

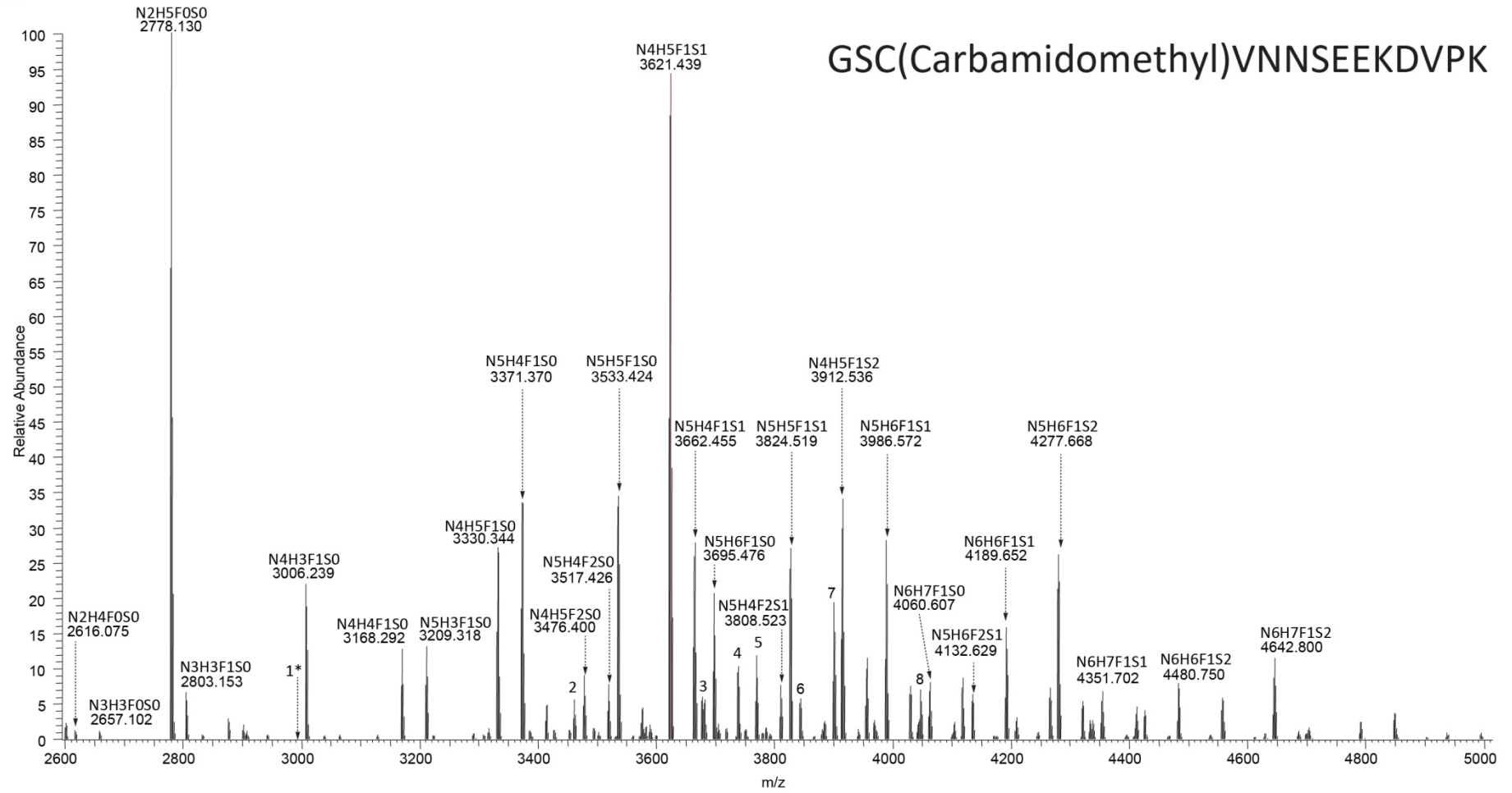
From: Glycoengineering of EphA4 Fc leads to a unique, long-acting and broad spectrum, Eph receptor therapeutic antagonist

Cassandra L. Pegg, Leanne T. Cooper, Jing Zhao, Michael Gerometta, Fiona M. Smith, Michael Yeh, Perry F. Bartlett, Jeffrey J. Gorman and Andrew W. Boyd

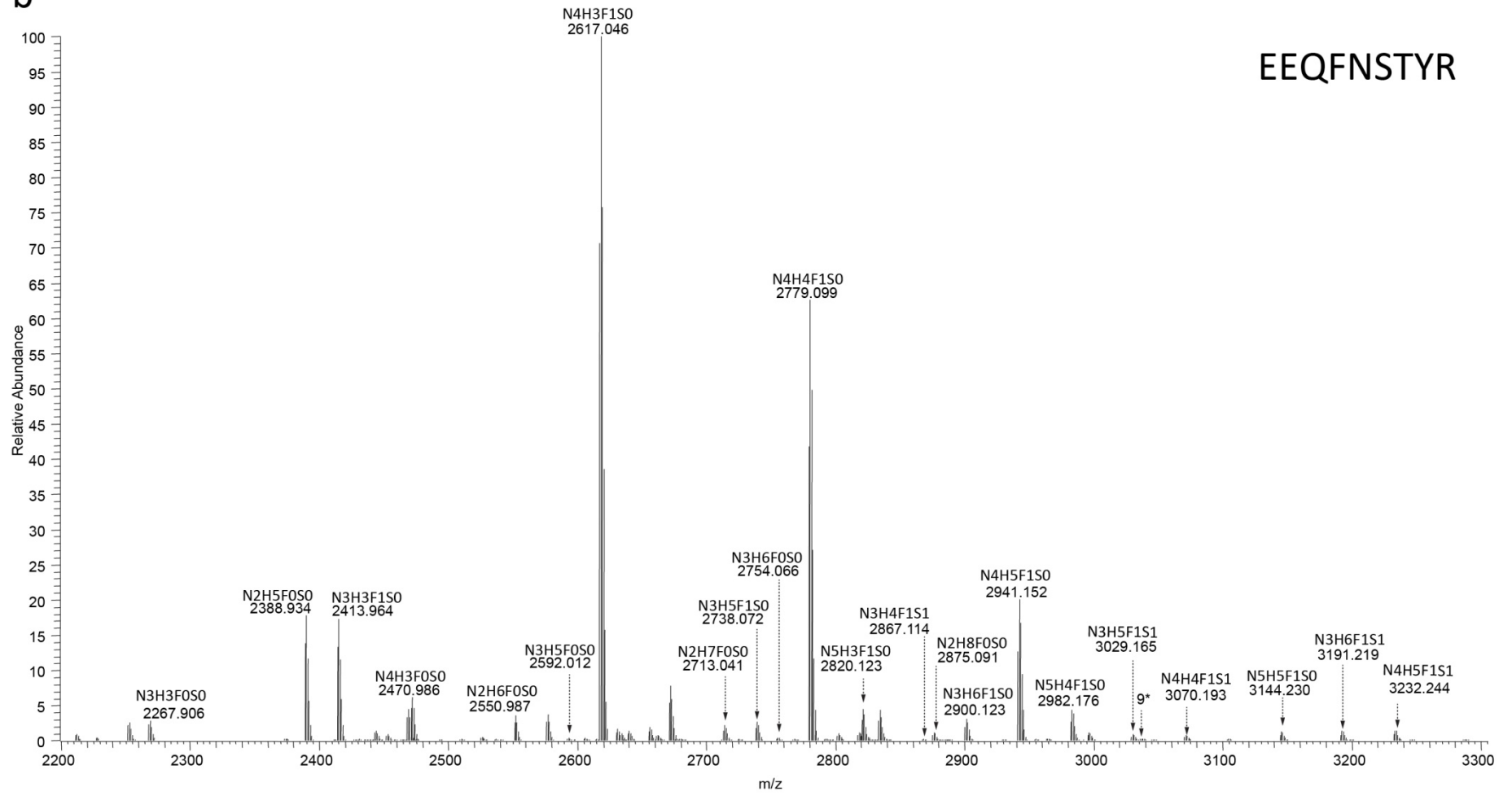
1 MAGIFYFALF SCLFGICDAV TGSRVYPANE VTLLDSRSVQ GELGWIASPL EGGWEEVSIM DEKNTPIRTY QVCNVMEPSQ
 81 NNWLRTDWIT REGAQRVYIE IKFTLRDCNS LPGVMGTCKE TFNLYYYESD NDKERFIREN QFVKIDTIAA DESFTQVDIG
 161 DRIMKLNTEI RDVGPLSKKG FYLAFQDVGA CIALVSVRVF YKCKPLTVRN LAQFPDITG ADTSSLVEVR GSCVNNSEEK
 241 DVPKMYCGAD GEWLVPIGNC LCNAGHEERS GECQACKIGY YKALSTDATC AKCPPHSYSV WEGATSCTCD RGFFRADNDA
 321 ASMPCTRPPS APLNLISNVN ETSVNLEWSS PQNTGGRQDI SYNVVCKKCG AGDPSKCRPC GSGVHYTPQQ NGLKTTKVISI
 401 TDLLAHTNYT FEIWA VNGVS KYNPNPDQSV SVTVTTNQA A PSSIALVQAK EVTRYSVALA WLEPDRPNGV ILEYEVKYYE
 481 KDQNERYSRI VRTAARNTDI KGLNPLTSYV FHVRARTAAG YGDFSEPLEV TTNTVPSRII GDGANSESKY GPPCPPCPAP
 561 EFLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSOEDPE VQFNWYVDGV EVHNAKTKPR EEQFNSTYRV VSVLTVLHQD
 641 WLNGKEYKCK VSNKGLPSSI EKTISKAKGQ PREPQVYTL PSEQEEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK
 721 TTPPVLDSDG SFFLYSRLTV DKSRWQEGNV FSCSVMHEAL HNHYTQKSL SLSLKG

Supplementary Figure S1. Sequence coverage of EphA4 Fc. Protein sequence coverage of EphA4 Fc based on identified tryptic peptides by HCD MS/MS. Sequence coverage derived from the Mascot search has been highlighted in dark grey.

a

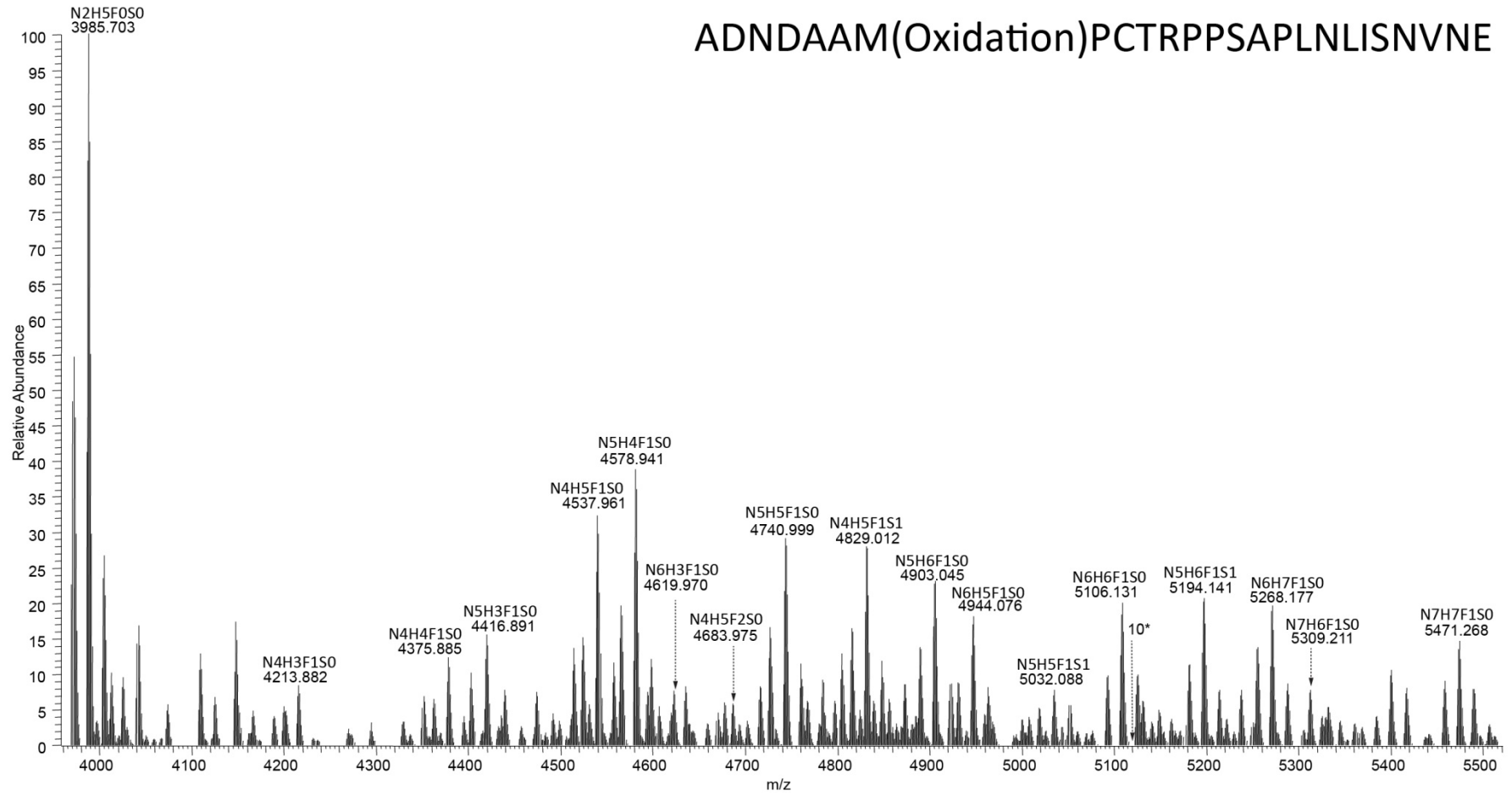


b

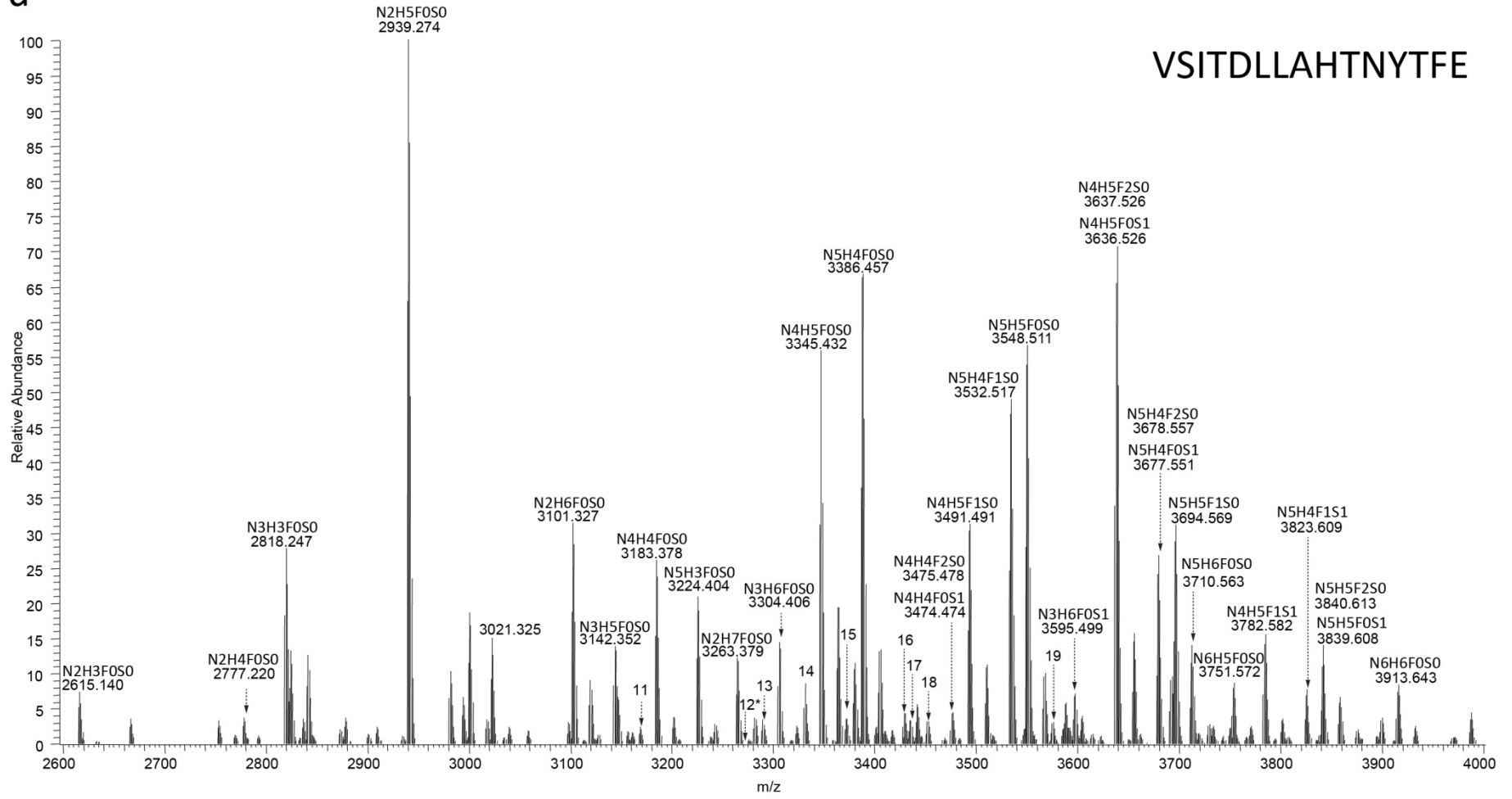


EEQFNSTYR

C

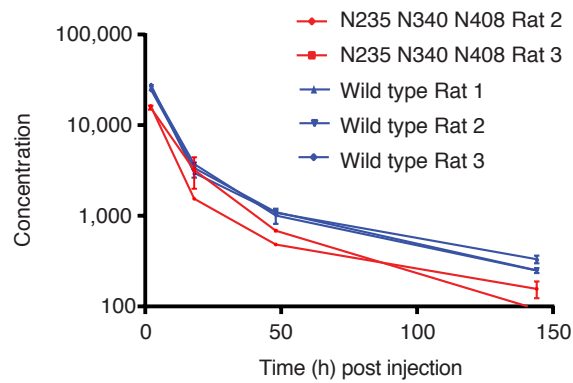


d



Supplementary Figure S2. Deconvoluted spectra identifying glycopeptides containing each N-linked site from wildtype EphA4 Fc.

Glycan heterogeneity observed on EphA4 Fc trypsin digested peptides GSC(Carbamidomethyl)VNNSEEKDVDPK (Panel. a) and EEQFNSTYR (Panel. b) and trypsin/Glu-C digested peptides ADNDAASM(oxidation)PCTRPPSAPLNLISNVNE (Panel. c) and VSITDLLAHTNYTFE (Panel. d) representing N-linked sites N235, N625, N340 and N408, respectively. MS precursor spectra were summed across the elution period for all glycoforms and deconvoluted from multiply charged signals to unprotonated monoisotopic masses using the Xtract feature within Xcalibur Qual Browser. Major signals representing glycopeptide precursors have been labelled with glycan compositions and precursor masses in the relevant sections of each spectrum. N= N-Acetylhexosamine, H= Hexose, F= Fucose (Deoxyhexose), S= Sialic acid (N-Acetylneuraminic acid). For ease of interpretation some of the minor signals have been labelled with numbers representing the corresponding glycan compositions: 1. N3H4F1S0; 2.N4H4F1S1; 3.N5H5F2S0; 4.N6H5F1S0; 5.N4H5F2S1; 6.N5H6F2S0; 7.N6H6F1S0; 8. N6H6F2S0; 9. N2H9F0S0; 10. N4H5F1S2; 11.N4H3F1S0; 12.N3H4F0S1; 13.N3H5F1S0; 14.N4H4F1S0; 15.N5H3F1S0; 16.N6H3F0S0; 17.N3H5F0S1; 18.N3H6F1S0; 19.N6H3F1S0. Numbers with an asterisk “*” denote precursors that were not present in the deconvoluted spectra. Please note that in one panel (Panel. d) four signals (~ m/z 3475, 3637, 3678 and 3840) have each been assigned two glycan compositions. The glycan compositions at each signal have the same number of HexNAc and Hex residues but one composition includes two fucose residues and the second a sialic acid residue. This occurs because the mass difference between two fucose (Fuc) residues and one sialic acid residue is 1.020 Da while the mass difference between ¹³C and ¹²C isotopic peaks is 1.003 Da, thus when summing and deconvoluting spectra the isotopic profiles overlap. Each composition was previously assigned based on retention time, precursor mass and the presence of relevant oxonium ions for Fuc and Sialic acid in the MS/MS spectra.



Supplementary Figure S3. Pharmacokinetic analysis of double mutant and wildtype EphA2 Fc proteins. Sandwich ELISA of the dual mutated EphA2 Fc (N407 and N435) and un-mutated EphA2 Fc (wild type) protein clearance in *Rattus norvegicus*. The numbering of asparagine residues that were mutated to glutamine corresponds to that of the amino acid sequence of human Ephrin type-A receptor 2 (UniProt identifier P29317). Data could not be obtained for Rat 1 injected with the double mutant of EphA2 Fc. The AUC_{last} was 431,000 ng.h/ml and 261,000 ng.h/ml for the wild type and triple mutant, respectively.