Thermostability of a recombinant G protein-coupled receptor expressed at high level in mammalian cell cultures

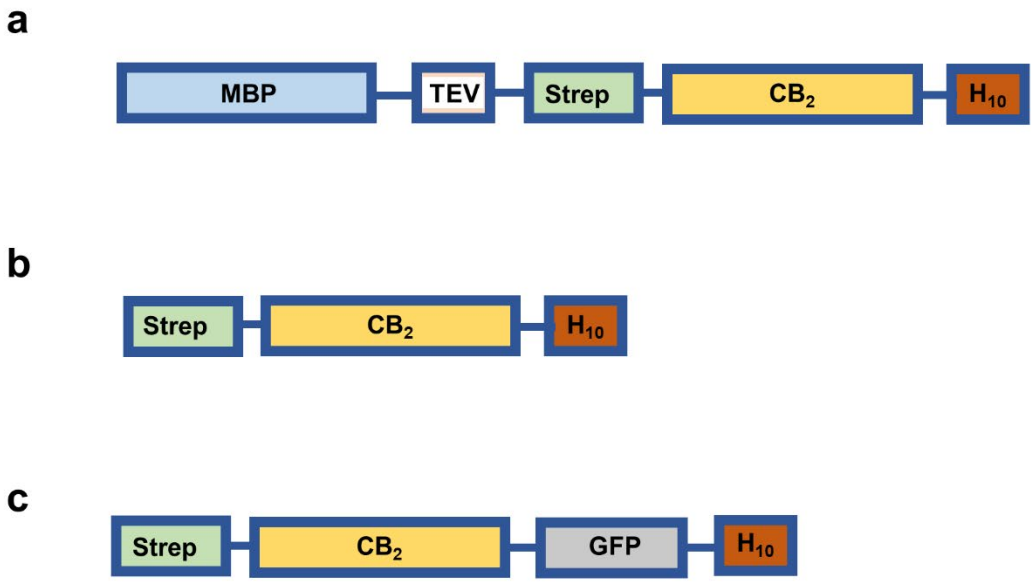
Alexei Yeliseev[1]*, Arjen van den Berg[2], Lioudmila Zoubak[1], Kirk Hines[1], Sam Stepnowski[2], Kyle Williston[2], Wanhua Yan[2], Klaus Gawrisch[1], Jonathan Zmuda[2]

[1] National Institute on Alcoholism and Alcohol Abuse, NIH, 5625 Fishers Lane, Room 3N11, Rockville, MD 20815, USA and

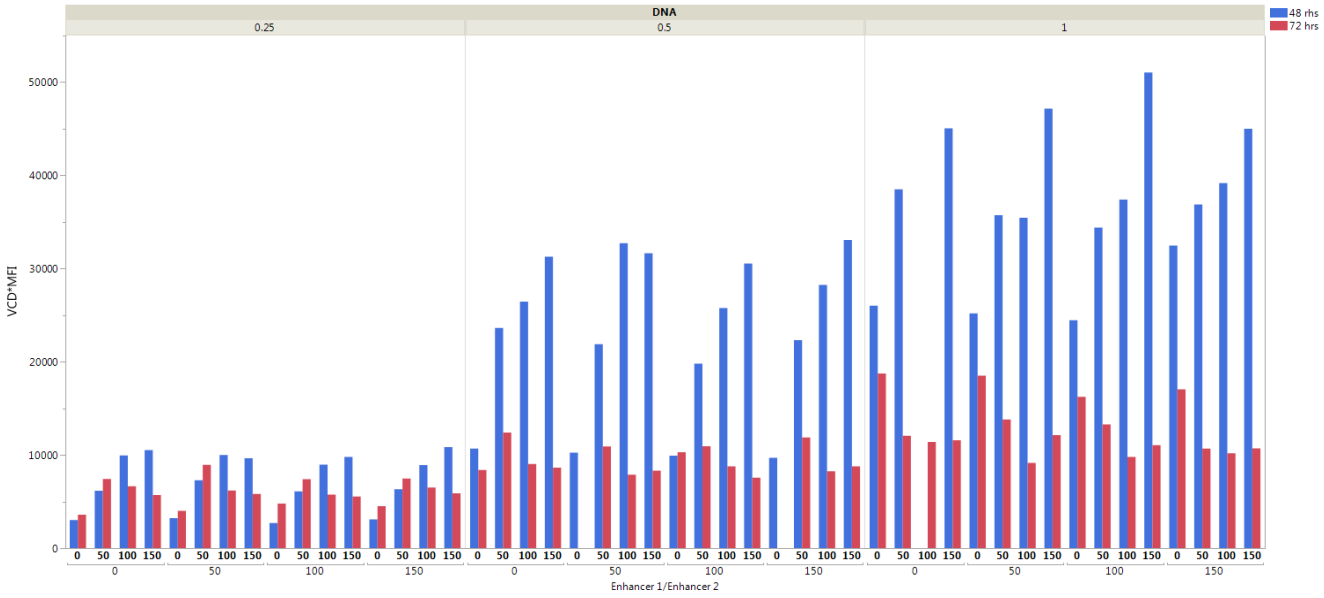
[2] ThermoFisher Scientific, 7335 Executive Way, Fredrick, MD 21704, USA

*Address correspondence to:
Alexei Yeliseev
National Institute on Alcoholism and Alcohol Abuse, NIH,
5625 Fishers Lane, Room 3N11, Rockville, MD 20815, USA
Phone: +1 3014430552
Email: yeliseeva@mail.nih.gov

Supplementary Data

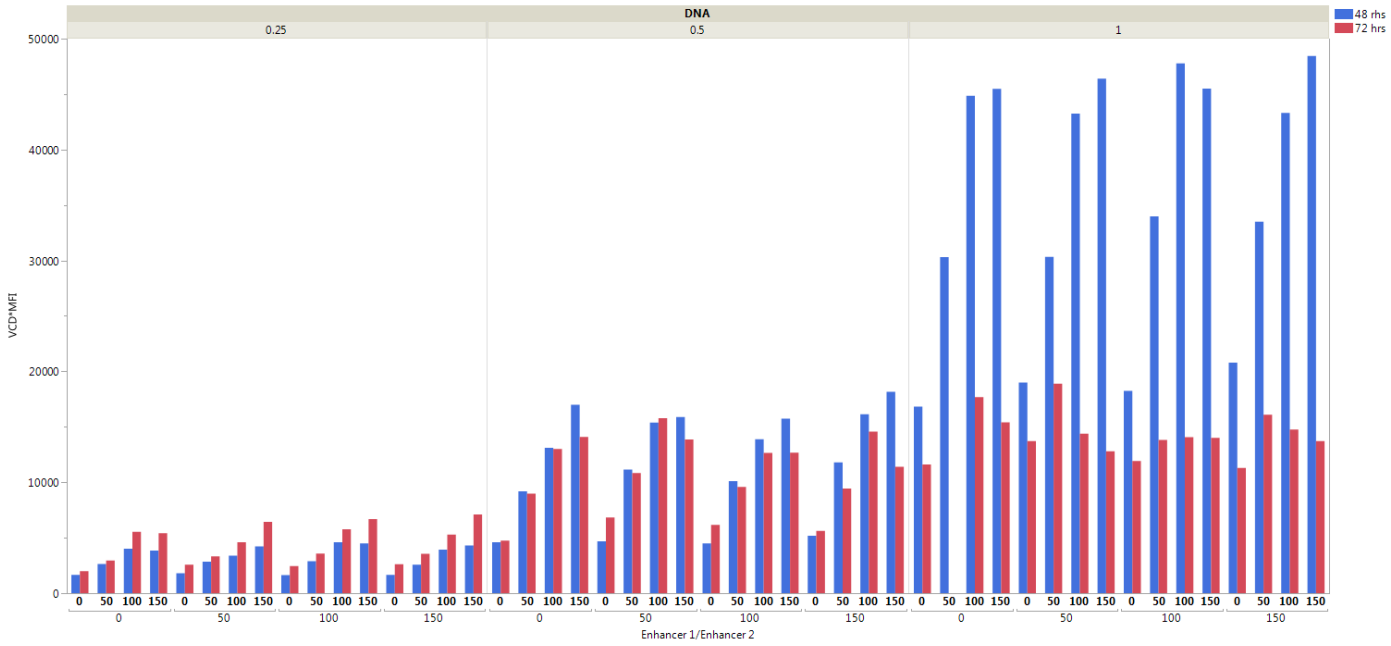


Supplementary Figure 1. **Constructs for expression of CB_2. a**, MBP-CB_2 fusion construct for expression in *E. coli* BL21(DE3).; **b**, CB_2 construct for expression in mammalian cell lines, in pCDNA3.4; **c**, CB_2-GFP construct for expression in Expi cell lines, in pCDNA3.4.



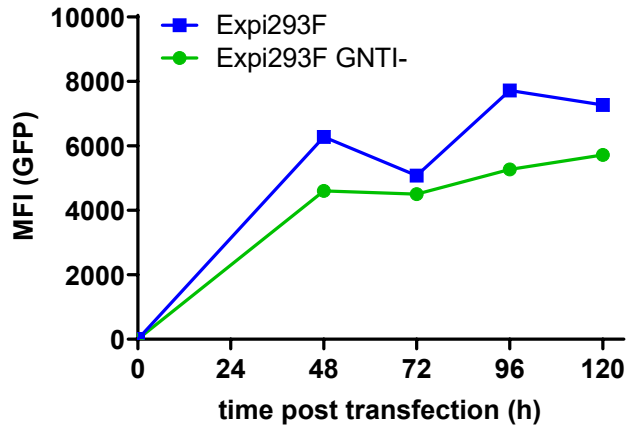
Supplementary Figure 2. DOE of CB_2 expression in Expi293F™. The Expi293F™ expression system was investigated for expression of CB_2-GFP by FACS. Three concentrations of DNA (top label) were combined with 4 amounts of enhancers 1 (x-axis upper bold label) and 2 (x-axis lower label). Data are presented as geometric mean of GFP MFI multiplied by VCD as determined by Vi-Cell XR to represent total harvestable CB_2 per volume of culture. For simplicity, only data from 48hrs (blue bars) and 72 hrs (red bars) are shown as signal clearly peaked at 48 hrs. Data presented are from a single experiment with single samples.

Expi293F™ GNTI-

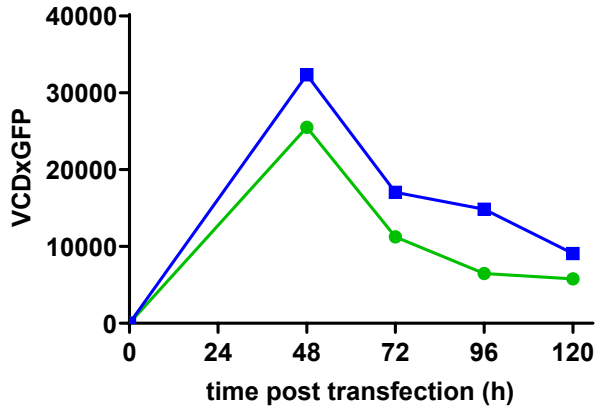


Supplementary Figure 3. DOE of CB_2 expression in Expi293F™ GNTI$^-$. The Expi293F™ GNTI$^-$ expression system was investigated for expression of CB_2-GFP by FACS. Three concentrations of DNA (top label) were combined with 4 amounts of enhancers 1 (x-axis upper bold label) and 2 (x-axis lower label). Data are presented as geometric mean of GFP MFI multiplied by VCD as determined by Vi-Cell XR to represent total harvestable CB_2 per volume of culture. For simplicity, only data from 48hrs (blue bars) and 72 hrs (red bars) are shown as signal clearly peaked at 48 hrs. Data presented are from a single experiment with single samples.

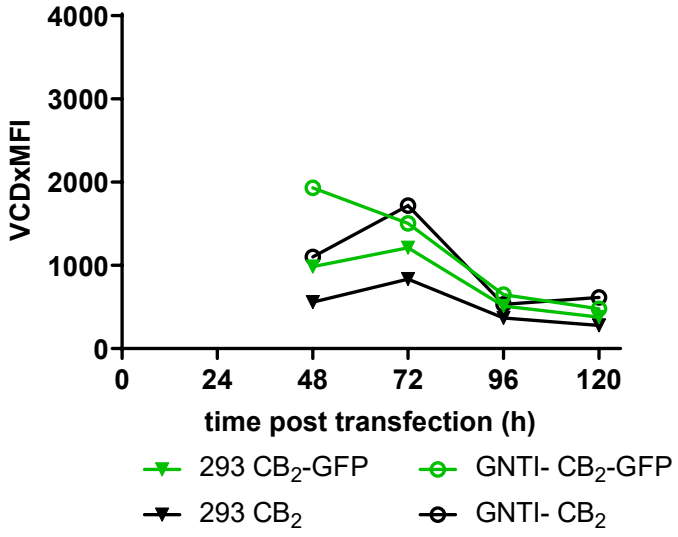
a



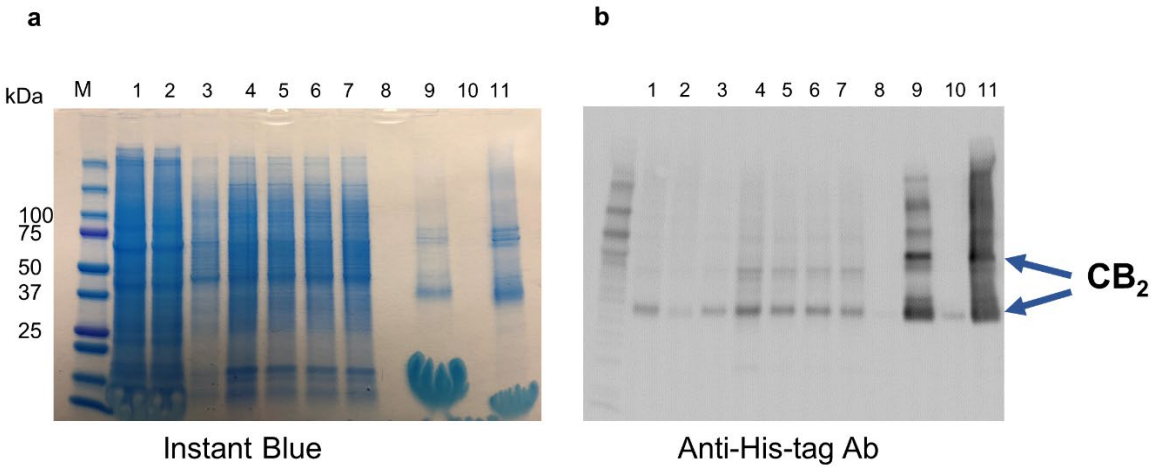
b



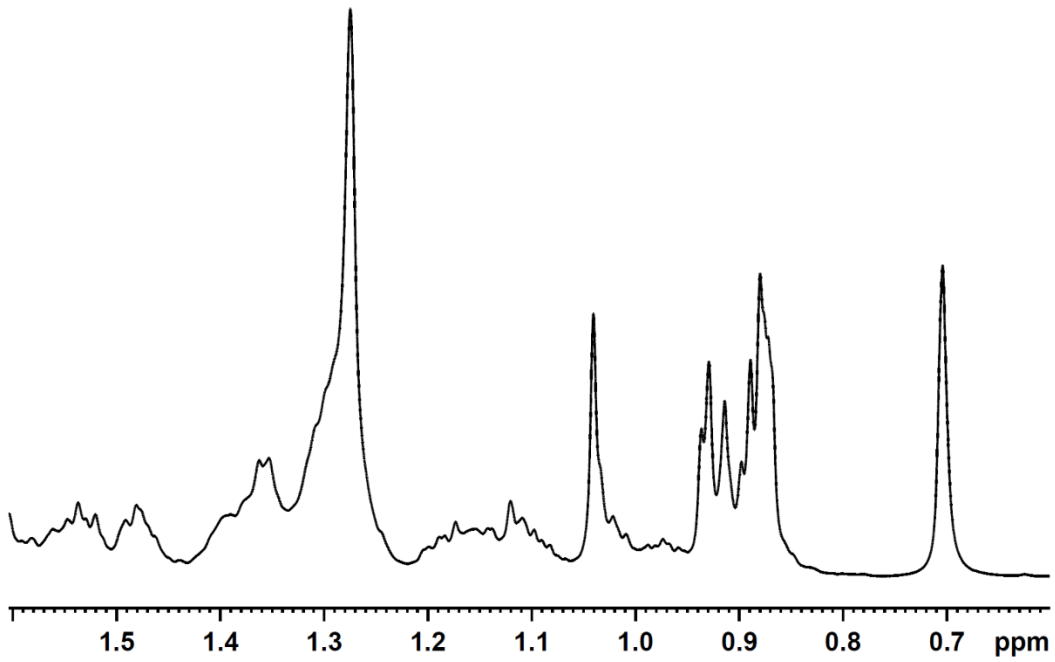
Supplementary Figure 4. Effect of addition of stabilizing ligand CP-55,940 on CB_2 expression. The mammalian cell systems were transfected using optimized conditions determined by the DOE shown in figure S2 and S3. Single cell GFP intensity is depicted in **a**, whereas **b** depicts the effect of multiplication of GFP MFI by VCD. Data presented are from a single experiment with single samples



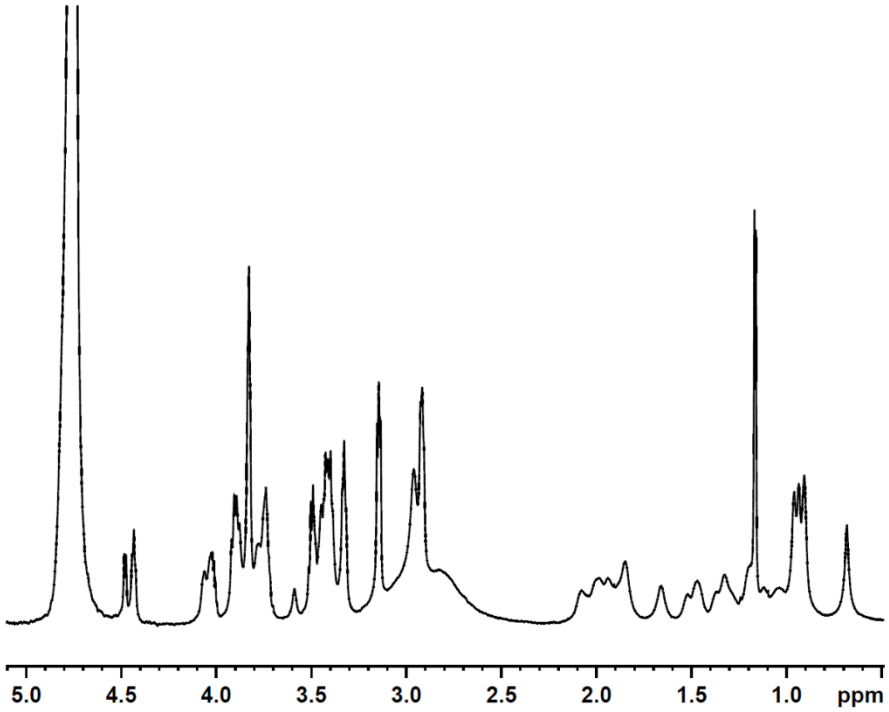
Supplementary Figure 5. Comparison of membrane expression of GFP-tagged CB_2 vs untagged CB_2. To assess whether CB_2 expressed as a GFP fusion protein followed similar behavior as untagged CB_2, Expi293F™ and Expi293F™ GNTI$^-$ cells were transfected with the 2 respective constructs and expression at the cell membrane was measured by FACS using an antibody raised against the extracellular domain of CB_2. Green lines represent GFP-fused CB_2, black lines the untagged CB_2. Data presented are from a single experiment with single samples.



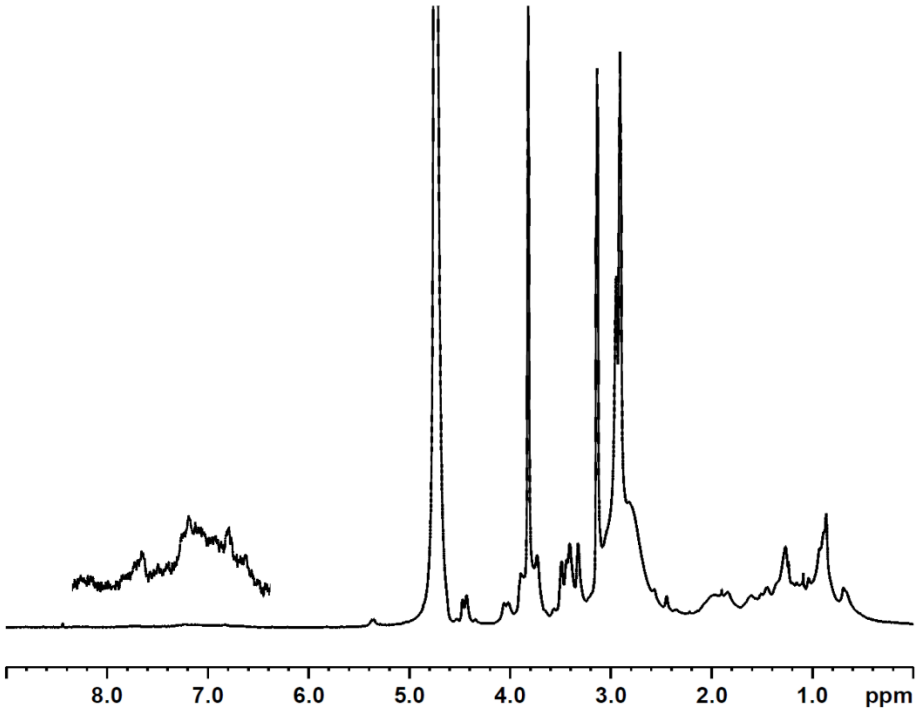
Supplementary Figure 6. Purification of CB_2 from Expi293F™ GNTI⁻ cells. **a**, Instant Blue-stained SDS-PAGE, 10%; **b**, Western blot probed with anti-Histag antibody. CB_2 was purified as described in Methods. Lane1, solubilized total membrane proteins (crude extract); lanes 2-7 elution fractions from Ni-NTA and StrepTactin XT columns; 8, 10- wash of the StrepTactin column; 9, 11 – fractions of purified CB2. Arrows indicate the position of CB_2 monomer and dimer. Higher immunoreactive bands correspond to the oligomeric forms of CB_2 that are formed during SDS-PAGE separation.



Supplementary Figure 7. Determination of detergent- and lipid composition of CB_2 micelles. ^{1}H NMR spectrum of 10µL of the CB_2 in Façade-TEG/phospholipid/CHS micelles, dissolved in deuterated chloroform/ methanol (1/1, vol/vol), recorded at 22ºC. Area of resonances at 0.7ppm (Façade-TEG + CHS), 1.04 ppm (CHS) and 1.28 ppm (phospholipid) were used to determine concentrations in the original sample: CB_2: 74 µM, Façade-TEG: 1.09 mM, phospholipid: 83 µM, CHS: 357 µM.

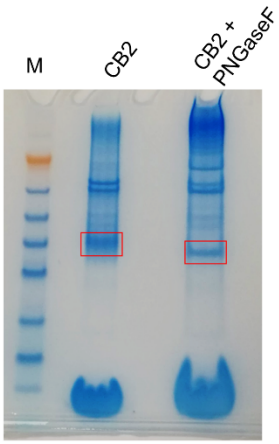


Supplementary Figure 8. Determination of diffusion rates of Façade-TEG micelles. ^{1}H NMR spectrum of Façade-TEG (250 μM) in D_2O recorded at 22°C. The methyl group resonance at 0.7 ppm was selected for measurement of diffusion rates of Façade-TEG molecules.

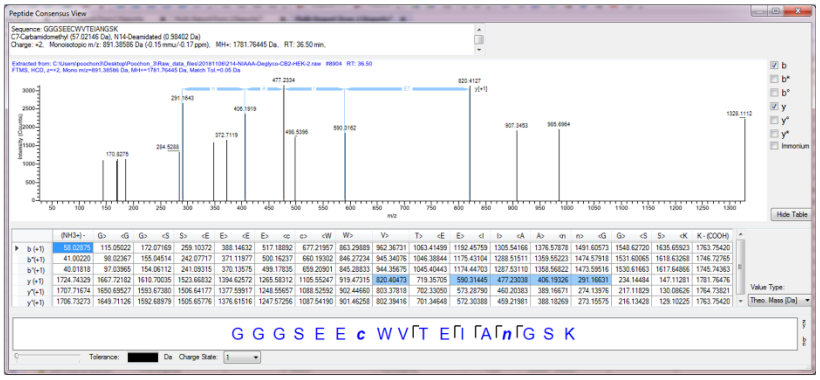


Supplementary Figure 9. Determination of diffusion rates of CB_2/Façade-TEG/CHS/phospholipids micelles. ^1H NMR spectrum of CB_2 solubilized in Façade-TEG/phospholipid/CHS micelles in deuterated Tris/D_2O buffer, pD 7.8, recorded at 22ºC. The band of amide resonances of CB_2 from 6.8-7.6 ppm was used to measure diffusion rates of CB_2-containing Façade-TEG micelles.

a



b



c

Result of analysis of CB2 band without deglycosylation treatment:

MWSHPQFEKG GGSGGGSGGG SAWSHPQFEK GGGSEECWVT EIANGSK **DGL DSNPMKDYMI LSGPQK** TAVA VLCTLLGLLS ALENVAVLYL ILSSHQLRRK

PSYLFIGSLA GADFLASVVF ACSFVNFHVF HGVDSKAVFL LKIGSVTMTF TASVGSLLLT AIDRYLCLR **Y PPSYKALLTR** GRALVTLGIM WVLSALVSYL

PLMGWTCCPR PCSELFPLIP NDYLLSWLLF IAFLFSGIIY TYGHVLWKAH QHVASLSGHQ DRQVPGMARM RLDVRLAKTL GLVLAVLLIC WFPVLALMAH

P PP

SLATTLSDQV KKAFAFCSML CLINSMVNPV IYALRSGEIR **SSAHHCLAHW K** KCVR **GLG SE AKEEAPR SS V TETEADGKIT PWPDSR** DLDL SDCGTHHHHH

HHHHH

Result of analysis of CB2 band with deglycosylation treatment:

Deamidated

MWSHPQFEKG GGSGGGSGGG SAWSHPQFEK **GGGSEECWVT EIA NGSKDGL DSNPMKDYMI LSGPQK** TAVA VLCTLLGLLS ALENVAVLYL ILSSHQLRRK

PSYLFIGSLA GADFLASVVF ACSFVNFHVF HGVDSKAVFL LKIGSVTMTF TASVGSLLLT AIDRYLCLR **Y PPSYK** ALLTR GRALVTLGIM WVLSALVSYL

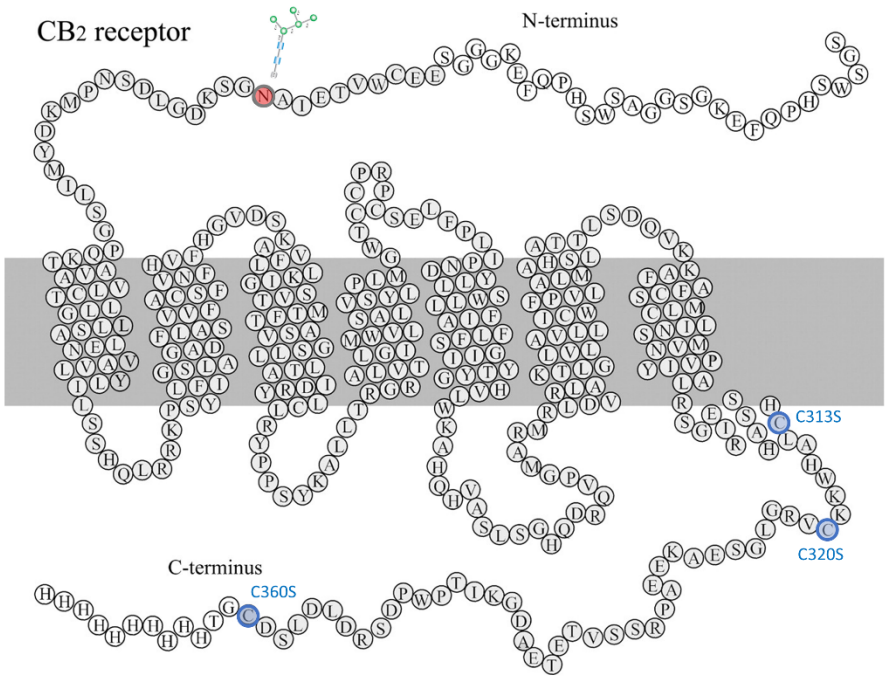
PLMGWTCCPR PCSELFPLIP NDYLLSWLLF IAFLFSGIIY TYGHVLWK **AH QHVASLSGHQ DRQVPGMAR** M RLDVRLAKTL GLVLAVLLIC WFPVLALMAH

P P P

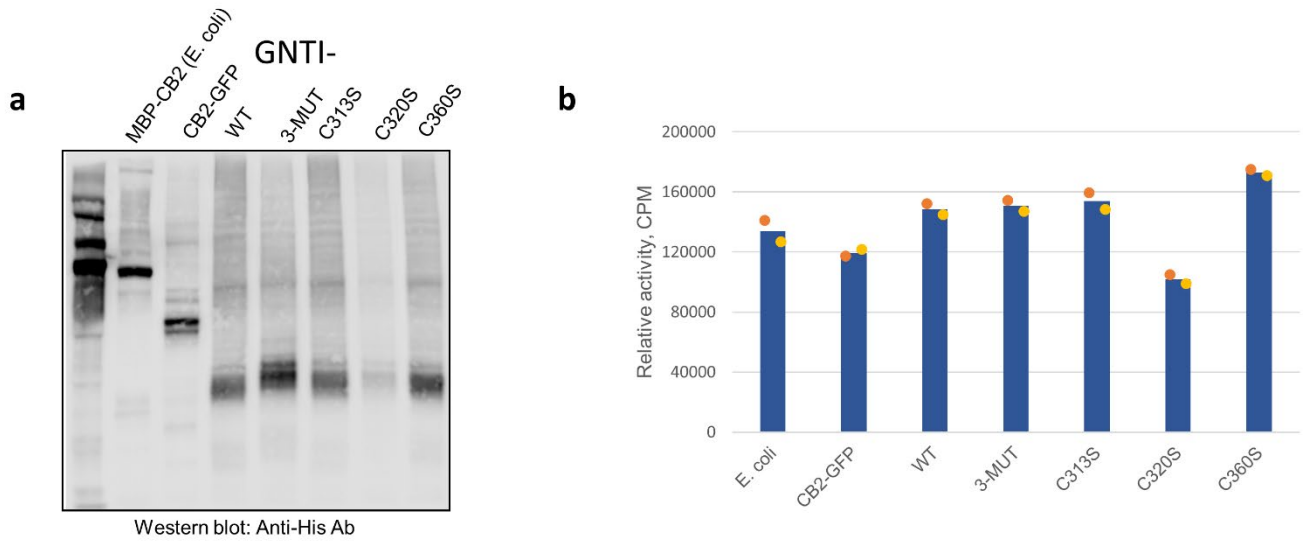
SLATTLSDQV KKAFAFCSML CLINSMVNPV IYALRSGEIR SS AHHCLAHW KKCVR **GLG SE AKEEAPRS SV TETEADGKIT PWPDSR** DLDL SDCGTHHHHH

HHHHH

Supplementary Figure 10. **Determination of glycosylation site by LC/MS/MS analysis of CB_2 purified from Expi293F™ GNTI⁻ cells. a**, Preparation of protein sample for LC/MS/MS. Samples were treated with Deglycosylation Mix II, separated on a 4-12% NuPAGE gel, and stained with SimplyBlue. The target protein gel-bands (red-squared) were excised for in-gel trypsin digest; **b**, LC/MS/MS analysis of tryptic digest of deglycosylated sample; **c**, Peptides detected by LC/MS/MS. Green colored sequence was detected from analysis of non-deglycosylated CB_2 band (upper panel) and deglycosylated CB_2 band (lower panel) after trypsin digest. Note: P indicates that S or T is phosphorylated; N11 is deamidated in the deglycosylated CB_2.

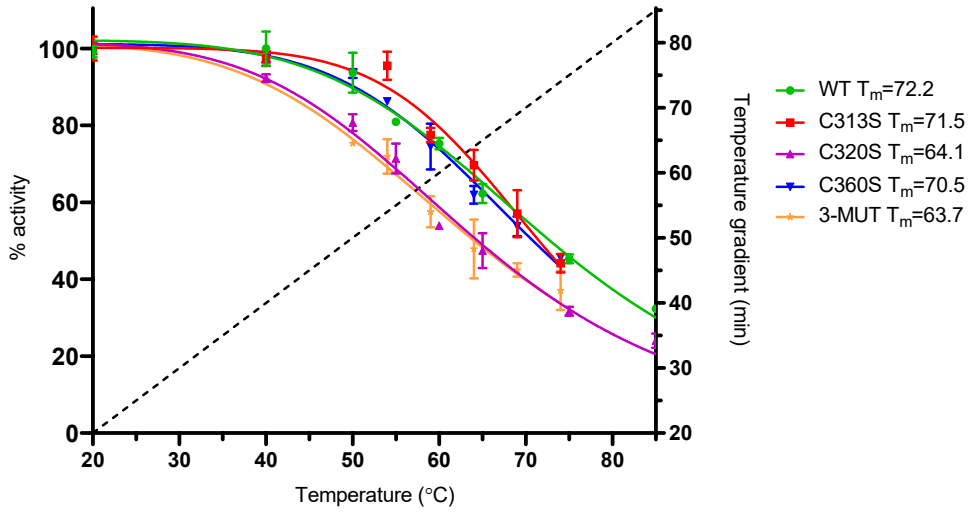


Supplementary Figure 11. **Schematic structure of the recombinant human CB_2 protein expressed in HEK cells.** Highlighted residues indicate possible sites for glycosylation and acylation.

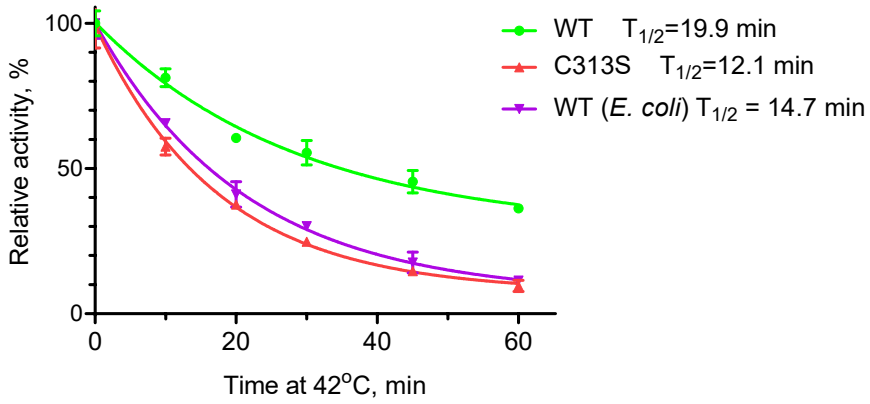


Supplementary Figure 12. **Expression of cysteine-replacement variants of CB_2 in Expi293F™ GNTI⁻ cells. a**, Western blot analysis of CB_2 levels in membrane preparations from GNTI- cells. 20 μg of membrane protein per lane. For comparison, MBP-CB_2 fusion expressed in *E. coli* cell membranes and CB_2-GFP fusion protein expressed in GNTI- cell membranes are shown. **b**, activity of CB_2 and its cysteine replacement variants as determined by G protein activation assay. 2 μg of membrane preparation per reaction performed in the presence of 5 μM CP-55,940. Results are an average of two independent measurement with individual data points shown.

a

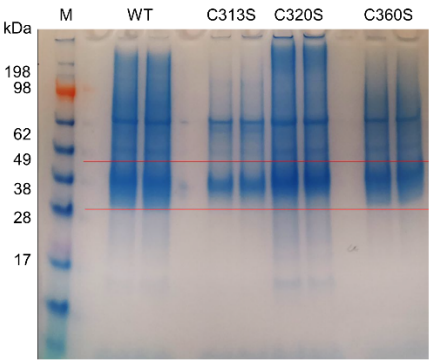


b



Supplementary Figure 13. **Stability of cysteine-replacement variants of CB_2 expressed in ExpiF™ GNTI⁻ cells. a**, thermoinactivation of CB_2 and variant constructs in membranes subjected to a temperature gradient of 1°C/ min; **b**, isothermal stability of purified CB_2 WT and C313S constructs in Façade-TEG/ CHS micelles at 42°C. Purified CB_2 from *E. coli* is shown for comparison. Activity of CB_2 in samples subjected to temperature treatment was determined by G protein activation test in the presence of saturating concentrations of agonist CP-55,940. Bars represent an average of two independent measurements and individual measurements are represented by dots.

a



b

Site	WT	C313S	C320S	C360S
	palmitoylation			
C4	ND	ND	ND	ND
C40	ND	ND	ND	ND
C134	ND	ND	ND	ND
C284	ND	ND	ND	ND
C313	partial	ND	ND	partial
C320	ND	ND	ND	ND
Note: ND=not detected				

c

Result of analysis of WT CB_2 (HEK):

MWSHPQFEKG GSGGGGSGGG SAWSHPQFEK GGGSERCWVT KIANGSKDGL DSNPMKDYMI LSGPQKTAVA VLCTLLGLLS ALENVAVLYL LLSSRQLRRK PSYLFIGSLA GADFLASVVF ACSFVNFHVF HGVDSKAVFL LKIGSVTMTF TASVGSLLLT AIDRYLCLRY PPSYKALLTR GRALVTLGIM WVLSALVSYL PLMGWTCCPR PCSELFPLIP NDYLLSWLLF IAFLFSGIIY TYGHVLWKAH QHVASLSGHQ DRQVPGMARM RLDVRLAKTL GLVLAVLLIC WFPVLALMAH SLATTLSDQV KKAFAFCSML CLINSMVNPV IYALRSGEIR SSAHHCLAHW KKCVRGLGSE AKEEAPRSSV TETEADGKIT PWPDSRDLDL SDCGTHHHHH HHHHH

Result of analysis of CB_2 variant C313S:

MWSHPQFEKG GSGGGGSGGG SAWSHPQFEK GGGSERCWVT KIANGSKDGL DSNPMKDYMI LSGPQKTAVA VLCTLLGLLS ALENVAVLYL LLSSRQLRRK PSYLFIGSLA GADFLASVVF ACSFVNFHVF HGVDSKAVFL LKIGSVTMTF TASVGSLLLT AIDRYLCLRY PPSYKALLTR GRALVTLGIM WVLSALVSYL PLMGWTCCPR PCSELFPLIP NDYLLSWLLF IAFLFSGIIY TYGHVLWKAH QHVASLSGHQ DRQVPGMARM RLDVRLAKTL GLVLAVLLIC WFPVLALMAH SLATTLSDQV KKAFAFCSML CLINSMVNPV IYALRSGEIR SSAHHSLAHW KKCVRGLGSE AKEEAPRSSV TETEADGKIT PWPDSRDLDL SDCGTHHHHH HHHHH

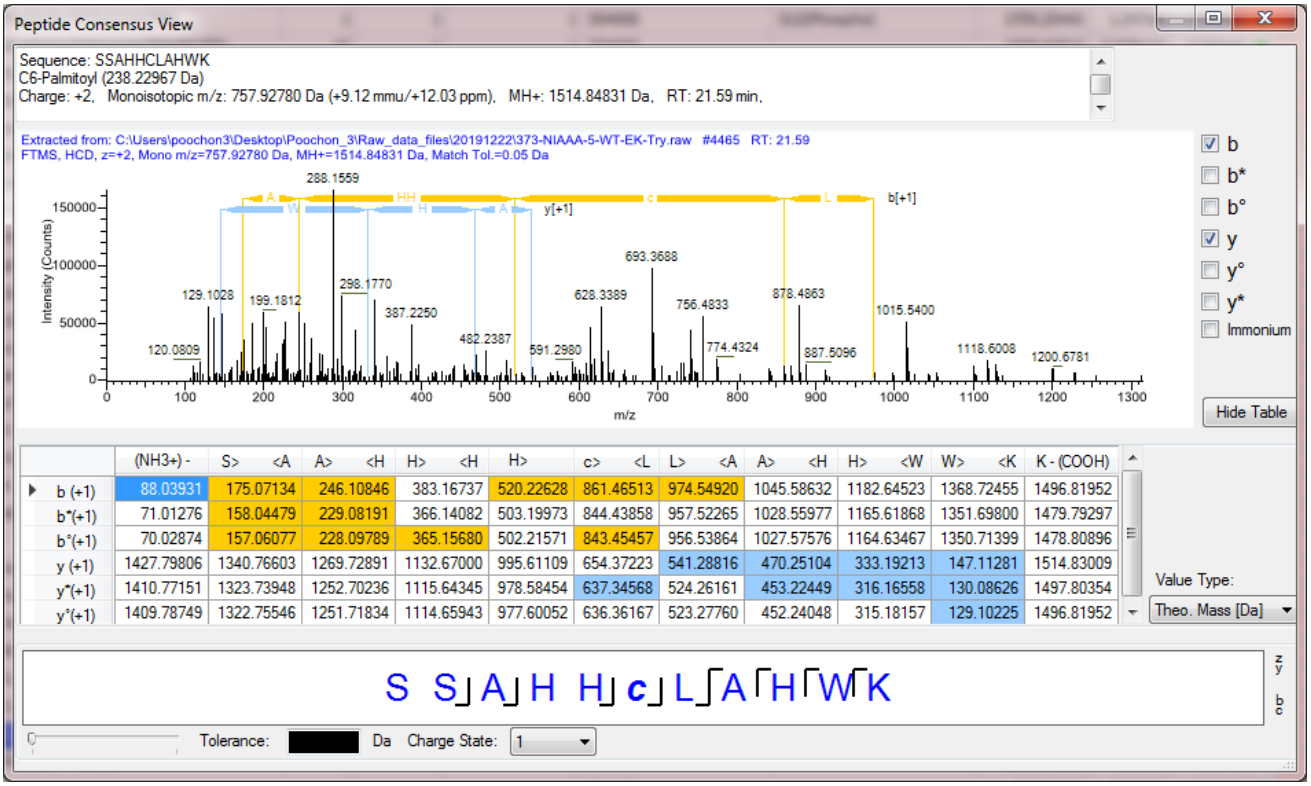
Result of analysis of CB_2 variant C320S:

Result of analysis of CB_2 variant C360S:

Illustration of peptide area detected by LC/MS/MS.

Green colored sequence was detected by LC/MS/MS from analysis of the samples digested by trypsin or chymotrypsin. Note: **P** indicates the S, T, or Y is phosphorylated; **Q** indicates the C is modified by Palmitoylation.

Supplementary Figure 15. **Determination of palmitoylation sites by LC/MS/MS analysis of CB_2 purified from Expi293F™ GNTI⁻ cells. a**, Preparation of protein sample for LC/MS/MS. Samples were separated on a 4-12% NuPAGE gel and stained with SimplyBlue. The target protein gel-bands (red-squared) were excised for in-gel trypsin digest; **b**, partial summary table; **c**, Peptides detected by LC/MS/MS. Green colored sequence was detected from analysis of CB_2. bands after trypsin digest. Note: P indicates the S or T is phosphorylated; Q indicates the cysteine residue modified by palmitoylation.



Sequence: SSAHHCLAHWK, **C6-Palmitoyl** (238.22967 Da)

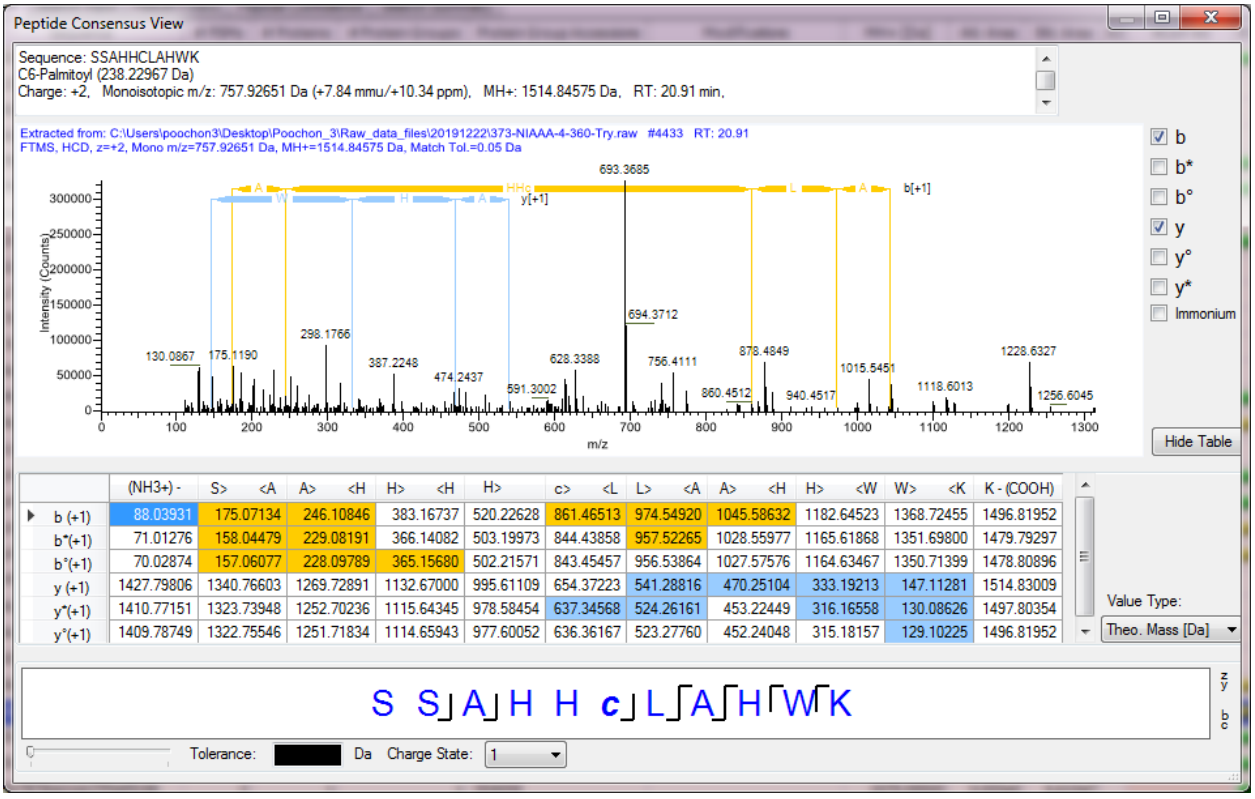
Charge: +2, Monoisotopic m/z: 757.92780 Da (+9.12 mmu/+12.03 ppm), MH+: 1514.84831 Da, RT: 21.59 min,

Identified with: Sequest HT (v1.3); XCorr:1.06, Ions matched by search engine: 0/0

Fragment match tolerance used for search: 0.05 Da; Fragments used for search: b; b-H_2O; y; y-H_2O; y-NH_3

Protein references (1): - CNR2_HUMAN Cannabinoid receptor 2, CB2-WT [Homo sapiens]

Supplementary Figure 16. Detection of palmitoylation at Cys313 by LC/MS/MS analysis of WT CB_2 purified from Expi293F™ GNTI⁻ cells. Samples were prepared and analyzed as described in Methods.



	(NH3+) -	S>	<A	A>	<H	H>	<H	H>	c>	<L	L>	<A	A>	<H	H>	<W	W>	<K	K - (COOH)
b (+1)	88.03931	175.07134		246.10846		383.16737		520.22628	861.46513		974.54920		1045.58632		1182.64523		1368.72455		1496.81952
b*(+1)	71.01276	158.04479		229.08191		366.14082		503.19973	844.43858		957.52265		1028.55977		1165.61868		1351.69800		1479.79297
b°(+1)	70.02874	157.06077		228.09789		365.15680		502.21571	843.45457		956.53864		1027.57576		1164.63467		1350.71399		1478.80896
y (+1)	1427.79806	1340.76603		1269.72891		1132.67000		995.61109	654.37223		541.28816		470.25104		333.19213		147.11281		1514.83009
y*(+1)	1410.77151	1323.73948		1252.70236		1115.64345		978.58454	637.34568		524.26161		453.22449		316.16558		130.08626		1497.80354
y°(+1)	1409.78749	1322.75546		1251.71834		1114.65943		977.60052	636.36167		523.27760		452.24048		315.18157		129.10225		1496.81952

Sequence: SSAHHCLAHWK, C6-Palmitoyl (238.22967 Da)

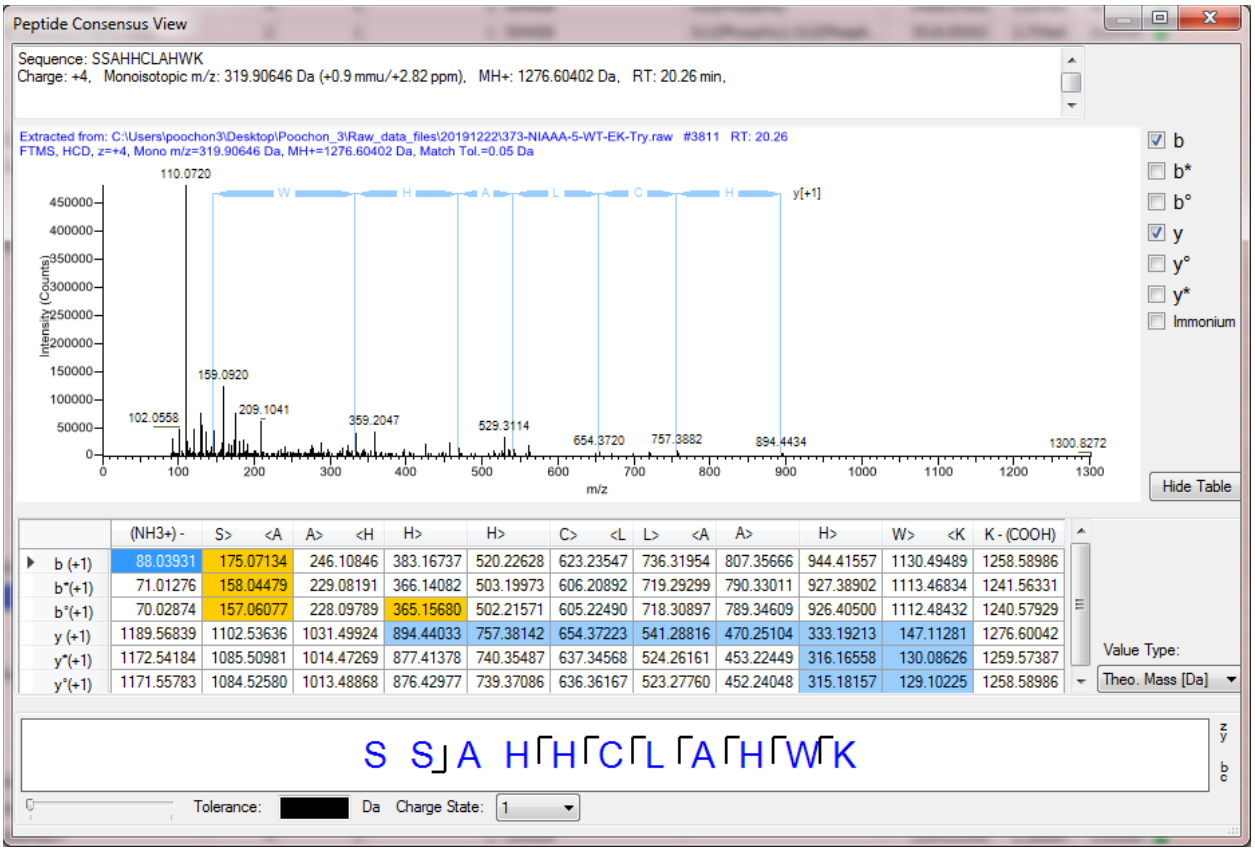
Charge: +2, Monoisotopic m/z: 757.92651 Da (+7.84 mmu/+10.34 ppm), MH+: 1514.84575 Da, RT: 20.91 min,

Identified with: Sequest HT (v1.3); XCorr:0.88, Ions matched by search engine: 0/0

Fragment match tolerance used for search: 0.05 Da; Fragments used for search: b; b-H_2O; y; y-H_2O; y-NH_3

Protein references (1): - CNR2_HUMAN Cannabinoid receptor 2, CB2-C360S [Homo sapiens]

Supplementary Figure 17. Detection of palmitoylation at Cys313 by LC/MS/MS analysis of CB_2 C360S purified from Expi293F™ GNTI⁻ cells. Samples were prepared and analyzed as described in Methods.



	(NH3+) -	S>	<A	A>	<H	H>	H>	C>	<L	L>	<A	A>	H>	W>	<K	K - (COOH)
b (+1)	88.03931	175.07134		246.10846		383.16737	520.22628	623.23547		736.31954		807.35666	944.41557	1130.49489		1258.58986
b*(+1)	71.01276	158.04479		229.08191		366.14082	503.19973	606.20892		719.29299		790.33011	927.38902	1113.46834		1241.56331
b°(+1)	70.02874	157.06077		228.09789		365.15680	502.21571	605.22490		718.30897		789.34609	926.40500	1112.48432		1240.57929
y (+1)	1189.56839	1102.53636		1031.49924		894.44033	757.38142	654.37223		541.28816		470.25104	333.19213	147.11281		1276.60042
y*(+1)	1172.54184	1085.50981		1014.47269		877.41378	740.35487	637.34568		524.26161		453.22449	316.16558	130.08626		1259.57387
y°(+1)	1171.55783	1084.52580		1013.48868		876.42977	739.37086	636.36167		523.27760		452.24048	315.18157	129.10225		1258.58986

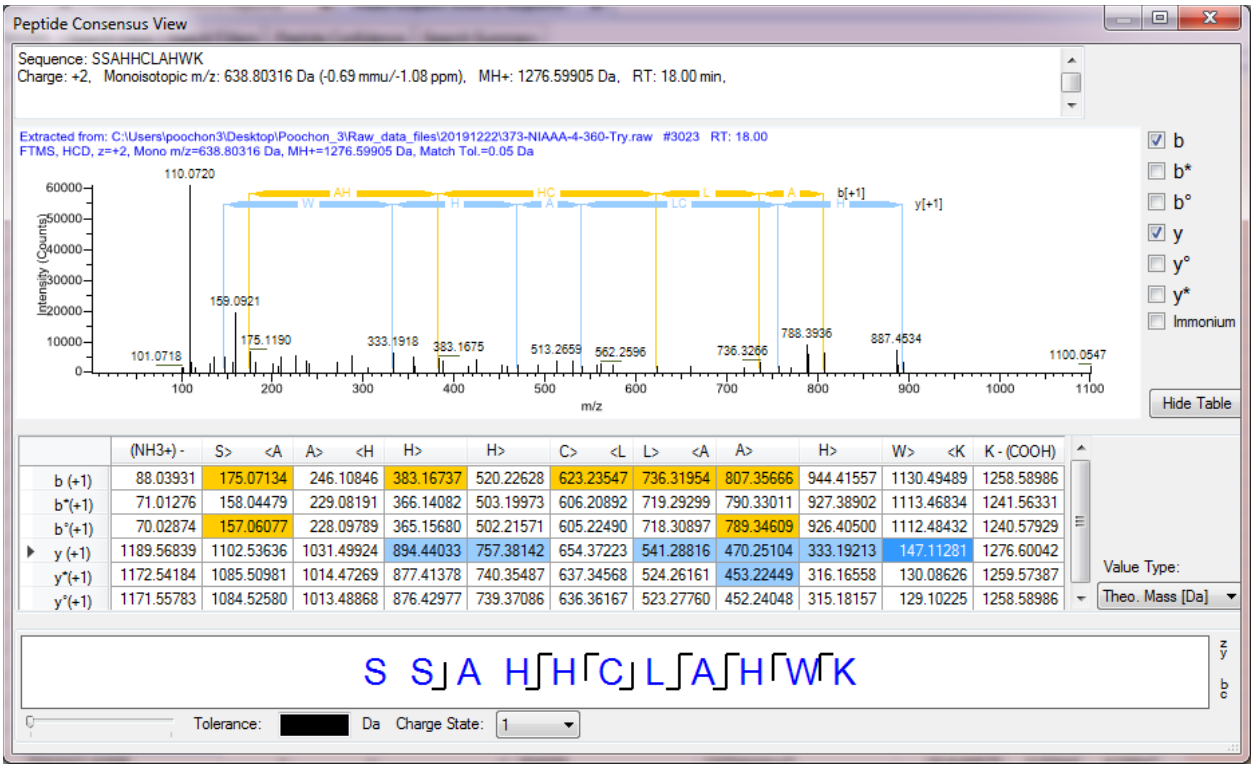
Sequence: SSAHHCLAHWK, Charge: +4, Monoisotopic m/z: 319.90646 Da (+0.9 mmu/+2.82 ppm), MH+: 1276.60402 Da, RT: 20.26 min,

Identified with: Sequest HT (v1.3); XCorr:3.12, Ions matched by search engine: 0/0

Fragment match tolerance used for search: 0.05 Da; Fragments used for search: b; b-H_2O; y; y-H_2O; y-NH_3

Protein references (1): - CNR2_HUMAN Cannabinoid receptor 2, CB2-WT [Homo sapiens]

Supplementary Figure 18. LC/MS/MS analysis of non-palmitoylated WT CB_2 purified from Expi293F™ GNTI⁻ cells. Samples were prepared and analyzed as described in Methods.



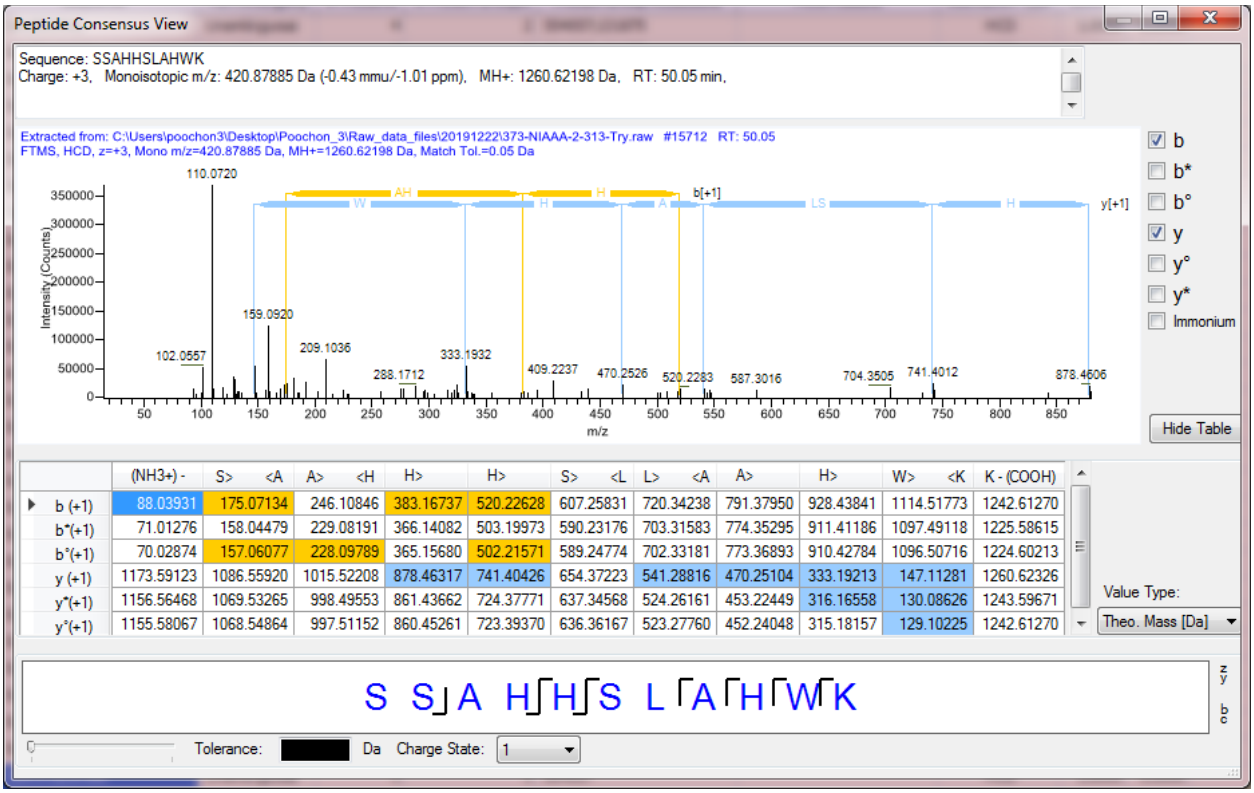
Sequence: SSAHHCLAHWK, Charge: +2, Monoisotopic m/z: 638.80316 Da (-0.69 mmu/-1.08 ppm), MH+: 1276.59905 Da, RT: 18.00 min,

Identified with: Sequest HT (v1.3); XCorr:1.94, Ions matched by search engine: 0/0

Fragment match tolerance used for search: 0.05 Da; Fragments used for search: b; b-H_2O; y; y-H_2O; y-NH_3

Protein references (1): - CNR2_HUMAN Cannabinoid receptor 2, CB2-C360S [Homo sapiens]

Supplementary Figure 19. LC/MS/MS analysis of non-palmitoylated CB_2 C360S purified from Expi293F™ GNTI⁻ cells. Samples were prepared and analyzed as described in Methods.



	(NH3+) -	S>	<A	A>	<H	H>	H>	S>	<L	L>	<A	A>	H>	W>	<K	K - (COOH)
b (+1)	88.03931	175.07134		246.10846		383.16737	520.22628	607.25831		720.34238		791.37950	928.43841	1114.51773		1242.61270
b*(+1)	71.01276	158.04479		229.08191		366.14082	503.19973	590.23176		703.31583		774.35295	911.41186	1097.49118		1225.58615
b°(+1)	70.02874	157.06077		228.09789		365.15680	502.21571	589.24774		702.33181		773.36893	910.42784	1096.50716		1224.60213
y (+1)	1173.59123	1086.55920		1015.52208		878.46317	741.40426	654.37223		541.28816		470.25104	333.19213	147.11281		1260.62326
y*(+1)	1156.56468	1069.53265		998.49553		861.43662	724.37771	637.34568		524.26161		453.22449	316.16558	130.08626		1243.59671
y°(+1)	1155.58067	1068.54864		997.51152		860.45261	723.39370	636.36167		523.27760		452.24048	315.18157	129.10225		1242.61270

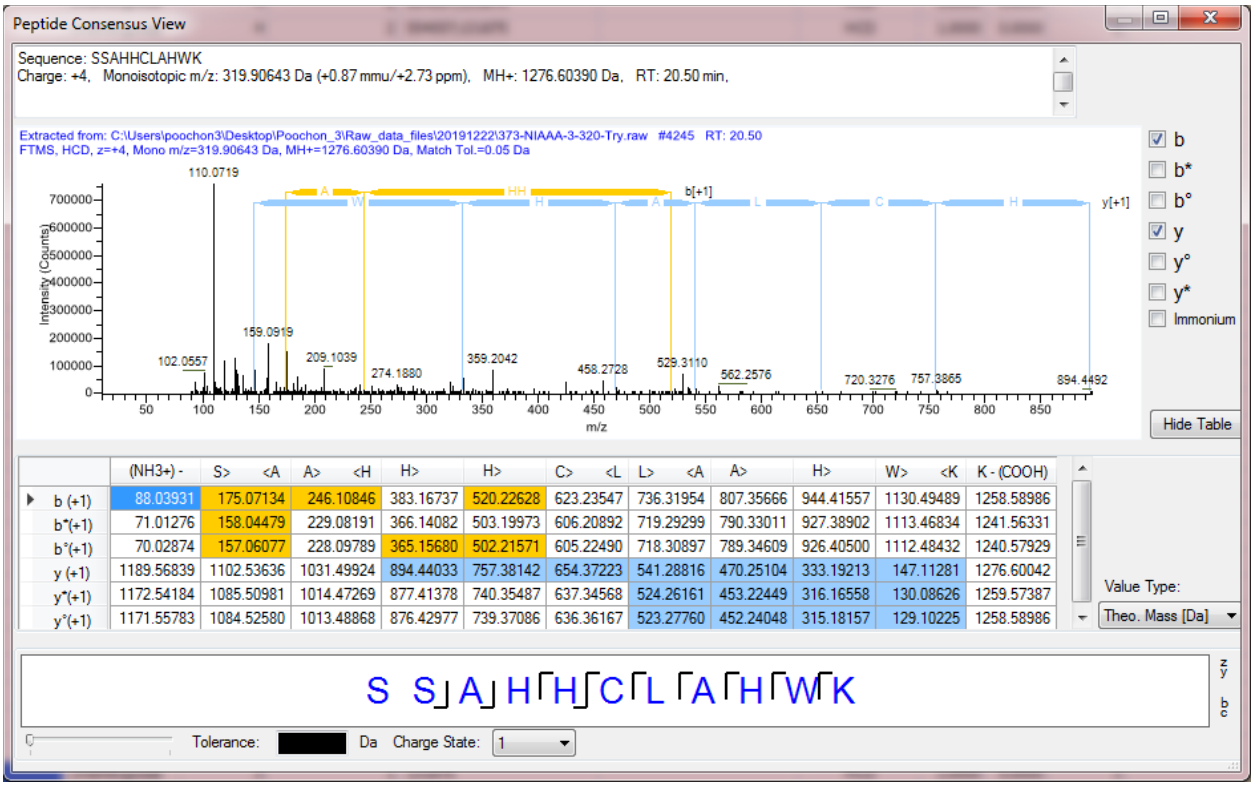
Sequence: SSAHHSLAHWK, Charge: +3, Monoisotopic m/z: 420.87885 Da (-0.43 mmu/-1.01 ppm), MH+: 1260.62198 Da, RT: 50.05 min,

Identified with: Sequest HT (v1.3); XCorr:3.14, Ions matched by search engine: 0/0

Fragment match tolerance used for search: 0.05 Da; Fragments used for search: b; b-H_2O; y; y-H_2O; y-NH_3

Protein references (1): - CNR2_HUMAN Cannabinoid receptor 2 C313S, CB2-C313S [Homo sapiens]

Supplementary Figure 20. LC/MS/MS analysis of non-palmitoylated CB_2 C313S purified from Expi293F™ GNTI⁻ cells. Samples were prepared and analyzed as described in Methods.



	(NH3+) -	S>	<A	A>	<H	H>	H>	C>	<L	L>	<A	A>	H>	W>	<K	K - (COOH)
b (+1)	88.03931	175.07134		246.10846		383.16737	520.22628	623.23547		736.31954		807.35666	944.41557	1130.49489		1258.58986
b*(+1)	71.01276	158.04479		229.08191		366.14082	503.19973	606.20892		719.29299		790.33011	927.38902	1113.46834		1241.56331
b°(+1)	70.02874	157.06077		228.09789		365.15680	502.21571	605.22490		718.30897		789.34609	926.40500	1112.48432		1240.57929
y (+1)	1189.56839	1102.53636		1031.49924		894.44033	757.38142	654.37223		541.28816		470.25104	333.19213	147.11281		1276.60042
y*(+1)	1172.54184	1085.50981		1014.47269		877.41378	740.35487	637.34568		524.26161		453.22449	316.16558	130.08626		1259.57387
y°(+1)	1171.55783	1084.52580		1013.48868		876.42977	739.37086	636.36167		523.27760		452.24048	315.18157	129.10225		1258.58986

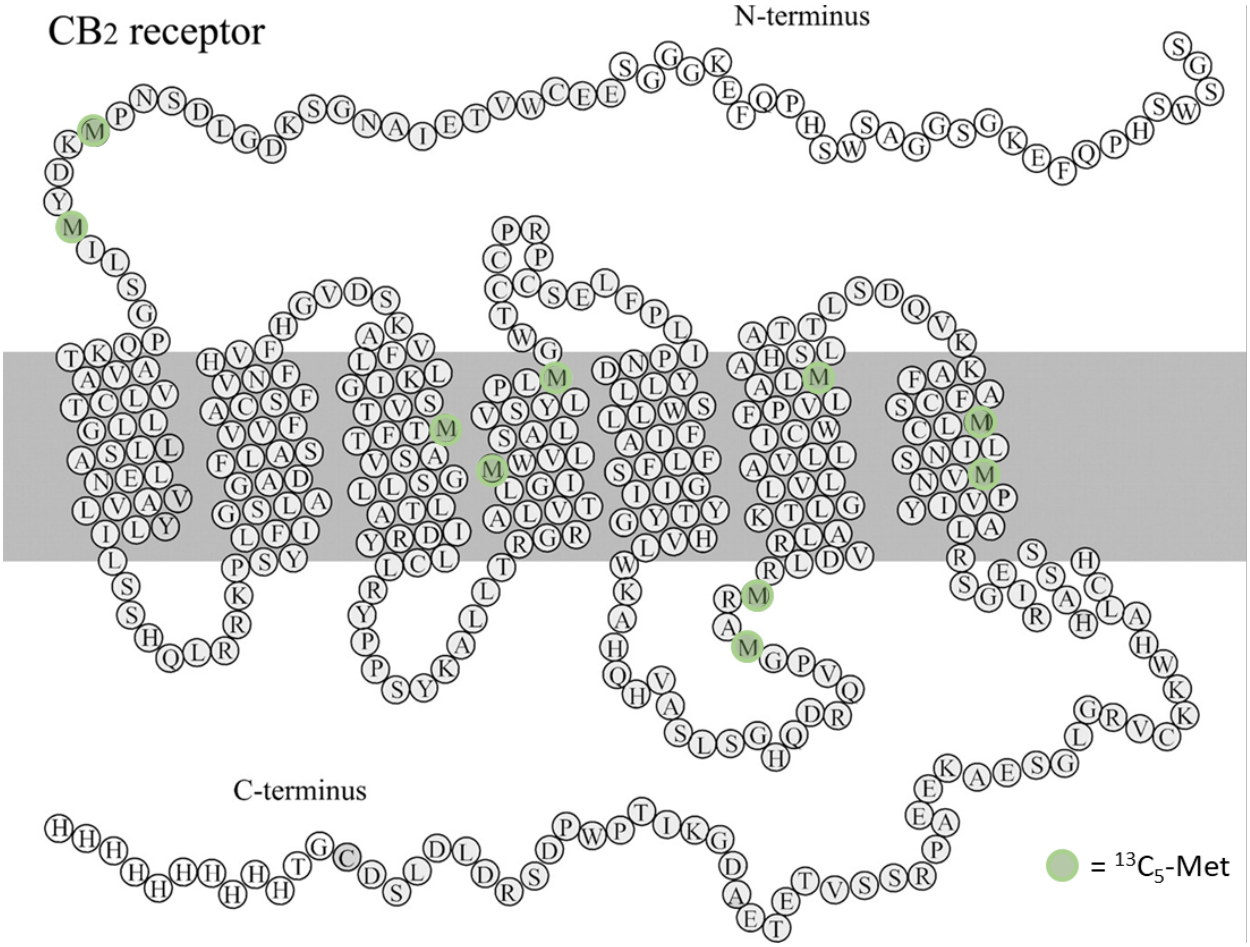
Sequence: SSAHHCLAHWK, Charge: +4, Monoisotopic m/z: 319.90643 Da (+0.87 mmu/+2.73 ppm), MH+: 1276.60390 Da, RT: 20.50 min,

Identified with: Sequest HT (v1.3); XCorr:3.24, Ions matched by search engine: 0/0

Fragment match tolerance used for search: 0.05 Da; Fragments used for search: b; b-H_2O; y; y-H_2O; y-NH_3

Protein references (3): - CNR2_HUMAN Cannabinoid receptor 2-C320S, CB2-C320S [Homo sapiens]

Supplementary Figure 21. LC/MS/MS analysis of non-palmitoylated CB_2 C320S purified from Expi293F™ GNTI⁻ cells. Samples were prepared and analyzed as described in Methods.



Supplementary Figure 22. Schematic structure of the recombinant CB_2 labeled with $^{13}C_5$-methionine. Highlighted residues indicate sites of incorporation of $^{13}C_5$-methionine.

sample	diffusion constant (m^2/s)	hydrodynamic radius (nm)	FA-TEG aggregation number
0.25 mM FA-TEG in D2O	(2.20 ± 0.02) x 10^-10	0.89 ± 0.02	1.8
1 mM FA-TEG in D2O	(1.35 ± 0.02) x 10^-10	1.45 ± 0.02	7.7
10 mM FA-TEG in D2O	(0.86 ± 0.01) x 10^-10	2.28 ± 0.03	30.3
74 µM CB2 in FA-TEG/lipid/CHS	(0.40 ± 0.03) x 10^-10	4.90 ± 0.06	
CB2 sample, second component	(0.03 ± 0.005) x 10^-10	65.4 0.89 ± 1.0	

Supplementary Table 1. Diffusion constants and derived hydrodynamic radii of Façade-TEG particles. Experiments were conducted at 22 ºC. The diffusion constants and derived hydrodynamic radii of FA-TEG particles indicate the critical micelle concentration of FA-TEG below 1 mM and an aggregation number in micelles at 10 mM FA-TEG of about 30. For the CB_2 sample, protein diffusion/hydrodynamic radie of micelles containing CB_2 is reported. The sample contained a second component with particles one order of magnitude larger in size.