Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Targeting SARS-CoV-2 Spike Protein by Stapled hACE2 Peptides

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1. Materials and methods

All reagents were obtained from commercial sources and used without further purifications. Water was purified using a Millipore Milli-Q water purification system (Merck-Millipore, Burlington, MA, USA). For HPLC the following buffers were used: Buffer A: H_2O (0.1% TFA); Buffer B: acetonitrile (0.1% TFA). Lyophilization of purified peptides was achieved using a VaCo 2 lyophilizer (Zirbus Technology GmbH, Bad Grund, Germany).

2. Synthesis and purification of ACE2 peptides

35-mer hACE2 peptides were chain assembled on Rink amide resin using microwave assisted SPPS on a Liberty Blue peptide synthesizer (CEM corporation, Matthews, NC, USA). All amino acid couplings were carried out with the equivalent ratio of [5]:[5]:[7.5] of [Fmoc-protected amino acid]:[DIC]:[Oxyma Pure] at 75 °C for 2 minutes or at 50 °C for 4 minutes for histidine.

Upon completion of the sequence, peptides were washed with DCM (3x), MeOH (3x) and Et_2O before being dried *in vacuo*. Subsequent cleavage from resin using 0.5% TIPS and 0.5% H_2O in conc. TFA. at room temperature for 4 h is followed by precipitation in cold Et_2O. The suspension was subjected to centrifugation for 5 minutes at 5000 rpm in an Eppendorf 5804R centrifuge (Eppendorf, Hamburg, Germany) after which the supernatant was decanted into the waste. The remaining white to yellow solid was washed twice by cold Et_2O and subjected to centrifugation after which the crude peptide was dissolved in a mixture of ACN and H_2O. The peptides were purified using preparative reverse-phase HPLC (RP-HPLC) using a gradient of buffer A and buffer B from 3% B to 70% over 40 minutes at 4 mL/min using a Gemini 10µm NX-C18 110Å LC column (Phenomenex, Torrance, CA, USA). Analytical RP-HPLC was carried out using a gradient of buffer A and buffer B from 5% B to 95% over 30 minutes at 1 mL/min on a Gemini 5µm C18 110Å LC column (Phenomenex). Analytical

injections were monitored at 222 nm. Peptides with purity of 90% and higher were used in subsequent experiments.

2.1 Stapling of hACE2 peptides

Fully protected linear hACE2 peptides on rink amide resin with incorporated Fmoc-Lys(Alloc)-OH and Fmoc-Glu(All)-OH at *i,i+4* positions were swollen with DCM under nitrogen flow for 10 min at room temperature. Phenylsilane (24 eq.) in DCM was added to the bubbling resin suspension for 2 min at room temperature. Subsequently addition of tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_4$) (1 eq.) to the bubbling suspension in DCM and continued agitation under nitrogen flow for 30 min at room temperature orthogonally removes alloc and allyl protection groups from the peptide. Washing of the resin with DCM (3x), DMF (3x) and sodiumdiethyldithiocarbamate (0.5% in DMF) was followed by a Kaiser test to monitor deprotection. HATU (1.5 eq.) and DIPEA (5 eq.) in DMF were added to the resin upon observing a positive (blue) Kaiser test, and the resin was agitated overnight at room temperature. The resin was washed with DMF (3x) and side-chain stapling was monitored with a Kaiser test, with a negative (yellow) Kaiser test being followed by removal of the N-terminal Fmoc protection group by treatment of the resin with 20% piperidine in DMF for 30 min at room temperature. Washes with DMF (3x) and another the observation of a positive Kaiser test is followed by extensive washing of the resin with DCM (3x), MeOH (3x) and Et_2O before being dried *in vacuo*. Stapled peptides are cleaved, purified and characterized following aforementioned methods.

Table S1. Characterization of hACE2 peptides

Entry	Peptide	Sequence	Formula	m/z Calc.	m/z Found	aHPLC rt
1	$hACE2_{21-55}$	IEEQAKTFLDKFNHEAEDLFYQSSLASWNYNTNIT	$C_{188}H_{273}N_{47}O_{61}$	4168.5	4169.3	12.4
2	$hACE2_{21-55}$A36K,F40E	IEEQAKTFLDKFNHEKEDLEYQSSLASWNYNTNIT	$C_{187}H_{278}N_{48}O_{63}$	4207.6	4207.9	12.1
3	$hACE2_{21-55}$F32K,A36E	IEEQAKTFLDKKNHEEEDLFYQSSLASWNYNTNIT	$C_{187}H_{278}N_{48}O_{63}$	4207.6	4207.7	12.2
4	$hACE2_{21-55}$F28K,F32E	IEEQAKTKLDKENHEAEDLFYQSSLASWNYNTNIT	$C_{181}H_{274}N_{48}O_{63}$	4131.5	4131.6	12.1
5	$hACE2_{21-55}$A36K-F40E	IEEQAKTFLDKFNHEKEDLEYQSSLASWNYNTNIT	$C_{187}H_{276}N_{48}O_{62}$	4189.6	4189.6	12.6
6	$hACE2_{21-55}$F32K-A36E	IEEQAKTFLDKKNHEEEDLFYQSSLASWNYNTNIT	$C_{187}H_{276}N_{48}O_{62}$	4189.6	4190.9	12.7
7	$hACE2_{21-55}$F28K-F32E	IEEQAKTKLDKENHEAEDLFYQSSLASWNYNTNIT	$C_{181}H_{272}N_{48}O_{62}$	4113.5	4114.1	11.8

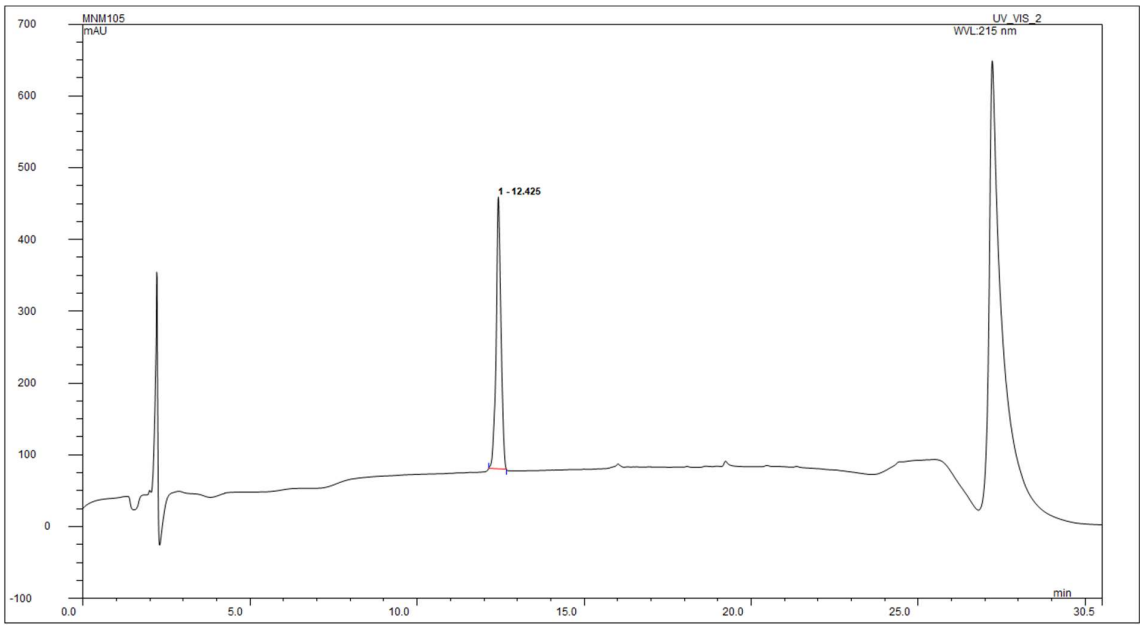


Figure S1. Analytical HPLC of the **1** peptide after RP-HPLC purification.

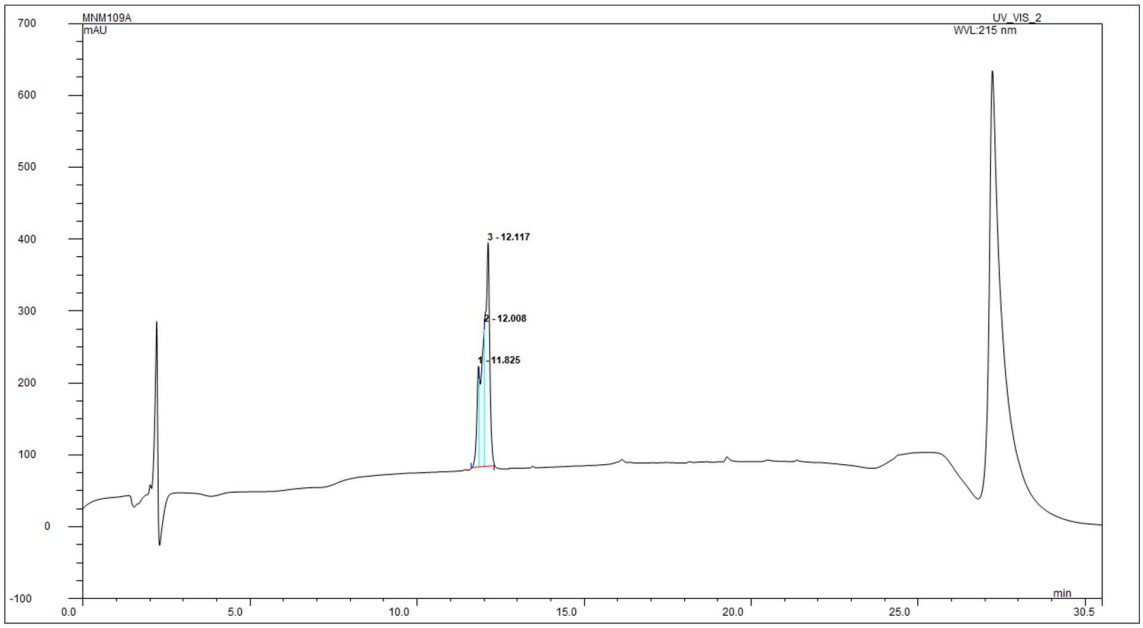


Figure S2. Analytical HPLC of the **2** peptide after RP-HPLC purification.

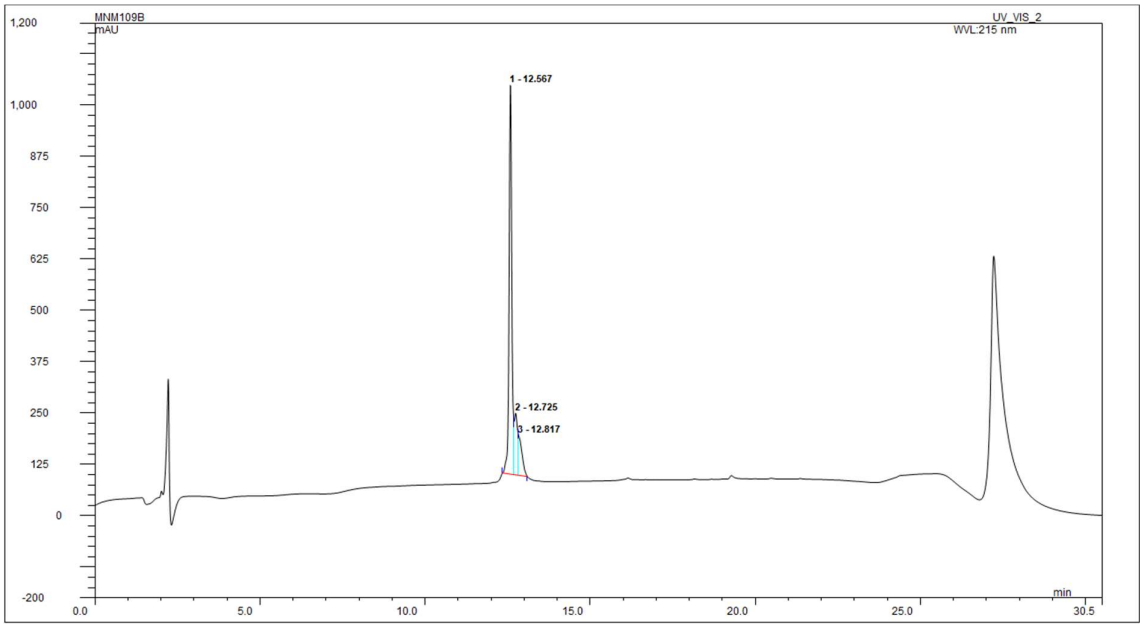


Figure S3. Analytical HPLC of the **3** peptide after RP-HPLC purification.

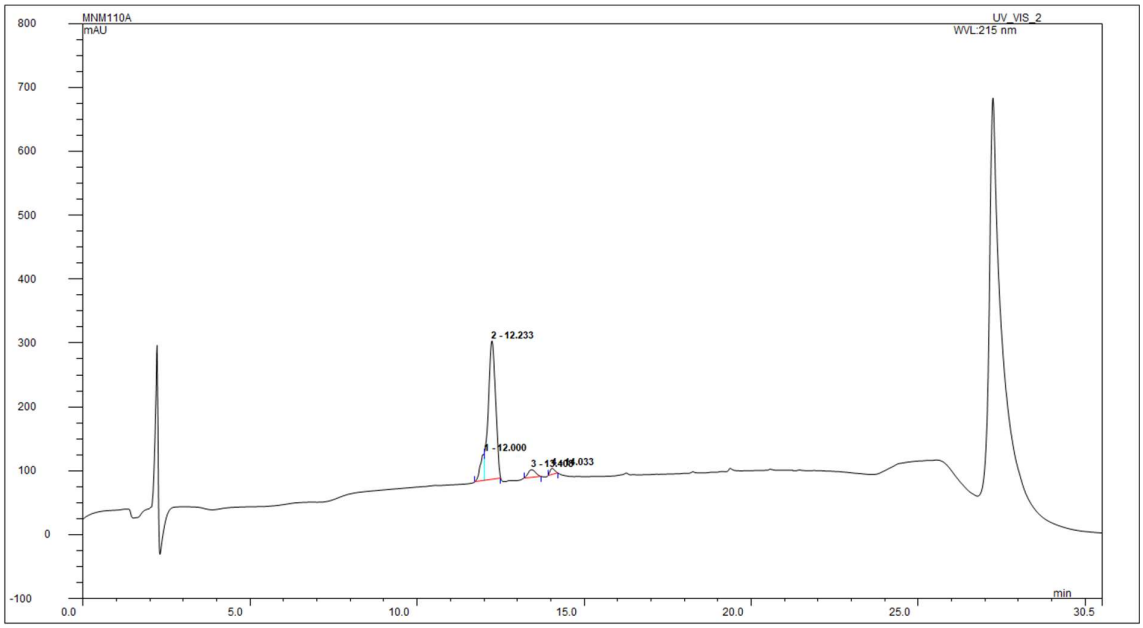


Figure S4. Analytical HPLC of the **4** peptide after RP-HPLC purification.

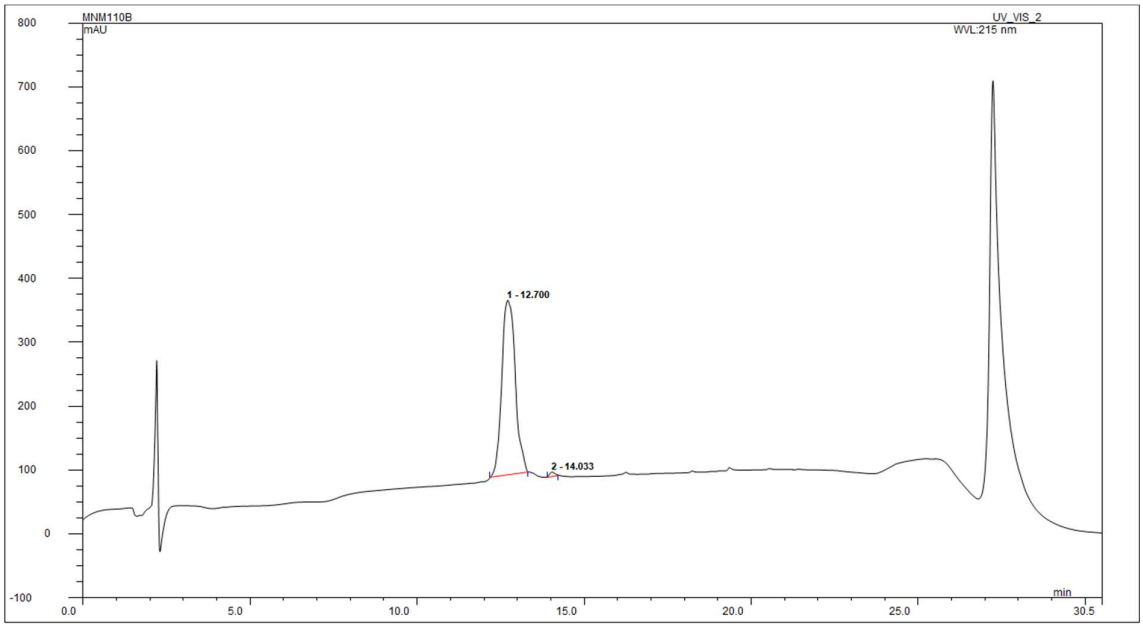


Figure S5. Analytical HPLC of the **5** peptide after RP-HPLC purification.

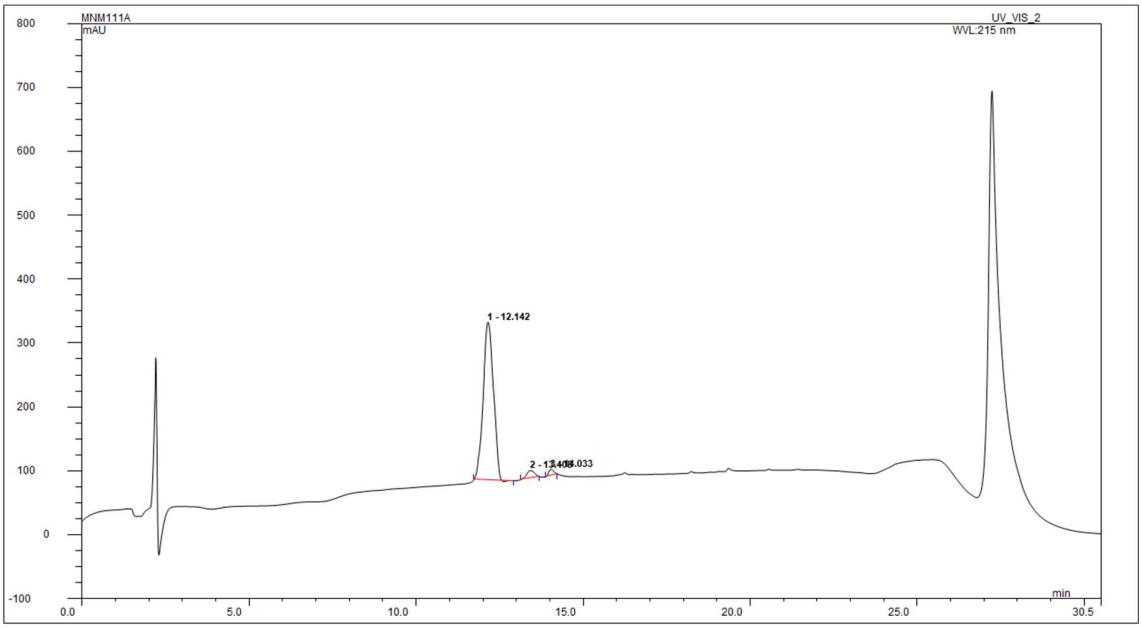


Figure S6. Analytical HPLC of the **6** peptide after RP-HPLC purification.

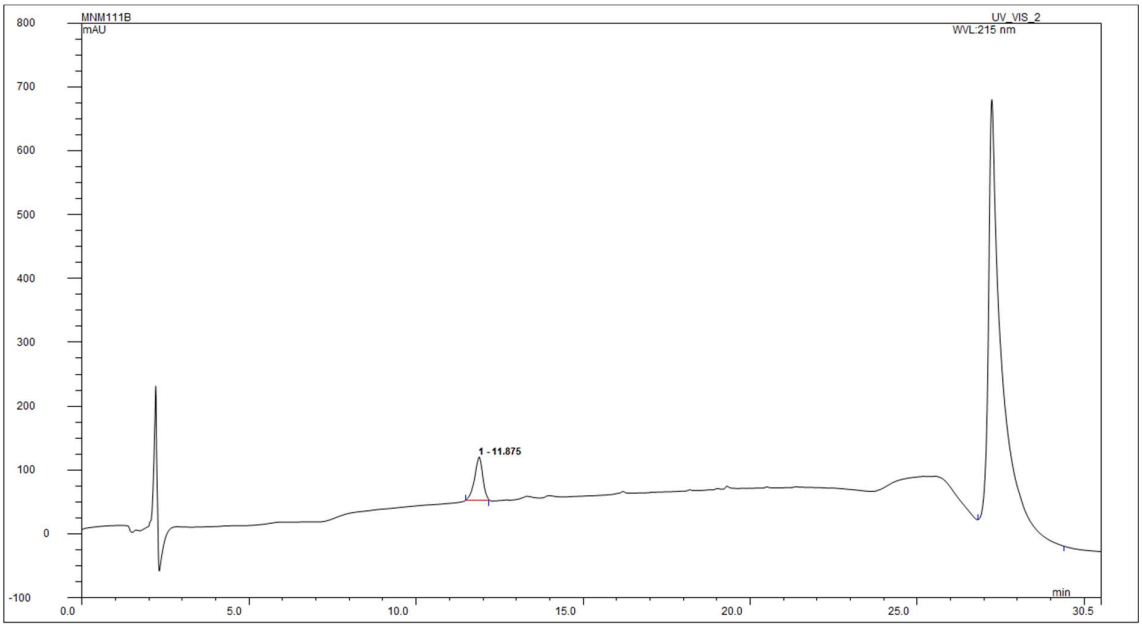


Figure S7. Analytical HPLC of the **7** peptide after RP-HPLC purification.

3. Circular Dichroism Spectroscopy

3.1 PSIPRED prediction of secondary structure of linear hACE2 peptides

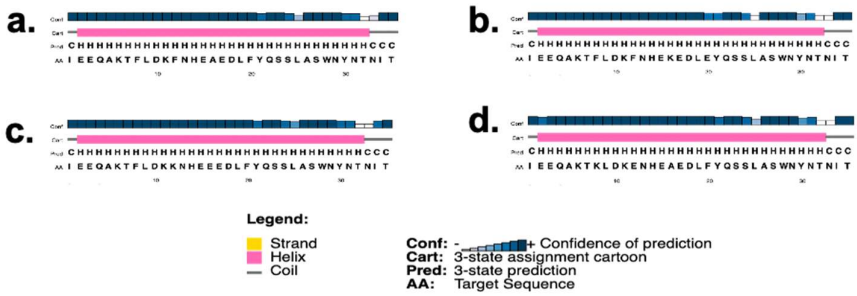


Figure S8. Prediction of the hACE2 peptide secondary structure was made using the PSIPRED 4.0 secondary structure prediction webserver.[1,2] The secondary structure prediction of linear hACE2 peptides a) **1**, b) **2**, c) **3**, and d) **4**.

3.2 Circular Dichroism Spectroscopy assay

To assess the peptide helicity, the hACE2 peptides (~30 μM) were dissolved in a 10 mM potassium phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) with varying concentrations of TFE (0% to 50%). The peptide concentration was determined using sequence-specific protein absorbance at 280 nm with a Nanodrop 2000 (Thermo Fischer Scientific, Waltham, MA, USA).[3] CD spectra were recorded on a Jasco J-815CD spectrometer (Jasco, Easton, MD, USA) using a 1 mm quartz cuvette (Hellma Analytics, Mullheim, Germany) at 25 °C in a wavelength range of 190 to 260 nm; Data Pitch: 0.5 nm; Scanning Speed: 20 nm/min; Response Time: 16 sec; Band Width; 1.5 nm; Accumulations: 5. Solvent background signal was subtracted for each wavelength. Helical content (fraction helicity (*fH*)) was quantified using poly-L-lysine hydrobromide (30-70 kDa, Merck, Darmstadt, Germany) in 0.1 M NaOH at pH 11.5 (100% alpha helical) as a reference for signal intensity at 222 nm.[4,5] fH can be calculated with $fH = (\theta_{222} - \theta_c)/(\theta_{222\infty} - \theta_c)$, where $\theta_{222\infty} = (-44{,}000 + 250T)\cdot(1 - k/Nr)$ and $\theta_c = 2220 - (53*T)$. K = 2.4 and T = 25 °C. Obtained

helicities were compared to estimates of helical content by DICHROWEB using the CDSSTR with reference set 4.

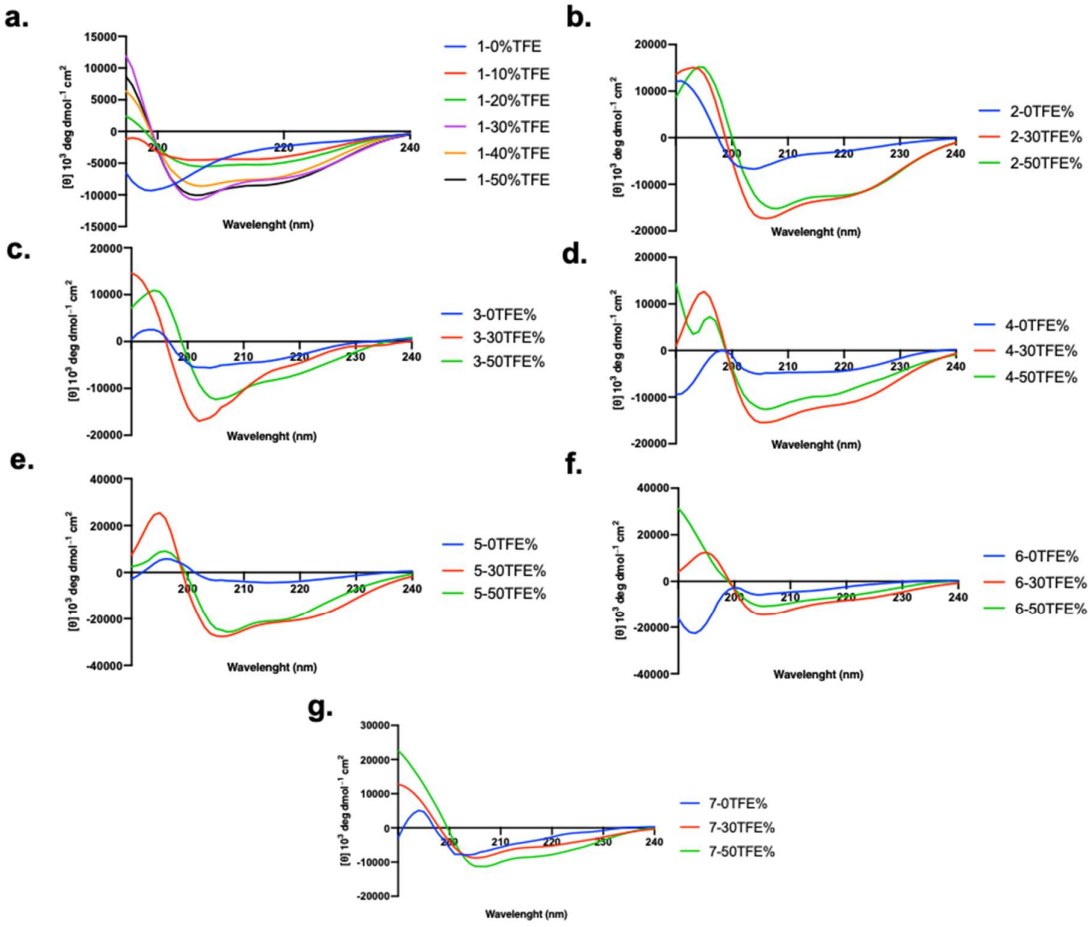


Figure S9. Circular Dichroism spectra of linear and stapled hACE2 peptides in molar ellipticity per residue. 30 μM peptide in 10 mM PBS at pH 7.4 varying percentages of TFE at 298 K. a) **1**, b) **2**, c) **3**, d) **4**, e) **5**, f) **6** and g) **7** at 0-50% TFE.

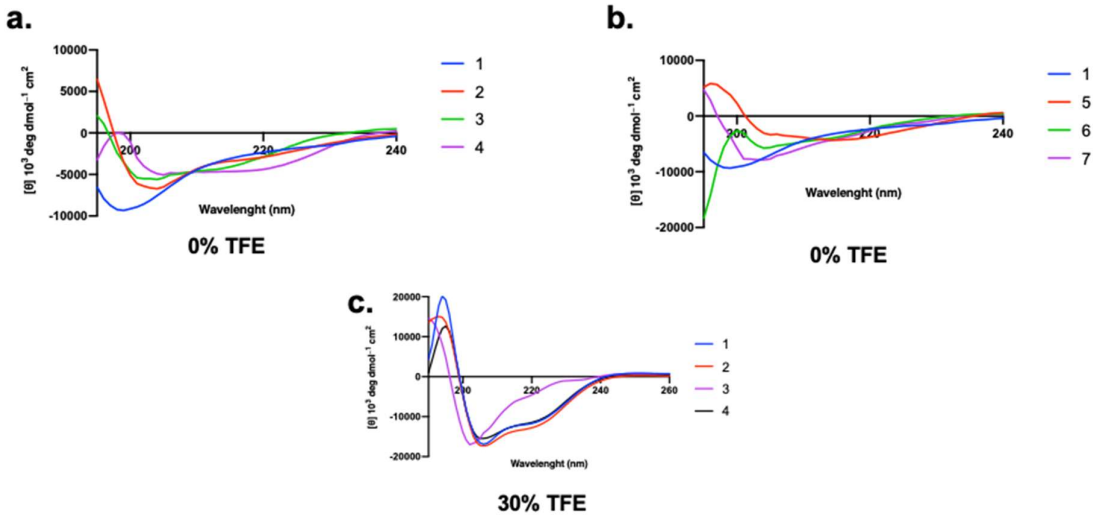


Figure S10. Circular Dichroism spectra of linear and stapled hACE2 peptides in molar ellipticity per residue. 30 µM peptide in 10 mM PBS at pH 7.4 at a-b) 0% TFE or c) 30% TFE at 298 K.

Table S2. θ222/θ208 (25 °C) ratios and determined helicities for the synthesized hACE2 peptides

	0% TFE							30% TFE						
		Fraction helicity							Fraction helicity					
	0222/0208 (25 °C)	(fH) normalized to PLL	CDSSTR (SET 4)	K2D2	CONTIN (SET4)	SELCON	AVG	0222/0208 (25 °C)	(fH) normalized to PLL	CDSSTR (SET 4)	K2D2	CONTIN (SET4)	SELCON (SET4)	AVG
1	0.41	16%	7%	7%	1%	0%	6%	0.69	58%	35%	26%	37%	33%	38%
2	0.51	4%	9%	9%	12%	15%	10%	0.70	65%	49%	29%	39%	45%	45%
3	0.46	13%	6%	7%	8%	20%	11%	0.25	19%	15%	7%	12%	2%	11%
4	0.84	15%	9%	13%	10%	5%	10%	0.73	56%	44%	28%	37%	39%	41%
5	0.92	17%	11%	17%	12%	7%	12%	0.58	79%	57%	37%	47%	42%	52%
6	0.35	20%	8%	8%	2%	0%	8%	0.57	41%	39%	27%	35%	26%	34%
7	0.31	24%	15%	7%	10%	8%	13%	0.60	27%	27%	15%	24%	27%	24%

4. SARS-CoV-2 Spike/hACE2 Inhibitor Screening Assays

The panel of stapled hACE2 N-terminal α1-helix peptides was screened for inhibition of the SARS-CoV-2 Spike/hACE2 complex formation using the commercially available SARS-CoV-2 Spike Inhibitor Screening Assay Kit (BPS Bioscience, Cat. Number #79931, San Diego, CA, USA) following the manufacturer's instructions. All peptides were screened for inhibition at a single concentration (10 μM) in PBS, before screening active peptides in a range of concentrations (0.3 μM - 300 μM) for IC50 determination. Additionally, a concentration-response curve of SARS-CoV-2 Spike Protein (RBD) Recombinant Human Monoclonal Antibody (T01KHu, Thermo Fischer Scientific, Cat. Number #703958) in PBS was made as a positive control for inhibition (0.6 nM - 200 μM). SARS-CoV-2 Spike protein (RBD), mFc Tag was thawed on ice and subsequently diluted to 1 μg/mL in PBS. Each well of a 96-well white microplate was filled with 50 μL of the diluted Spike solution and was incubated overnight at 4 °C. The microplate was washed three times with 1x Immuno Buffer 1 before blocking the wells with 100 μL Blocking Buffer 2 for 1 h at room temperature with slow shaking. The microplate was washed three times with 1x Immuno Buffer 1 and supernatant was removed. 20 μL of 1x Immuno Buffer 1 was added to each well. 10 μL of the inhibitor solution was added to each designated well, and 10 μL of PBS was added to wells designated as "positive control" and "blank" before incubating for 1 h at room temperature with slow shaking. Incubation was followed by the addition of 20 μL thawed ACE2-His (2.5 ng/uL, 30 nM) to each well except for "blank". An additional 20 μL of 1x Immuno Buffer 1 was added to "blank" instead. The reaction was incubated for 1 h at room temperature with slow shaking before the microplate was washed three times with 1x Immuno Buffer 1. The wells were blocked with 100 μL Blocking Buffer 2 for 10 min at room temperature with slow shaking and were washed three times with 1x Immuno Buffer 1 afterwards. Anti-His-HRP was diluted 1000x in 1x Immuno Buffer 1 and 100 μL was added to each well to incubate for 1 h at room temperature with slow

shaking. The wells were blocked with 100 µL Blocking Buffer 2 for 10 min at room temperature with slow shaking after 3 washes with 1x Immuno Buffer 1. The supernatant was decanted, and the wells were dried by tapping the microplate onto clean paper towels. Elisa ECL substrate A and B were mixed 1:1, and 100 uL of the substrate mixture was added to each well before measuring chemiluminescence using a FluoStar Omaga microplate reader (Ramcon A/S, Birkerød, Denmark). Data was normalized to luminescence values for the negative control and the curves were fitted with the variable slope non-linear regression.

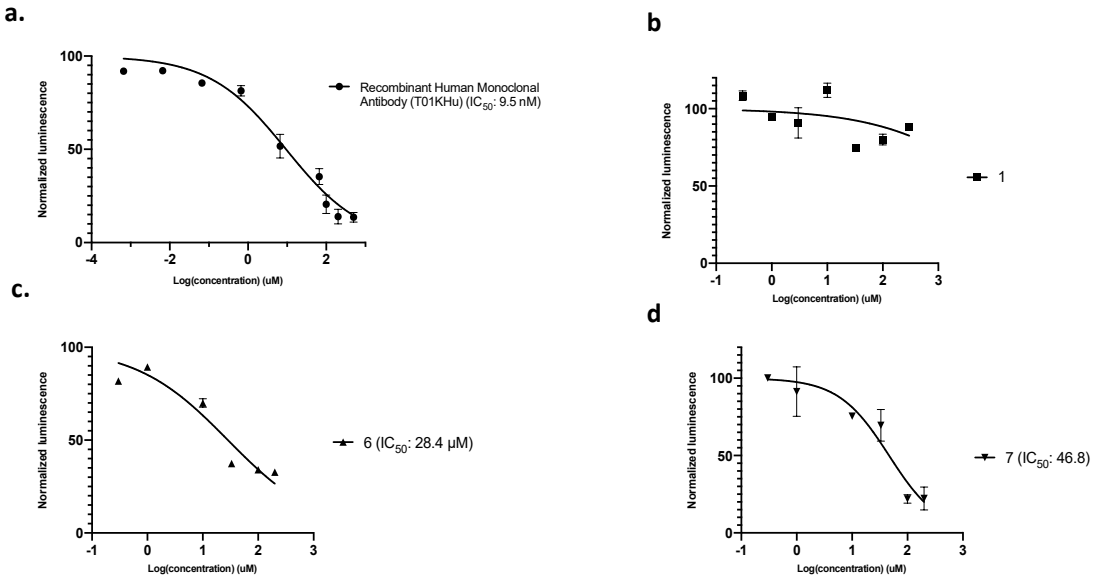


Figure S11. SARS-CoV-2 S protein-hACE2 inhibitor screening control. Luminescence was recorded with a Fluo-Star Omega fluorescent microplate reader and was normalized to the negative control. Data is mean ± SD, n = 2. a) Recombinant Human Monoclonal Antibody (IC_{50}: 9.5 nM) was screened (0.6 nM - 200 µM) to validate the assay. b-d) Concentration dependent assay of **1**, **6** (IC_{50}: 28.4 µM) and **7** (IC_{50}: 46.8 µM), n = 2.

5. Surface Plasmon Resonance (SPR) Binding Assay

SPR sensorgrams were recorded on a Biacore™ X100 system using an C19RBDHC30M sensor chip (Xantec GmbH), running in 10 mM sodium phosphate, pH 7.4 at 25 °C, flow 30 µl/min, unless indicated

otherwise). The RBD-S1 domain immobilized by the manufacturer was inactivated in flow channel 1 ('Referencer solution', Xantec GmBH, 20 min, 5 µl/min) and left intact in channel 2. The surface was conditioned and regenerated using 10 mM Glycine pH 2 (4×20 s, 100 µl/min and 2x30 s 60µl/min, respectively). Prior to analyte injection, both surfaces were conditioned using running buffer (60 s, 100 µl/min) and a high salt buffer 50 mM sodium phosphate pH 6.8, 1 M NaCl (contact 60 s, stabilization 60 s). Peptide samples (5 µM) were injected (contact time 90 s, dissociation time 360 s), then the surface was regenerated as mentioned above. Peptides (5 µM, dissolved in running buffer) were injected in triplicate. The resulting sensorgrams were double referenced by subtracting the reference signal and a reference sensorgram in absence of peptide. The resulting sensorgrams where fitted based on a 1:1 binding model using the 'single-curve analysis' function in the Anabel 2.0 software[6] and resulting values and standard errors of fit were averaged from triplicates.

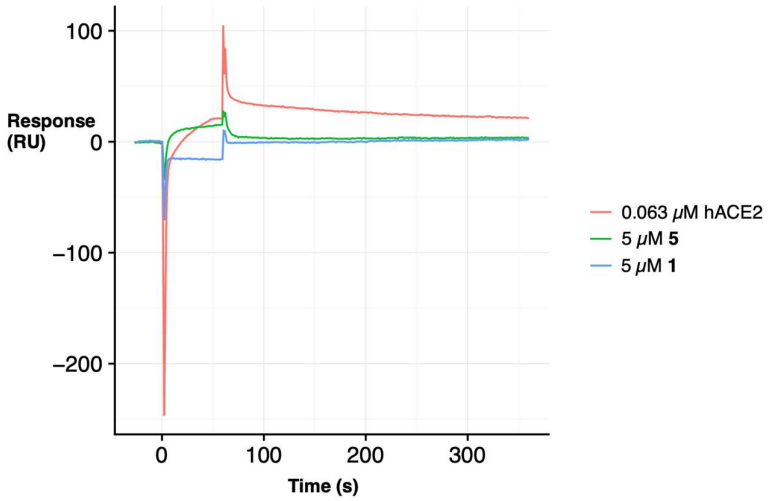


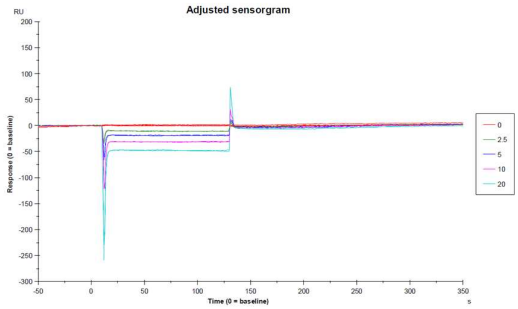
Figure S12. SPR Sensorgram overlay. In reference subtracted sensorgrams compound 1 exhibited no binding signal, whereas a 63 nM solution of hACE2 protein showed strong binding, reaching the equilibrium response level withing the 90s contact time and serving as a positive control for the SPR chip setup. The sensorgram spikes result from the physical separation between the reference flow cell which are in series in the microfluidic system (resulting in a 1 s delay for the 30 µl/min flow speed). The negative bulk-shift of the binding

curves resulted from a slightly higher signal from the bulk refractive index change in the reference cell.

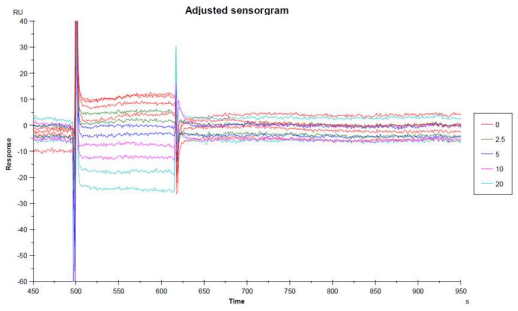
Table S3. **Determination of the binding affinity and kinetic parameters of c-hACE221-55A36K-F40E (5) binding to immobilized SARS-CoV-2 Spike protein with SPR.** *Conditions: 10 mM sodium phosphate, pH 7.4, 25 °C, n = 3. Reference subtracted SPR sensorgrams of* ***5.***

Curve	c(Reagent) [M]	kobs	StErr[Kobs]	kdiss	StErr[kdiss]	kass [1/M]	StErr(kass) [1/M]	KD [M]	StErr(KD) [M]
Cycle.12_Fc.2.1_Y	0.000005	0.128553	0.007405	0.032581	0.002091	19194.29	1538.983	1.69745E-06	1.74E-07
Cycle.19_Fc.2.1_Y	0.000005	0.118359	0.00655	0.041209	0.002613	15429.88	1410.406	2.67075E-06	2.97E-07
Cycle.27_Fc.2.1_Y	0.000005	0.104313	0.006472	0.029692	0.001735	14924.11	1340.029	1.98955E-06	2.13E-07
Average		**0.117075**	**0.006809**	**0.034494**	**0.002147**	**16516.09**	**1429.806**	**2.11925E-06**	**2.28E-07**

a. 1, $hACE2_{21\text{-}55}$



b. 6, $hACE2_{21\text{-}55F32K\text{-}A36E}$



c. 7, $hACE2_{21\text{-}55F28K\text{-}F32E}$

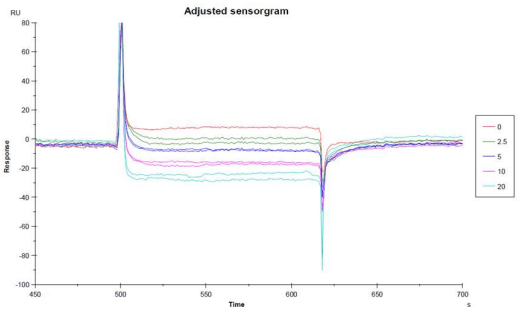


Figure S13. Determination of binding of $hACE2_{21\text{-}55}$ peptides to immobilized SARS-CoV-2 Spike protein with SPR. *Conditions: 10 mM sodium phosphate, pH 7.4, 25 °C, n = 2. A-D) Reference subtracted SPR sensorgrams of **1**, **5**, **6** and **7** at 0-20 µM.*

6. Serum Stability Assay

Human serum (Merck, Darmstadt, Germany) was thawed at 37 °C before being centrifugated at 5000 RPM for 5 min to remove the lipids from the solution. Supernatant was subsequently diluted in PBS (1:4 v/v). An aqueous peptide stock (500 μM) was diluted with 25% human serum (1:8, v/v, peptide/serum). Peptide solutions were incubated at 37 °C 750 RPM using an Eppendorf ThermoMixer G (Eppendorf, Hamburg, Germany), and reactions were quenched at 0, 10, 30, 60 and 180 min with ice cold acetonitrile (1:1 v/v). Quenched samples were incubated on ice for 30 min before centrifugation at 14000 g for 25 min. Supernatant was analyzed with analytical RP-HPLC using a gradient of buffer A and buffer B from 5% B to 95% over 30 minutes at 1 mL/min on a Gemini 5μm C18 110Å LC column (Phenomenex). Analytical injections were monitored at 222 nm.

7. References

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