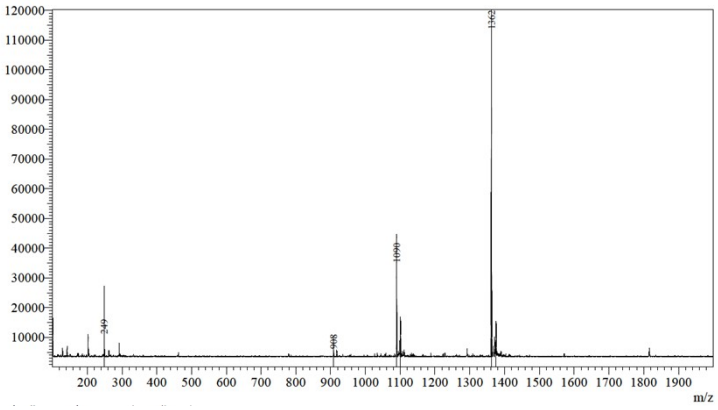
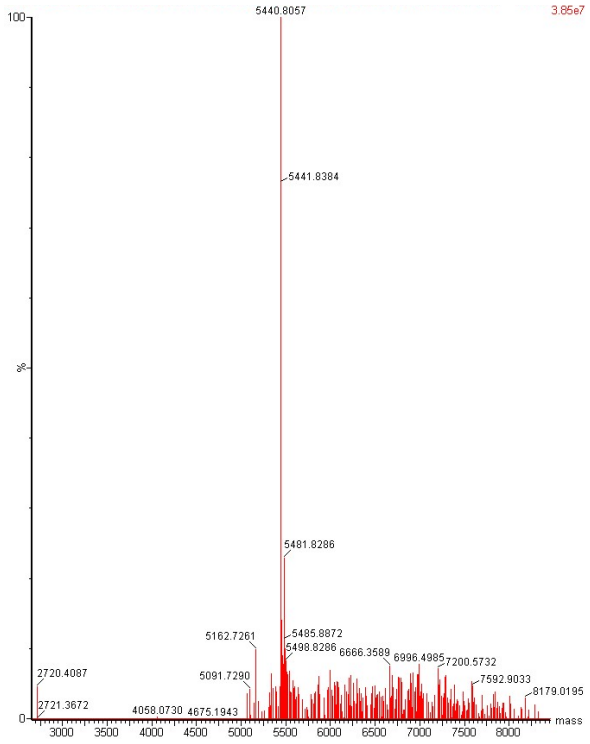


TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	33.077	10414	0.11
2	33.887	67776	0.75
3	34.484	388785	4.29
4	34.710	8570339	94.56
5	35.822	13047	0.14
6	36.965	8138	0.09
7	37.643	4504	0.05





P2-L4

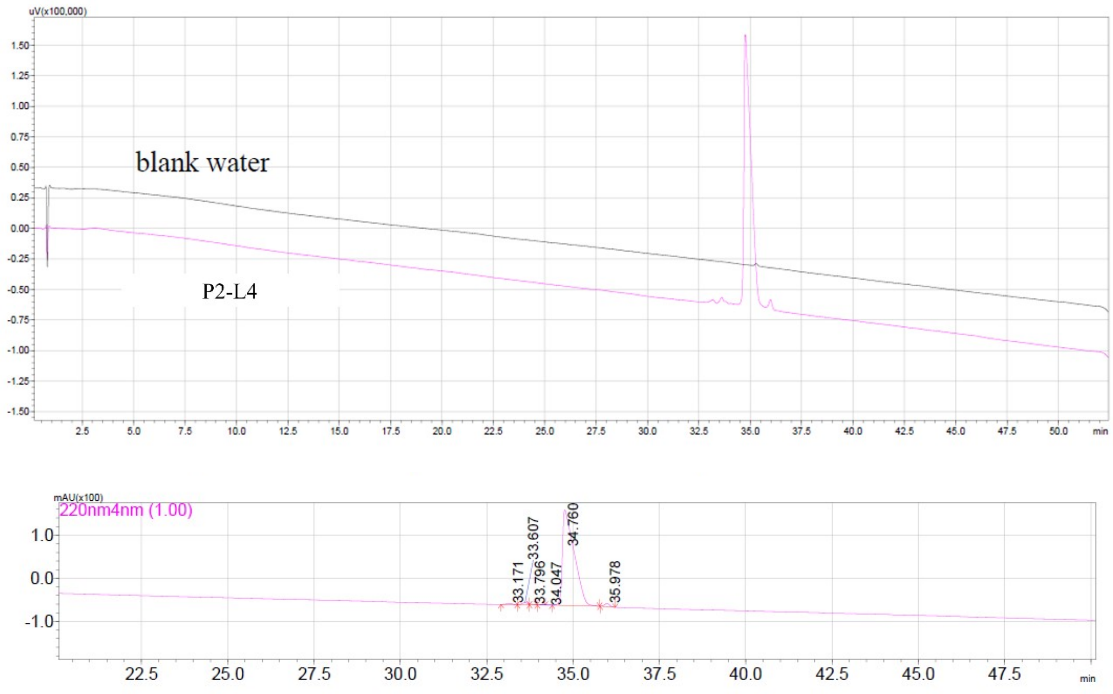
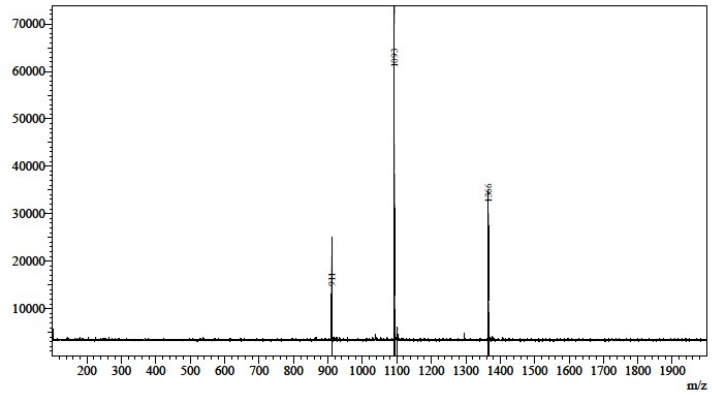


TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	33.171	28211	0.52
2	33.607	52699	0.98
3	33.796	13615	0.25
4	34.047	3057	0.06
5	34.760	52178818	96.79
6	35.978	75458	1.40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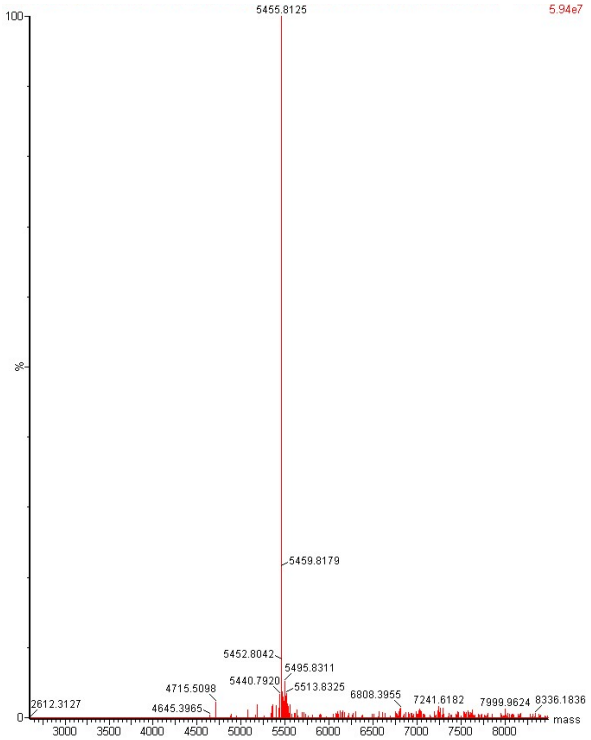
60000
50000
40000
30000
20000
10000
200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900
m/z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5.94e7
100
%
0
5459.8179
5452.8042
5495.8311
5440.7920
5513.8325
4715.5098
2612.3127
4645.3965
6808.3955
7241.6182
7999.9624
8336.1836
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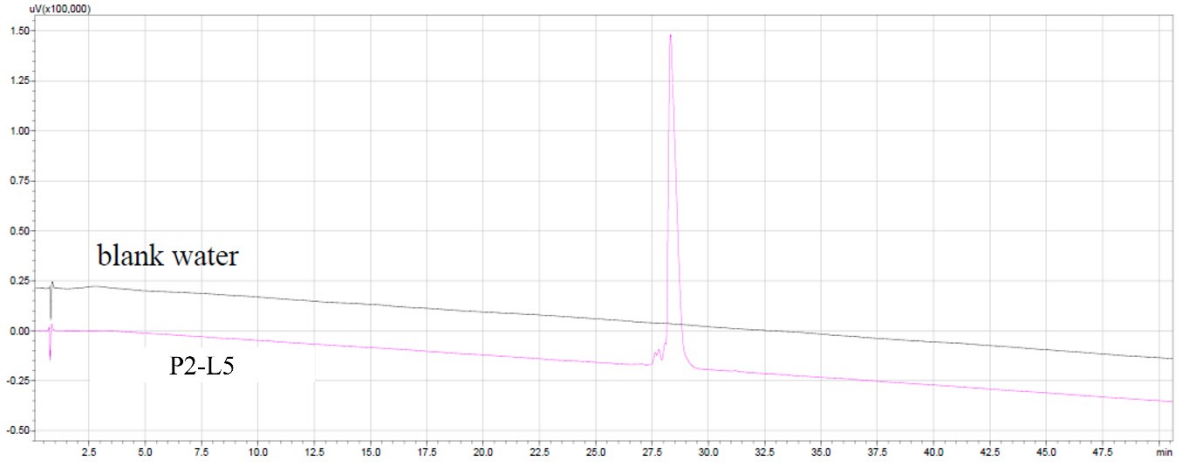
mass

P2-L5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1.25
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
blank water
P2-L5
2.5 5.0 7.5 10.0 12.5 15.0 17.5 20.0 22.5 25.0 27.5 30.0 32.5 35.0 37.5 40.0 42.5 45.0 47.5
min

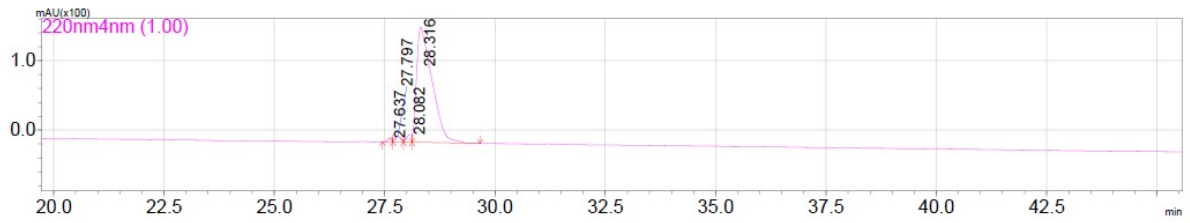
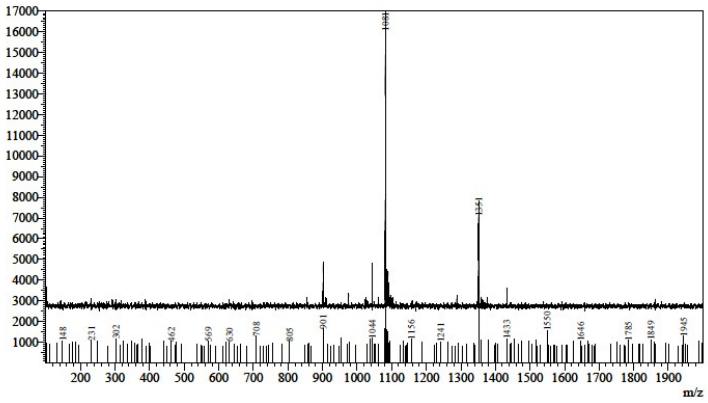
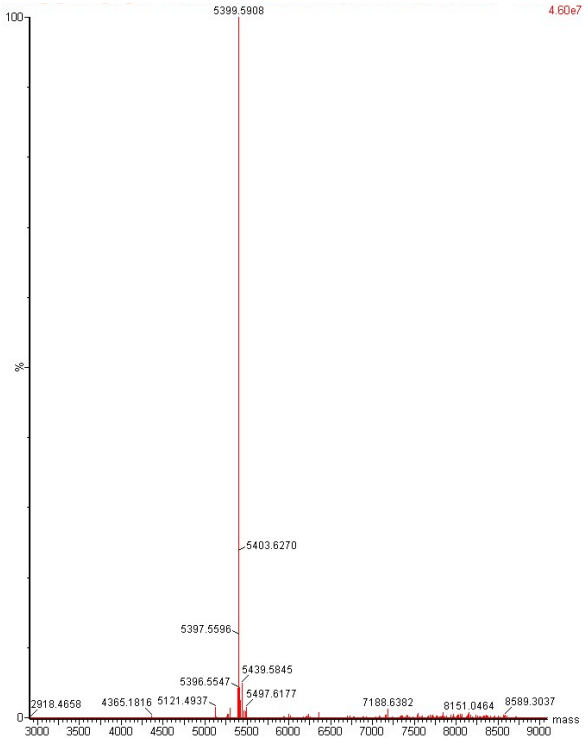


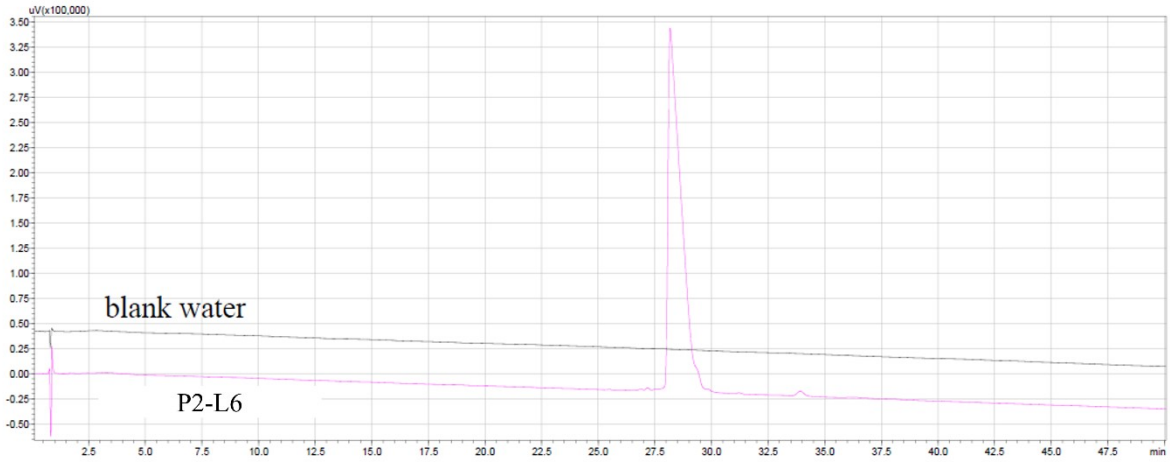
TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	27.637	45103	1.07
2	27.797	80830	1.91
3	28.082	89629	2.12
4	28.316	4018476	94.91





P2-L6



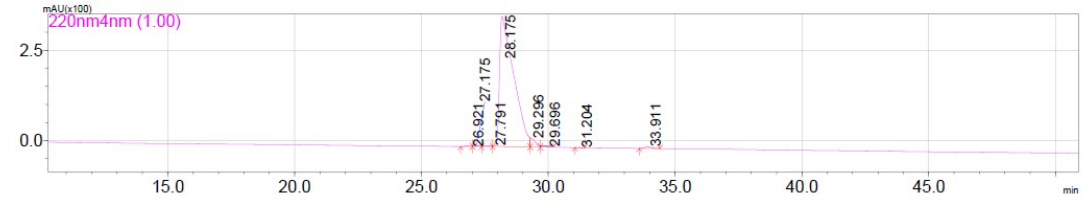
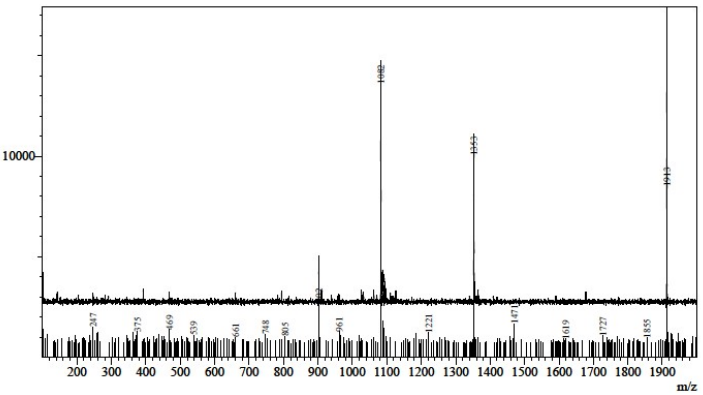
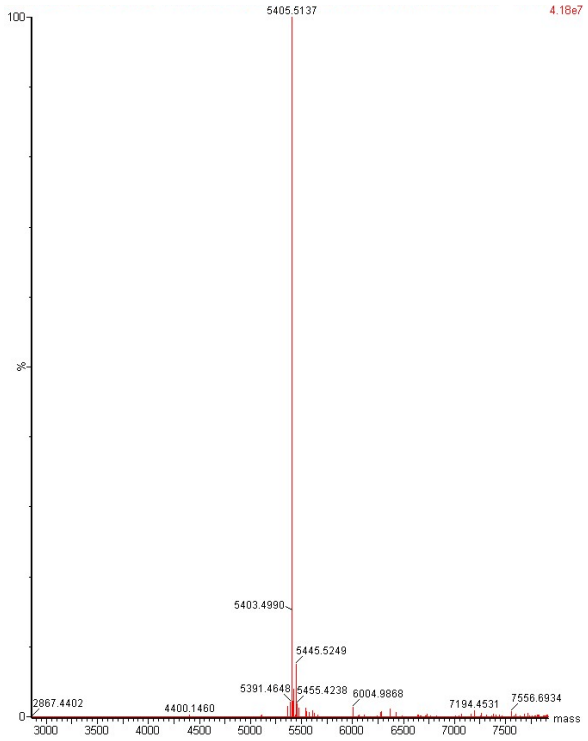


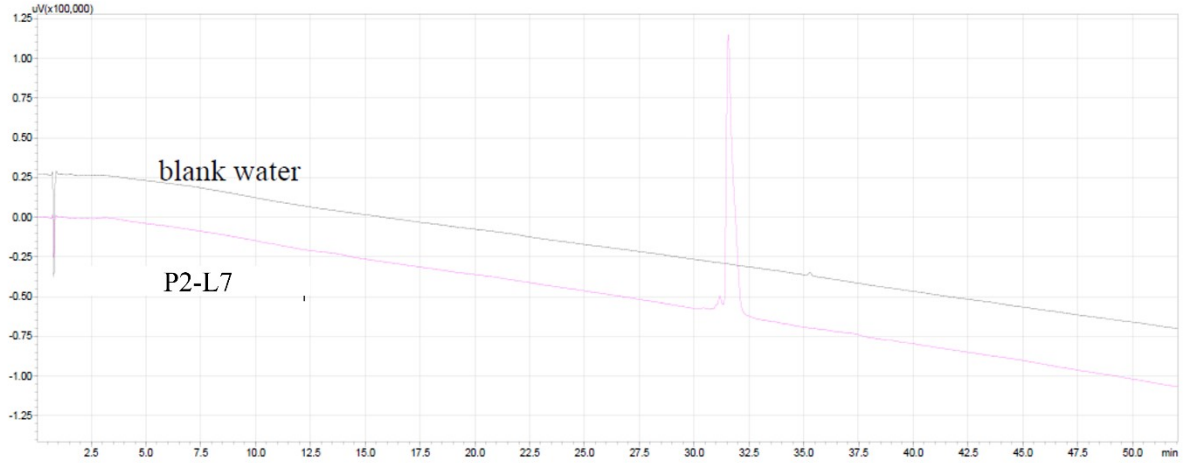
TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	26.921	8567	0.06
2	27.175	19873	0.14
3	27.791	24102	0.17
4	28.175	13944051	96.36
5	29.296	321848	2.22
6	29.696	51212	0.35
7	31.204	10551	0.07
8	33.911	91139	0.63





P2-L7



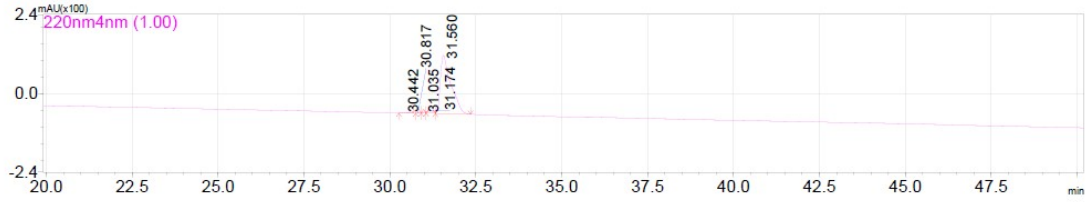
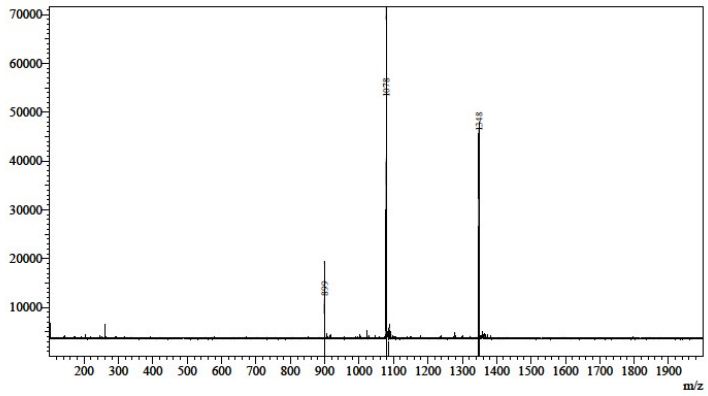


TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	30.442	9794	0.26
2	30.817	1669	0.04
3	31.035	17328	0.45
4	31.174	94767	2.48
5	31.560	3704610	96.77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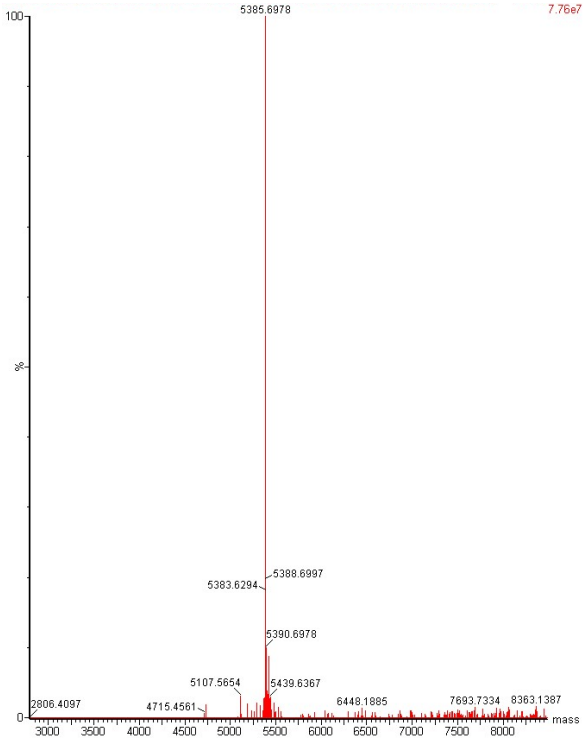
60000
50000
40000
30000
20000
10000
200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900
m/z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%
0
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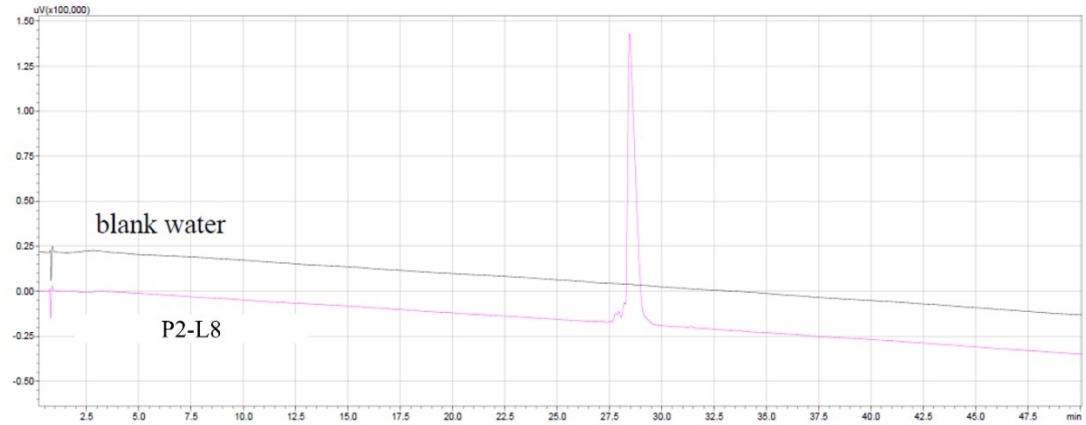
7.76e7
5388.6997
5383.6294
5390.6978
5107.5654
5439.6367
2806.4097
4715.4561
6448.1885
7693.7334
8363.1387
3000 3500 4000 4500 5000 5500 6000 6500 7000 7500 8000
mass

P2-L8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1.25
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
blank water
P2-L8
2.5 5.0 7.5 10.0 12.5 15.0 17.5 20.0 22.5 25.0 27.5 30.0 32.5 35.0 37.5 40.0 42.5 45.0 47.5
min

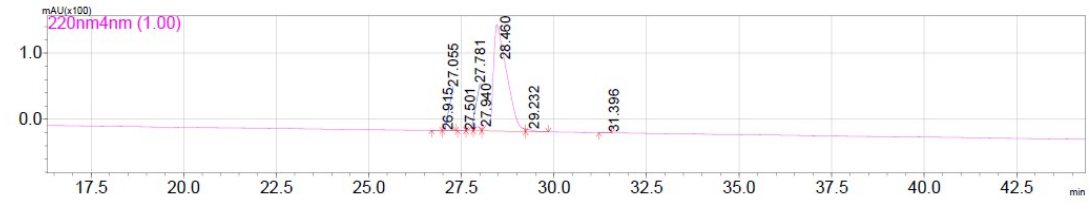
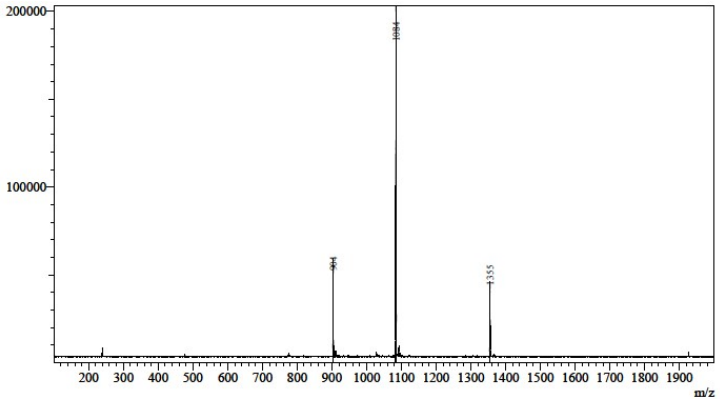
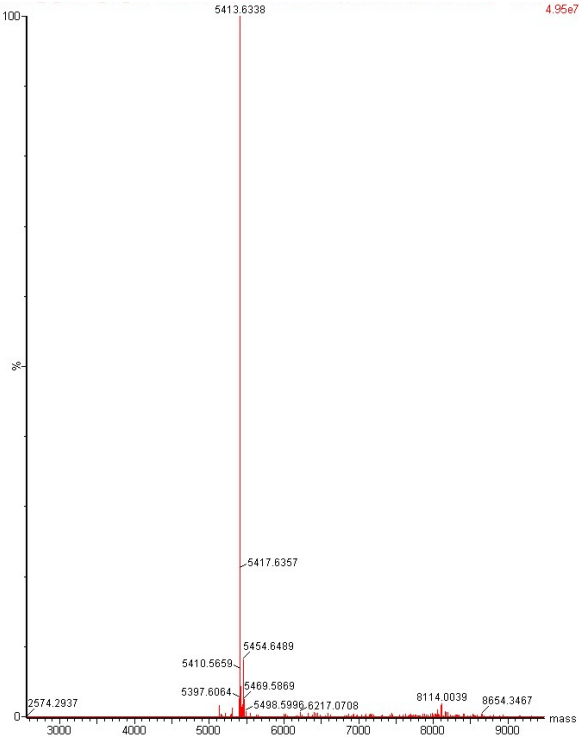


TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	26.915	2330	0.06
2	27.055	4575	0.11
3	27.501	539	0.01
4	27.781	31724	0.75
5	27.940	62508	1.48
6	28.460	4061972	96.48
7	29.232	41506	0.98
8	31.396	4991	0.12





P2-L9

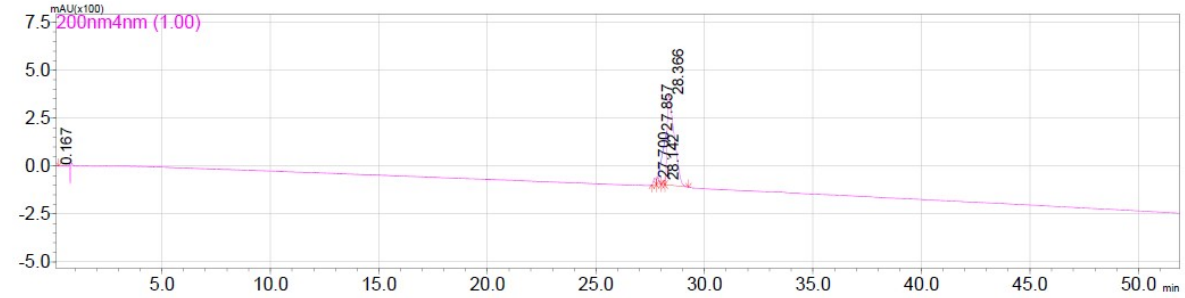
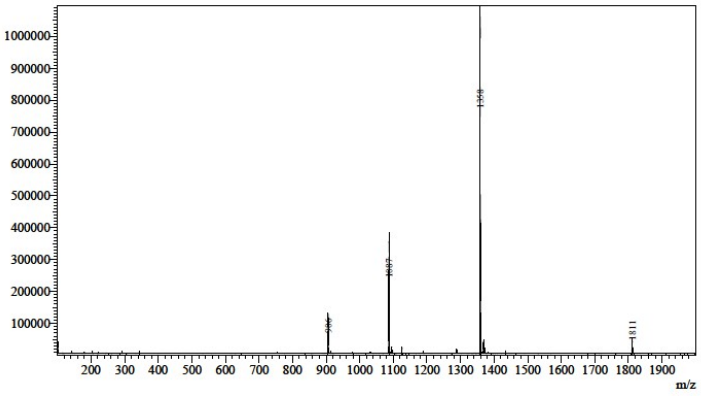
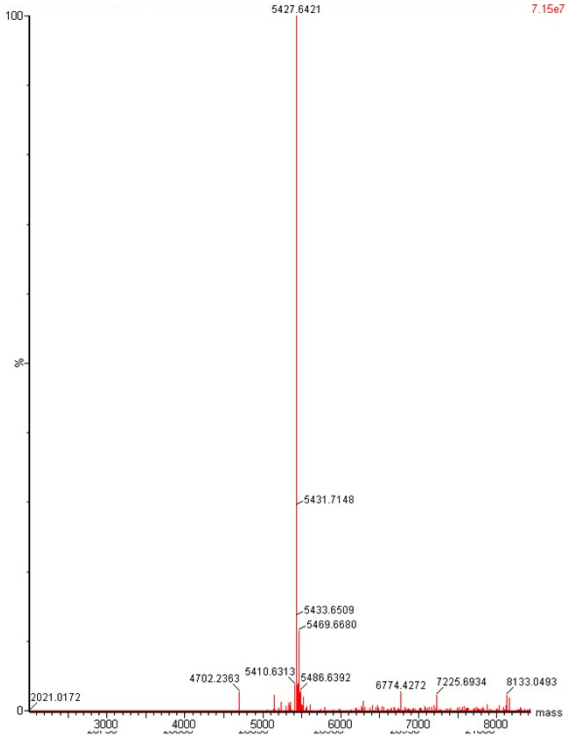


TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	27.700	268322	2.34
2	27.857	289930	2.52
3	28.142	124130	1.08
4	28.366	10799857	94.06





P2-L10

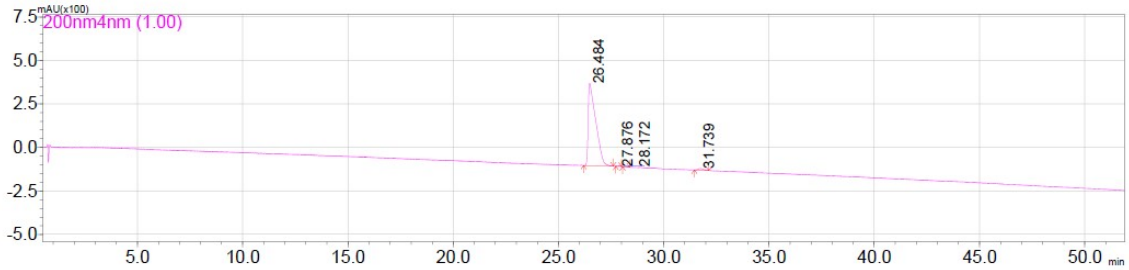
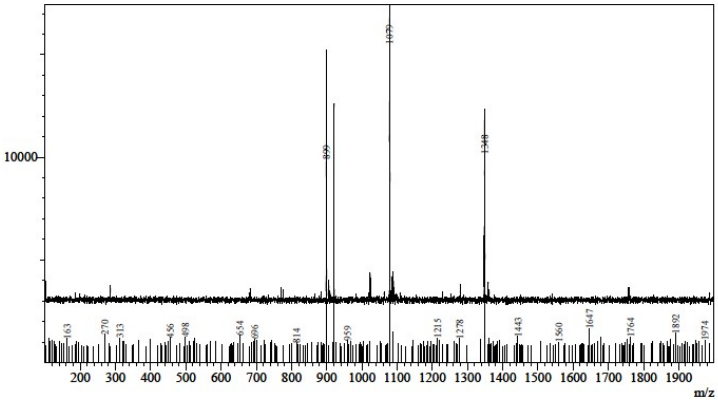
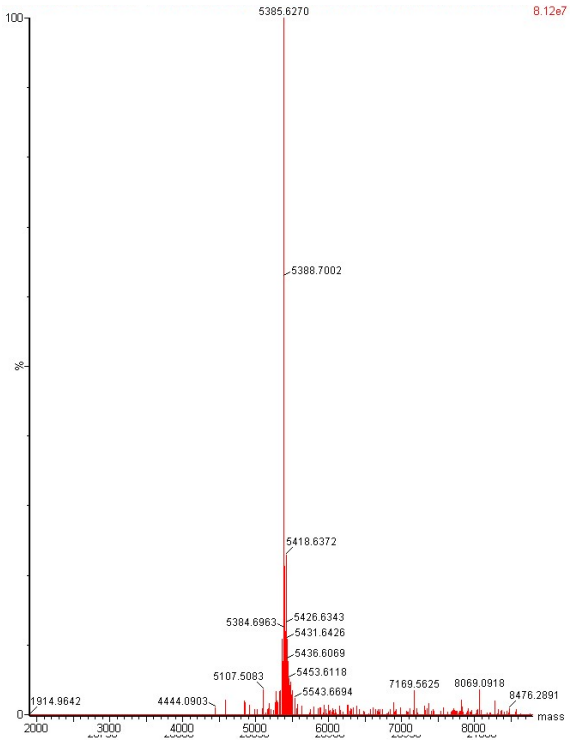


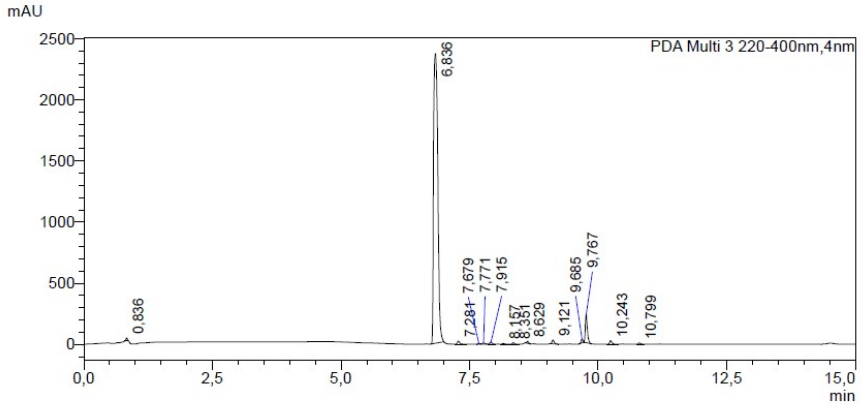
TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	26.484	11763860	98.16
2	27.876	16657	0.14
3	28.172	34487	0.29
4	31.739	169820	1.42





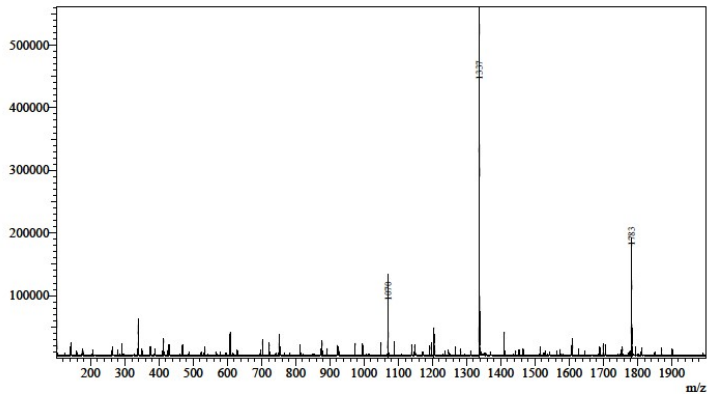
P3-L1

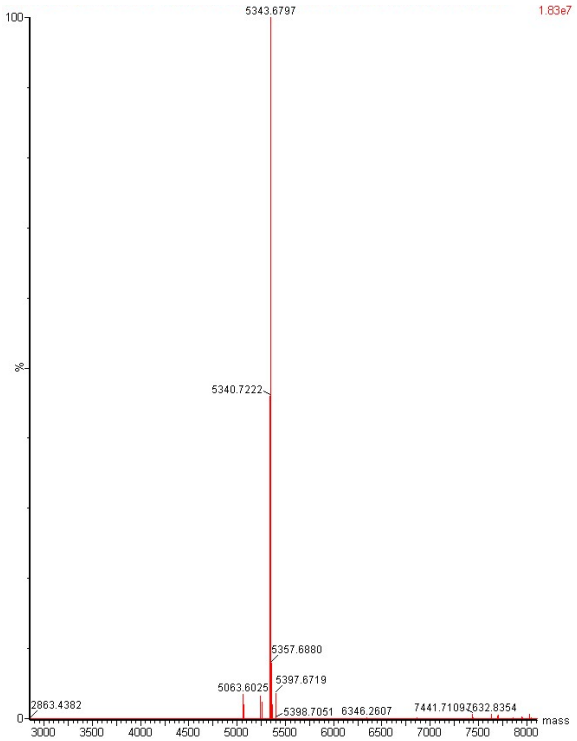


Peak Table

PDA Ch3 220-400nm

Peak#	Ret. Time	Area	Area%
1	0,836	39036	0,265
2	6,836	13507729	91,644
3	7,281	77167	0,524
4	7,679	13360	0,091
5	7,771	31018	0,210
6	7,915	51427	0,349
7	8,157	19885	0,135
8	8,351	35257	0,239
9	8,629	37020	0,251
10	9,121	94248	0,639
11	9,685	72825	0,494
12	9,767	638132	4,329
13	10,243	88477	0,600
14	10,799	33794	0,229
Total		14739375	100,000





P3-L11

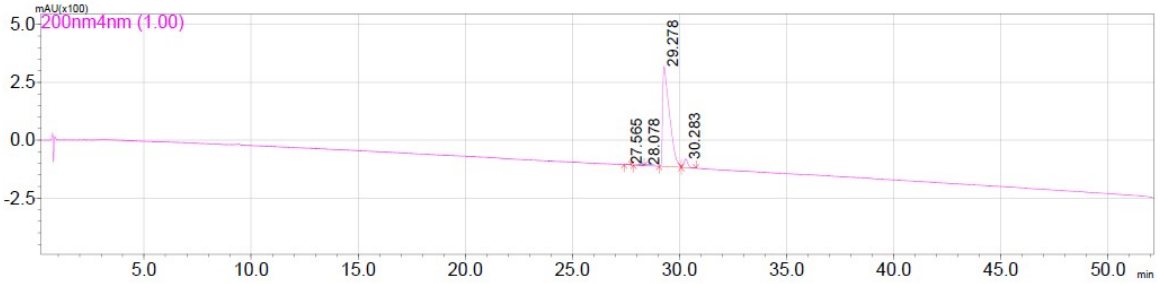
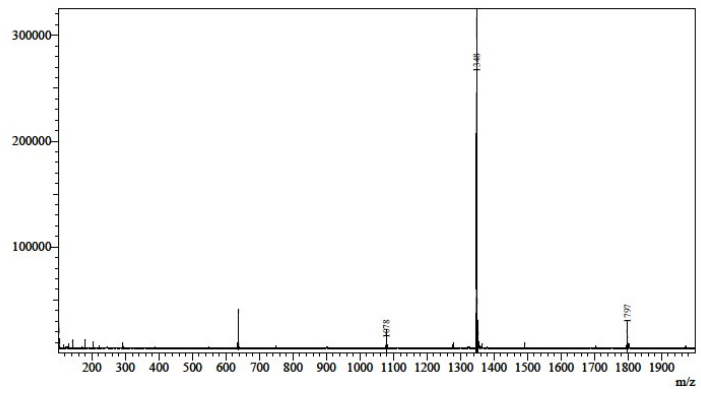
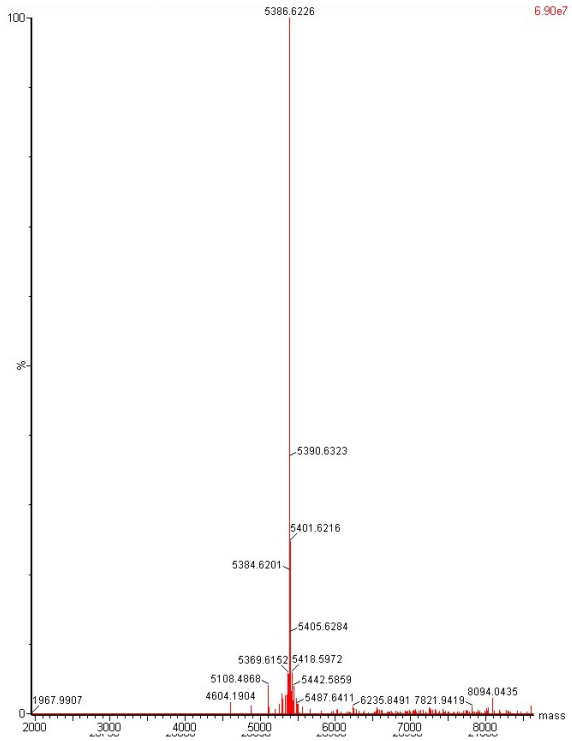


TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	27.585	5010	0.05
2	28.078	16608	0.17
3	29.078	9509587	95.16
4	30.283	462244	4.63





P3-L12

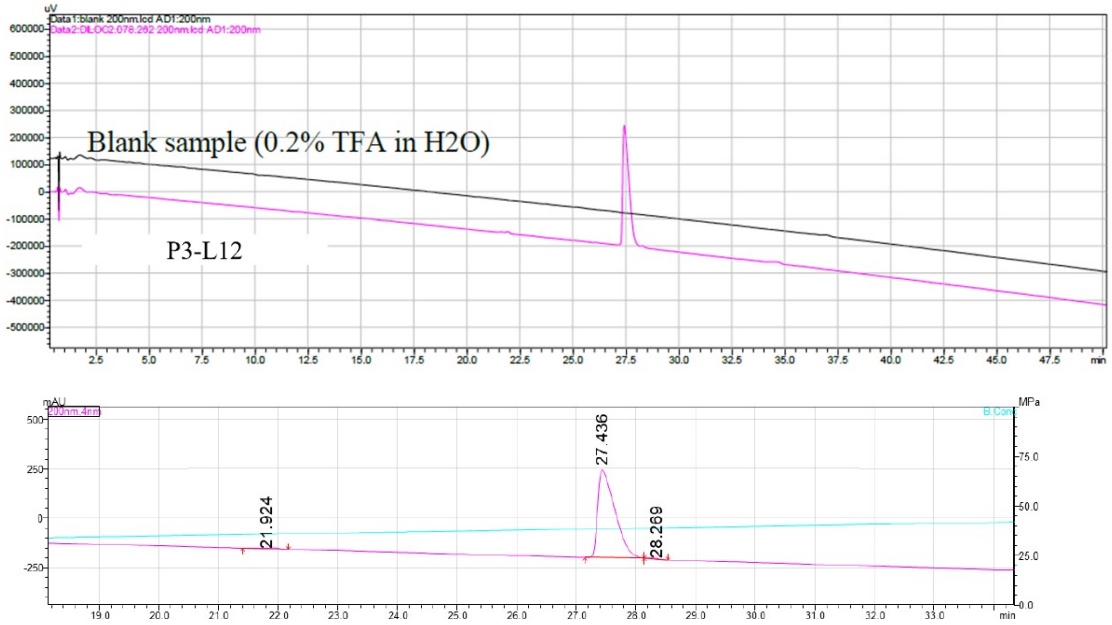
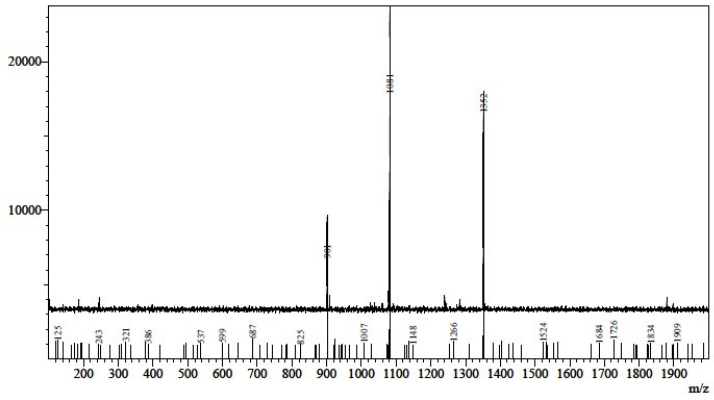
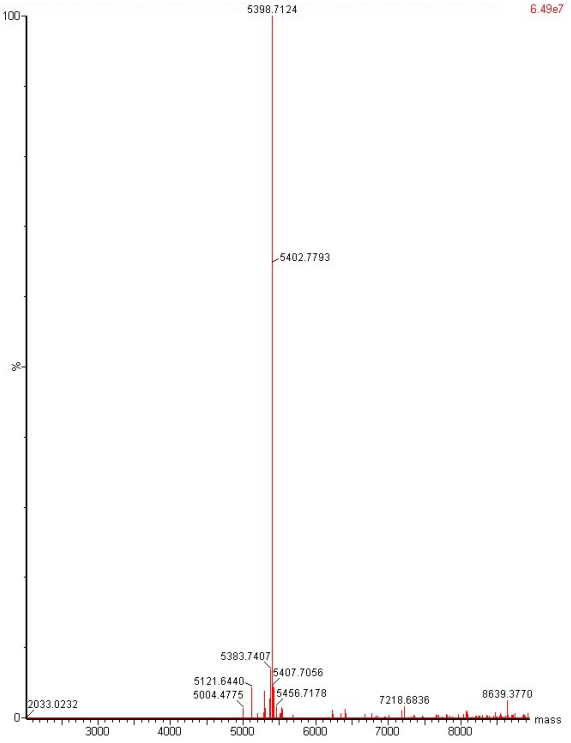


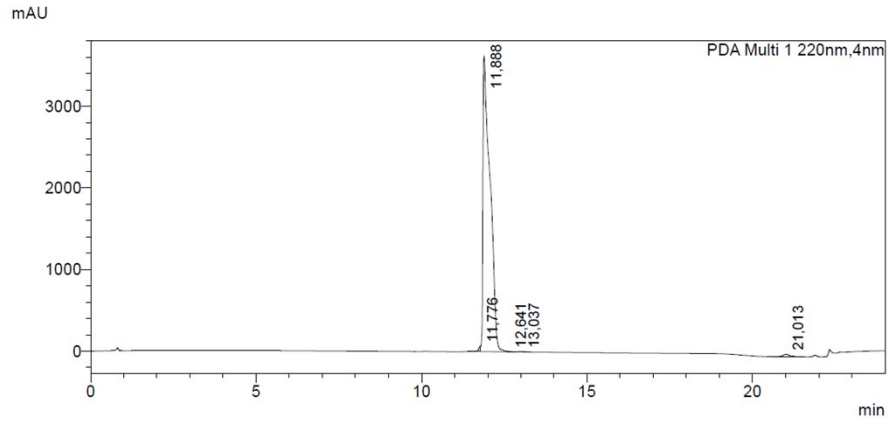
TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	21.924	114664	1.28
2	27.436	88146650	98.31
3	28.269	37023	0.41





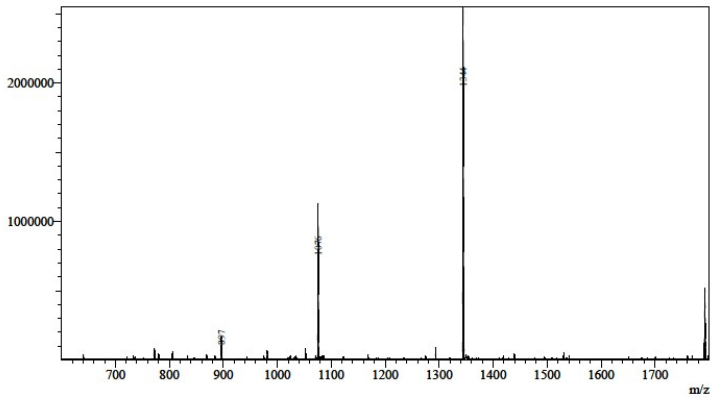
P3-L10



Peak Table

PDA Ch1 220nm

Peak#	Ret. Time	Area	Area%
1	11.776	240583	0.440
2	11.888	53903513	98.649
3	12.641	29450	0.054
4	13.037	35386	0.065
5	21.013	432772	0.792
Total		54641705	100.000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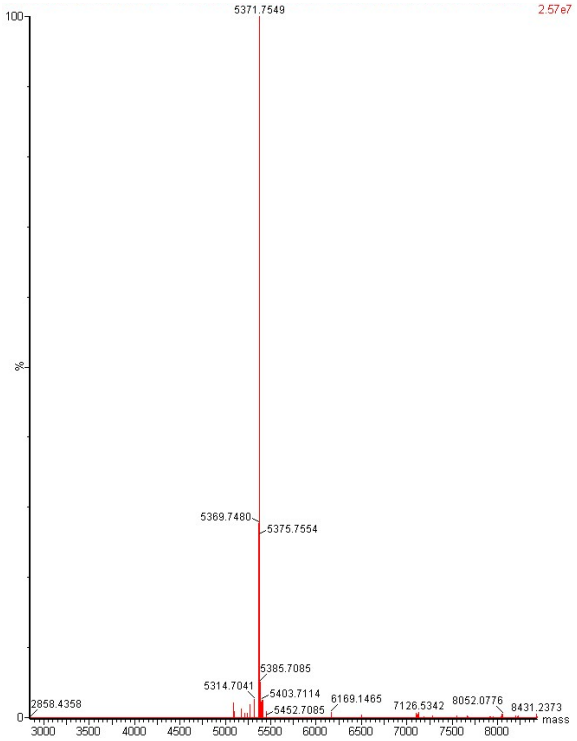
1000000
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
m/z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%
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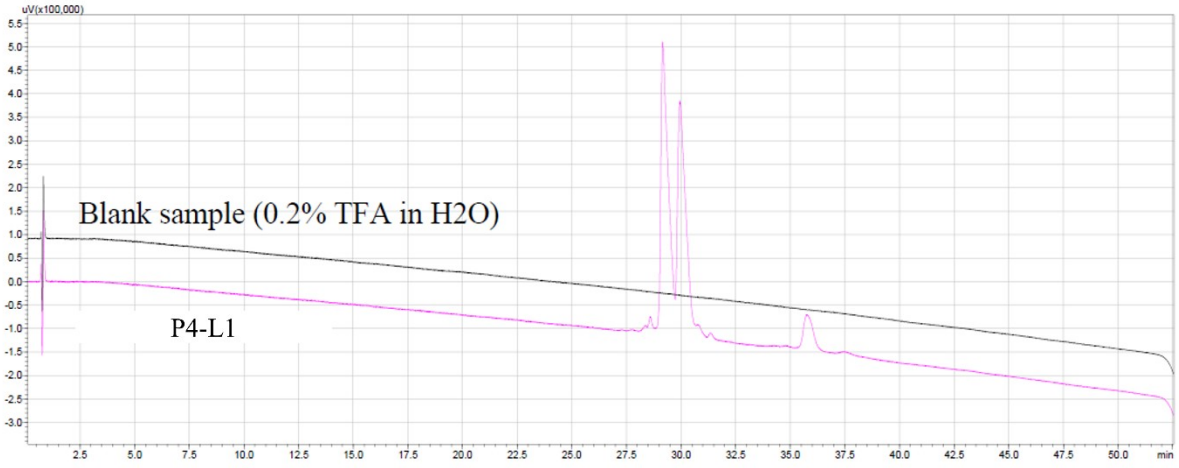
2.57e7
5369.7480
5375.7554
5385.7085
5314.7041
5403.7114
6169.1465
5452.7085
7126.5342
8052.0776
8431.2373
2858.4358
3000
3500
4000
4500
5000
5500
6000
6500
7000
7500
8000
mass

P4-L1


uV(x100,000)
Blank sample (0.2% TFA in H2O)
P4-L1
min

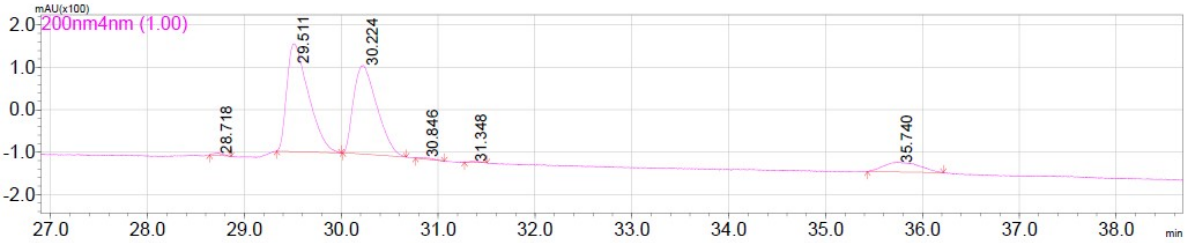
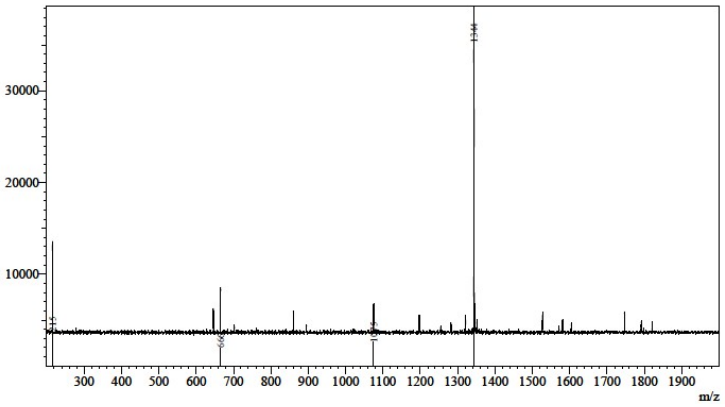
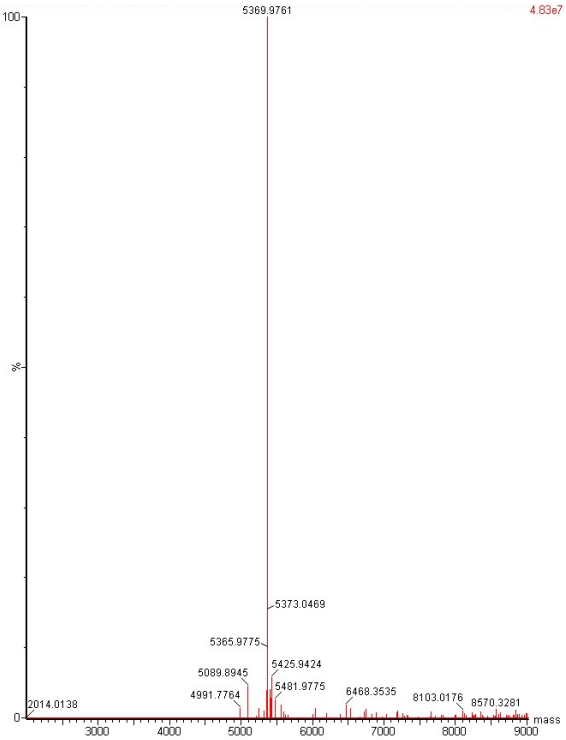


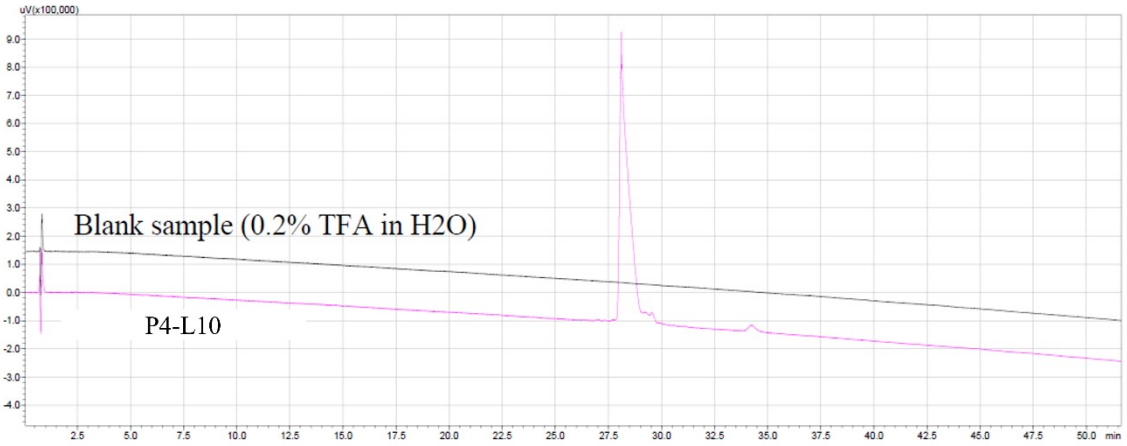
TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	28.718	36479	0.45
2	29.511	3944174	48.50
3	30.224	3505858	43.11
4	30.846	32779	0.40
5	31.348	19805	0.24
6	35.740	590956	7.27





P4-L10



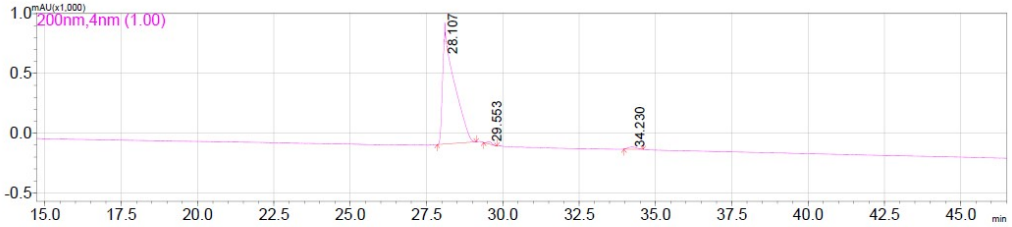
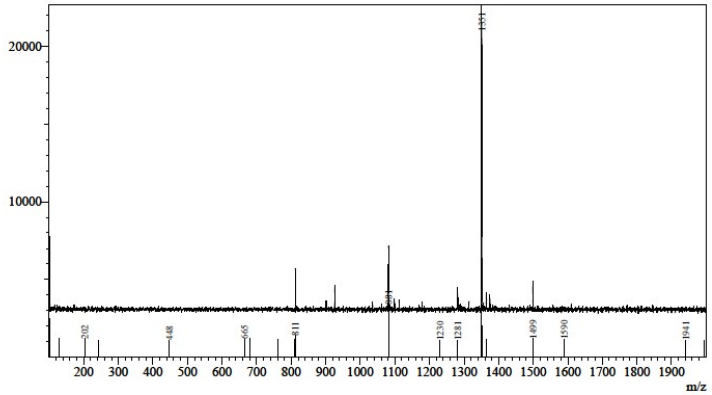
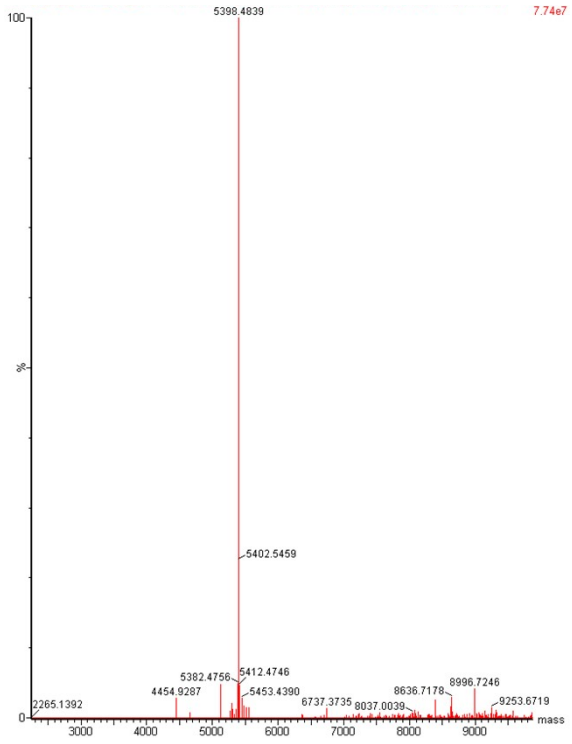


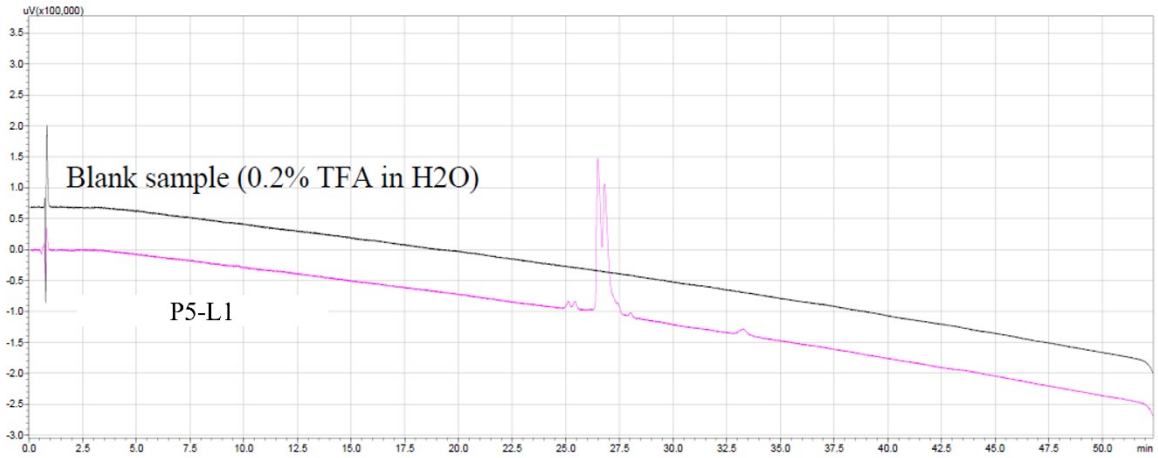
TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	28.107	25937432	97.83
2	29.553	202905	0.77
3	34.230	372624	1.41





P5-L1



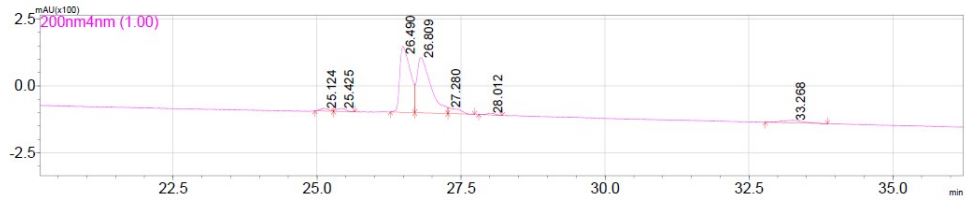
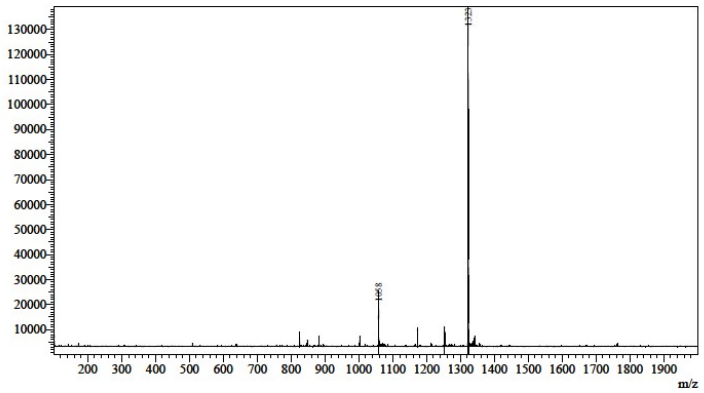
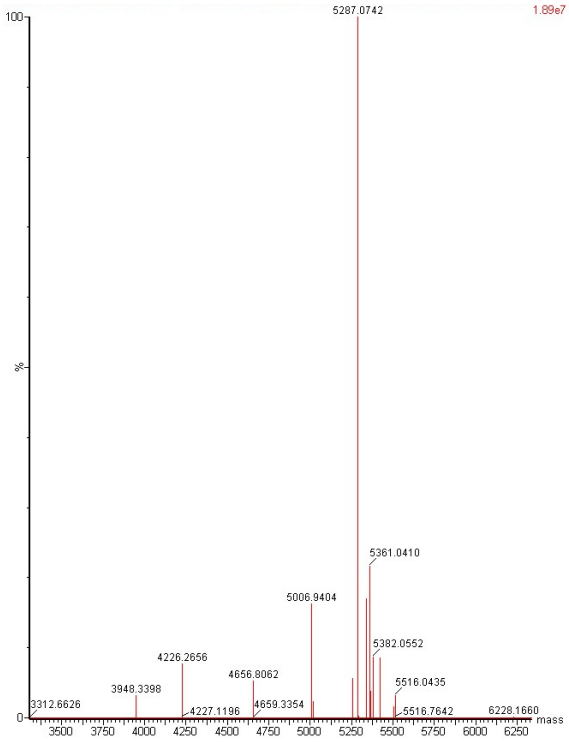


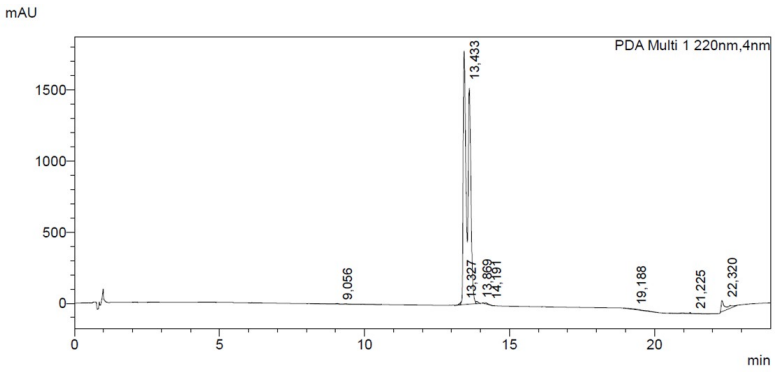
TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	25.124	101927	1.38
2	25.425	126294	1.71
3	26.490	3108165	41.99
4	26.809	3476873	46.97
5	27.280	292296	3.95
6	28.012	64220	0.87
7	33.268	224955	3.04





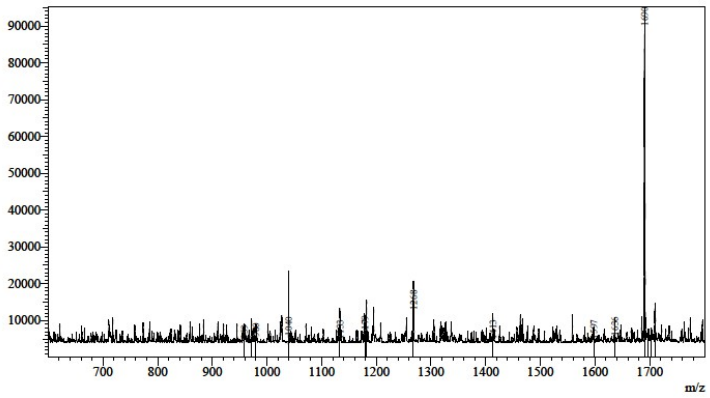
P6-L1

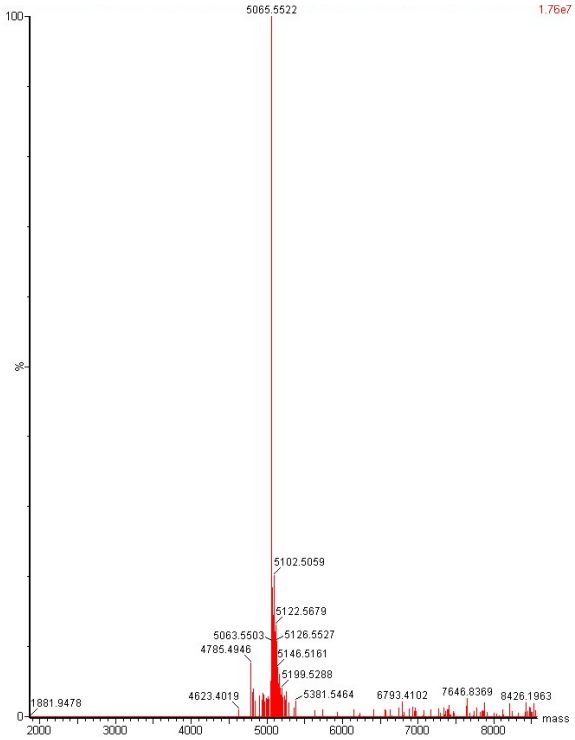


Peak Table

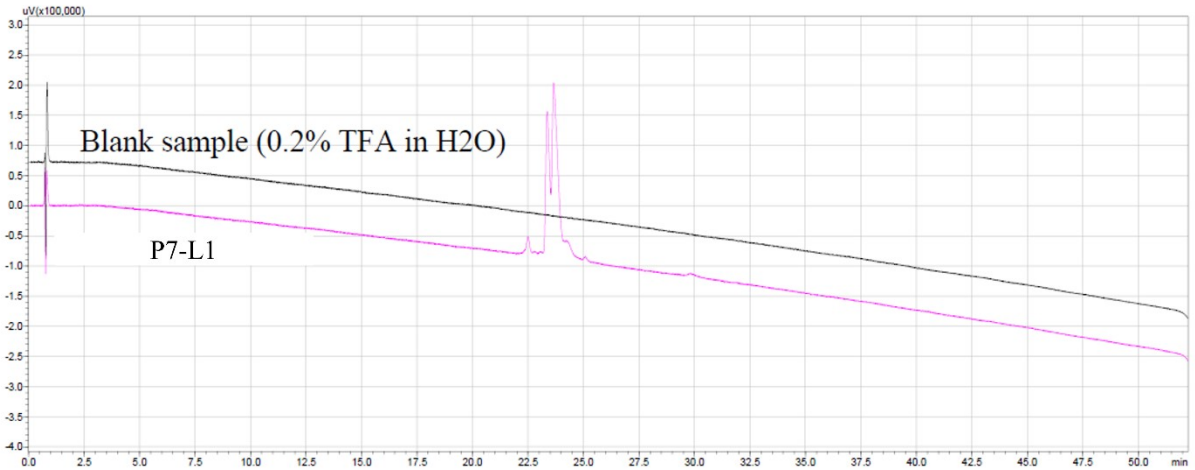
PDA Ch1 220nm

Peak#	Ret. Time	Area	Area%
1	9.056	34377	0.154
2	13.327	49273	0.221
3	13.433	21183755	94.845
4	13.869	77764	0.348
5	14.191	75917	0.340
6	19.188	146223	0.655
7	21.225	15122	0.068
8	22.320	752781	3.370
Total		22335212	100.000


m/z


5065.5522
1.76e7
%
5102.5059
5122.5679
5063.5503
5126.5527
4785.4946
5146.5161
5199.5288
4623.4019
5381.5464
6793.4102
7646.8369
8426.1963
1881.9478
mass

P7-L1


uV(x100,000)
Blank sample (0.2% TFA in H2O)
P7-L1
min

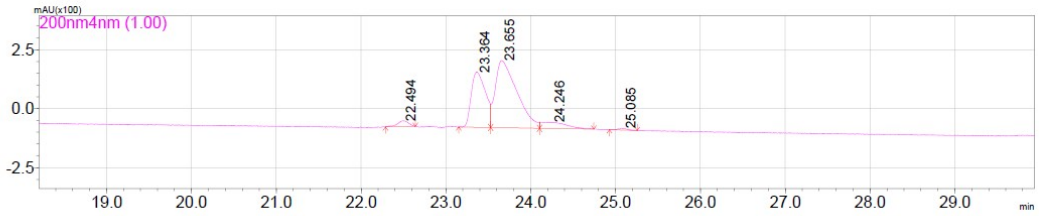
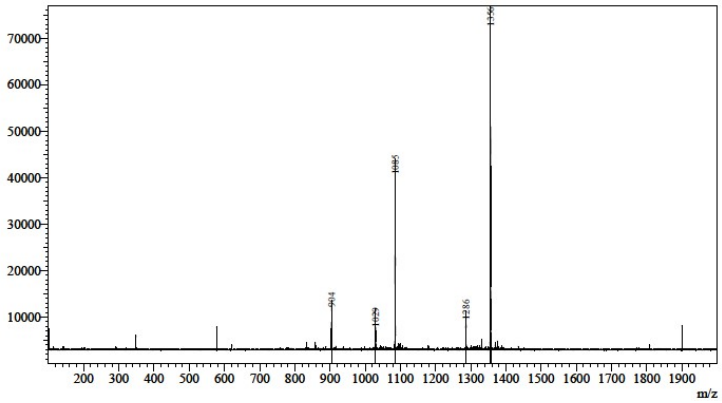
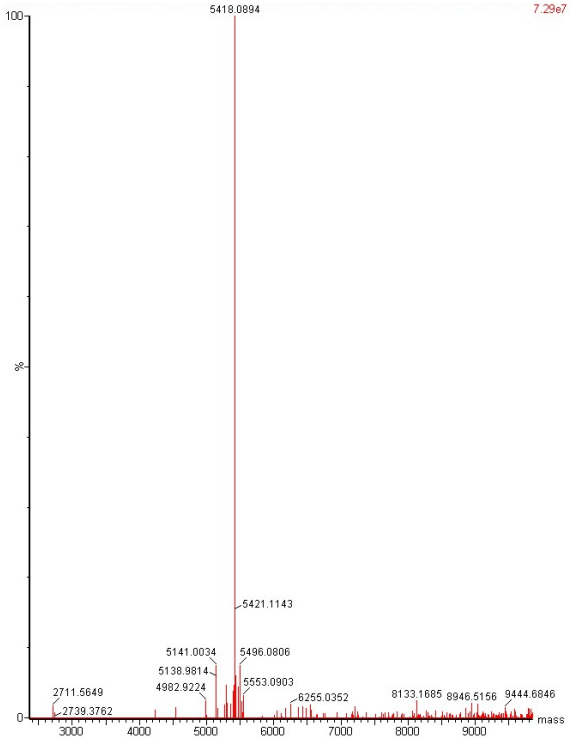


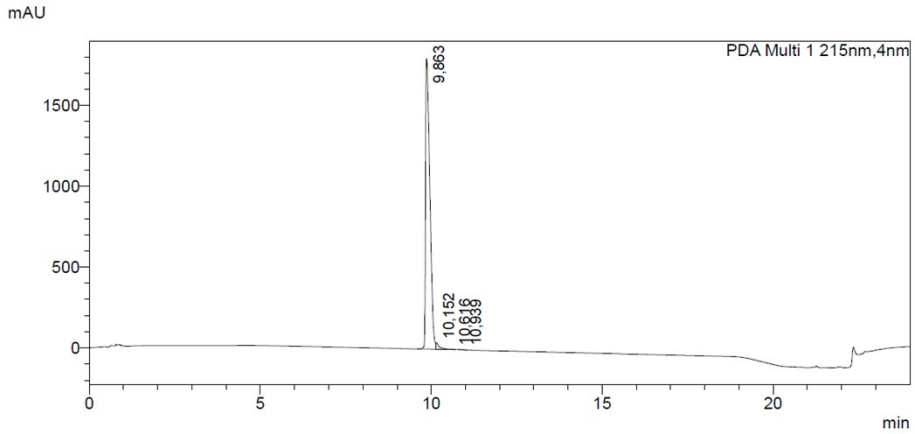
TABLE 1 PURITY

Peak no.	RT [min]	Area	%Area
1	22.494	223841	2.60
2	23.364	2731416	31.77
3	23.655	5046674	58.71
4	24.246	540256	6.28
5	25.085	54003	0.63





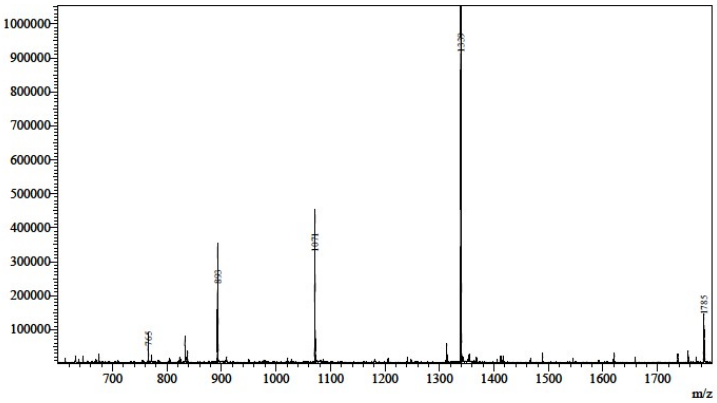
P8-L13

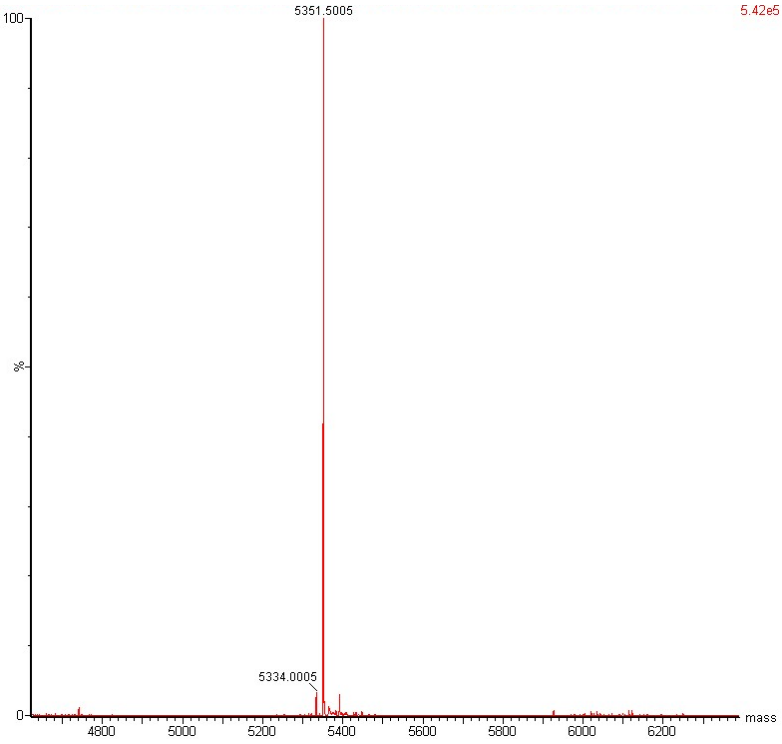


Peak Table

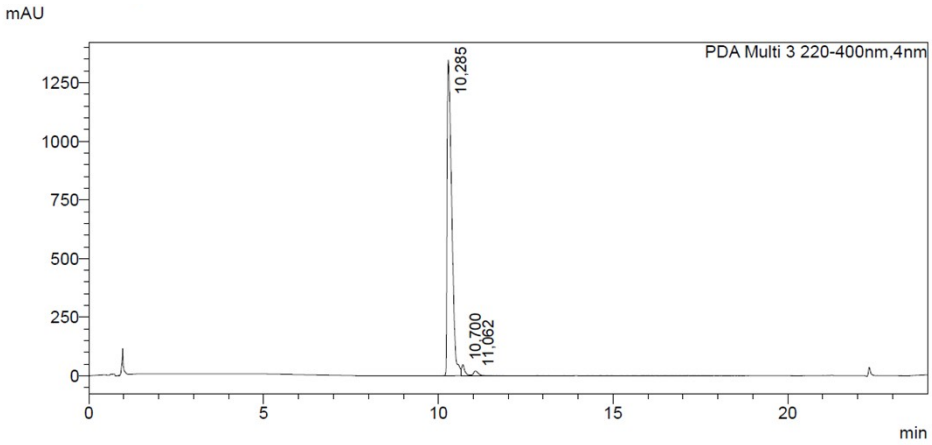
PDA Ch1 215nm

Peak#	Ret. Time	Area	Area%
1	9.863	15714122	98.332
2	10.152	255566	1.599
3	10.616	4804	0.030
4	10.939	6266	0.039
Total		15980758	100.000





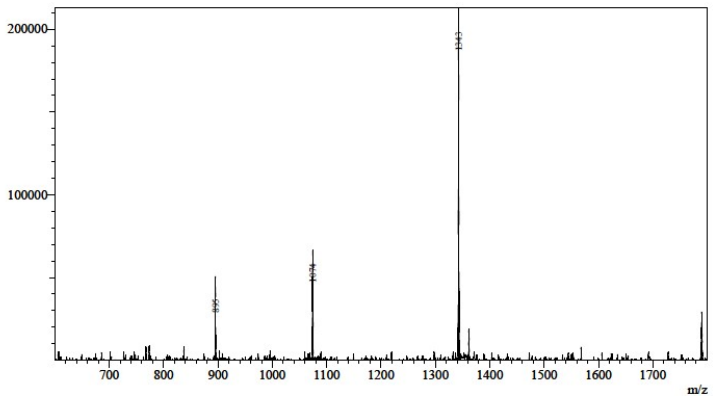
P8-L14

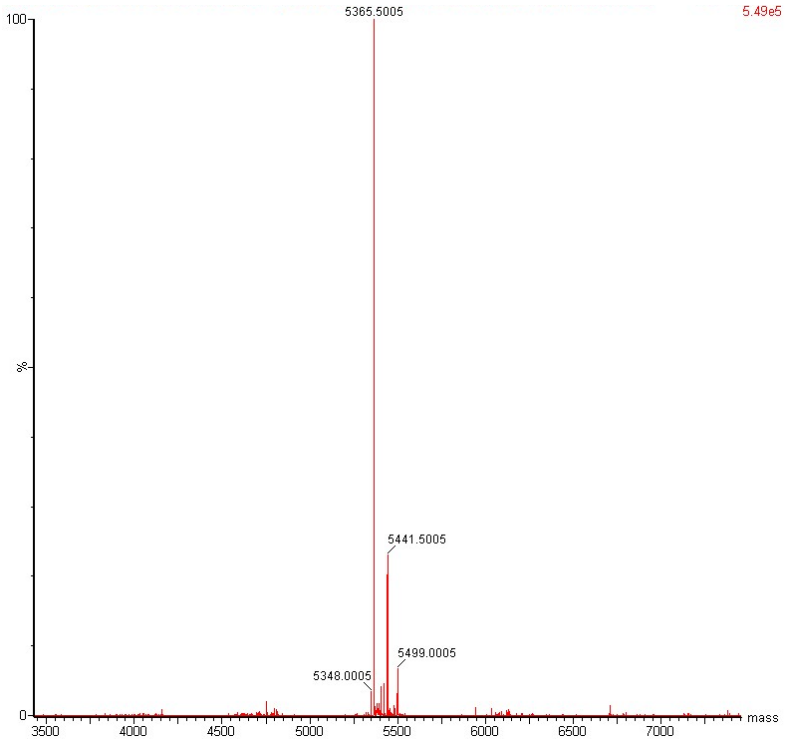


Peak Table

PDA Ch3 220-400nm

Peak#	Ret. Time	Area	Area%
1	10,285	12269526	95,985
2	10,700	311187	2,434
3	11,062	202074	1,581
Total		12782787	100,000




5365.5005
5.49e5
100
%
0
5441.5005
5499.0005
5348.0005
3500
4000
4500
5000
5500
6000
6500
7000
mass