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# Supplementary Materials: Effects of Dimerization, Dendrimerization, and Chirality in p-BthTX-I Peptide Analogs on the Antibacterial Activity and Enzymatic Inhibition of the SARS-CoV-2 PL$^{pro}$ Protein

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**Synthesis and characterization of the peptides**

The peptides were manually synthesized by solid-phase using the Fmoc (9-fluorenylmethyloxycarbonyl) protocol. The synthesis was performed using Rink Amide resin (0.55 mmol g-1, AAPPTEC) to obtain the peptide in alpha-carboxyamide form after the cleavage step. Briefly, deprotection of the Fmoc group was performed in 20% 4-methylpiperidine in dimethylformamide (DMF) by 1 and 20 min. Fmoc-amino acids were coupled using diisopropylcarbodiimide (DIC)/N-hydroxybenzotriazole (HOBt) in DMF for 2 h stirring, with 2-fold excess for all coupling reagents. If necessary, O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU)/diisopropylethylamine (DIEA) in DMF was used. The dimer and tetramer analogs (L and D isomers) were obtained using the previously described strategy [1] using one (for dimers) or 3 (for tetramers) Fmoc-Lys(Fmoc)-OH in the C-terminal region. The chains of peptides were simultaneously elongated grown up in $\alpha$- and ε-amino groups, obtaining homo dimers and tetramers. The cleavage of the peptide from the resin was performed for 2 h using 95% TFA, 2.5 TIS (triisopropylsilane) and 2.5 water, at a ratio of 10 mL/g resin. The crude peptides were precipitated with chilled ethyl ether, separated from soluble nonpeptide material by centrifugation, and lyophilized. Purification of the synthetic peptides was performed in a semi-preparative mode using a C18 reverse-phase column (AAPPTEC 1.0 × 25 cm). The purity was determined on a C18 reverse-phase (0.46 × 25 cm) analytical column (Agilent, Santa Clara, CA, USA). The solvents were 0.045% TFA in deionized water and 0.036% TFA in acetonitrile with 1 mL/min with a linear gradient of 5–95% solvent B (0.036% (v/v) TFA/acetonitrile) for 30 min. The chromatograms and mass spectra of the peptides are showed in Figure S1-S6. The mass spectrometry which indicated the molecular mass of the cation/charge ratio of the identified peptides' analysis was carried out. The mass spectra of these peptides were evaluated, and the synthesis was confirmed (Table S1).

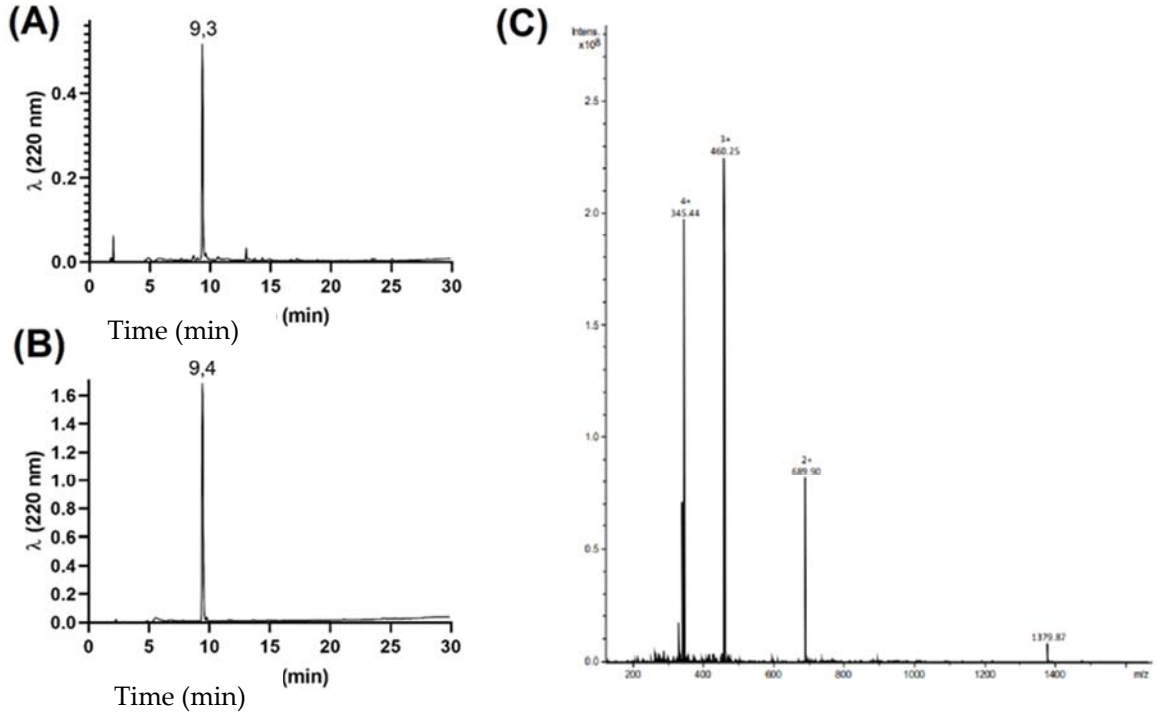


**Figure S1.** Chromatogram of the crude (a) and purified KKYRYHLKPF (L-monomer) peptide (b) - analytical reverse phase Phenomenex Jupiter C18 column (150 x 4.6 mm), packed with spherical 5 mm particle and 300 Å pore size, using a linear gradient of 5–95% (v/v) of solvent B for 30 min, at a flow rate of 1.0 mL/min and UV detection at 220 nm. Mass spectrum of the peptide (c) - Thermo LCQ-fleet mass spectrometer, with ESI-IT-MS configuration. Direct infusion of the sample solutions was carried out at a concentration of about 10 ppm in acetonitrile/water containing 0.1% v/v formic acid. The infusion flow was adjusted to 5.0 μL/min and the electrospray source was operated in positive mode, applying 4.5 kV to the electrospray capillary.

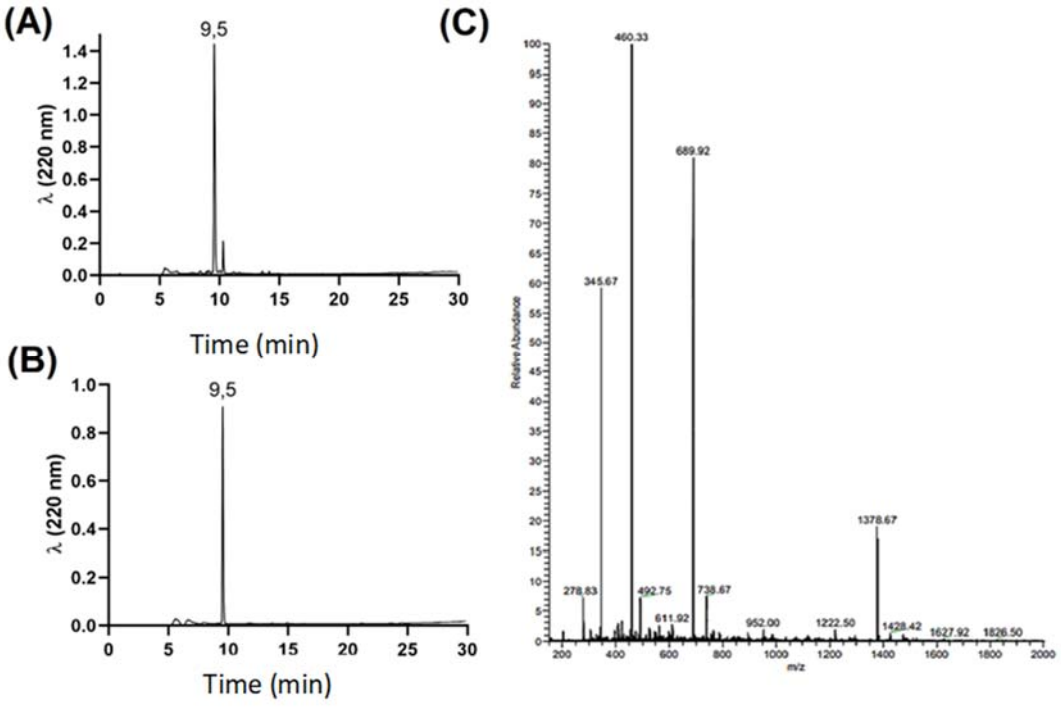


**Figure S2.** Chromatogram of the crude (a) and purified kkyryhlkpf (D-monomer) peptide (b) - analytical reverse phase Phenomenex Jupiter C18 column (150 x 4.6 mm), packed with spherical 5 mm

particle and 300 Å pore size, using a linear gradient of 5–95% (v/v) of solvent B for 30 min, at a flow rate of 1.0 mL/min and UV detection at 220 nm. Mass spectrum of the peptide (c) - Thermo LCQ-fleet mass spectrometer, with ESI-IT-MS configuration. Direct infusion of the sample solutions was carried out at a concentration of about 10 ppm in acetonitrile/water containing 0.1% v/v formic acid. The infusion flow was adjusted to 5.0 μL/min and the electrospray source was operated in positive mode, applying 4.5 kV to the electrospray capillary.

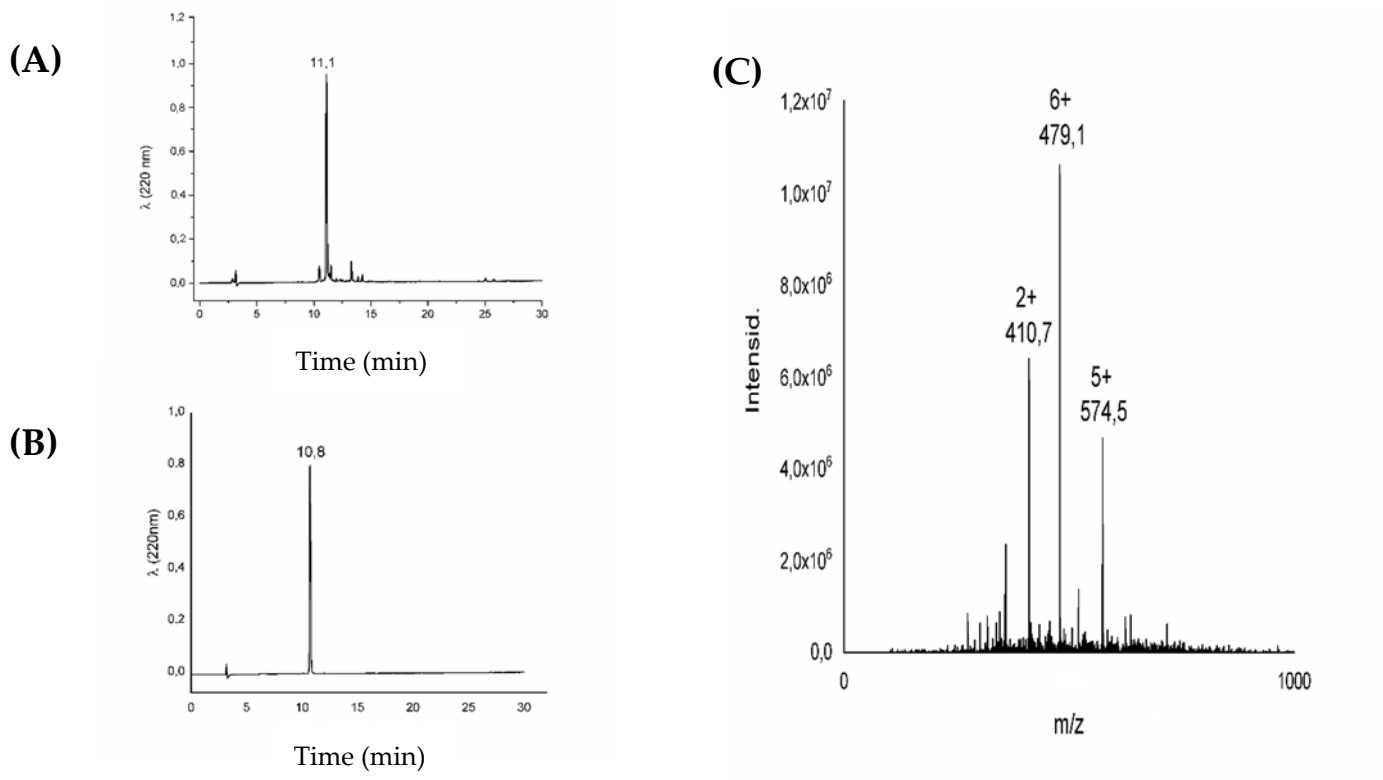


**Figure S3.** Chromatogram of the crude (a) and purified (KKYRYHLKPF)$_2$K (L-dimer) peptide (b) - analytical reverse phase Phenomenex Jupiter C18 column (150 x 4.6 mm), packed with spherical 5 mm particle and 300 Å pore size, using a linear gradient of 5–95% (v/v) of solvent B for 30 min, at a flow rate of 1.0 mL/min and UV detection at 220 nm. Mass spectrum of the peptide (c) - Thermo LCQ-fleet mass spectrometer, with ESI-IT-MS configuration. Direct infusion of the sample solutions was carried out at a concentration of about 10 ppm in acetonitrile/water containing 0.1% v/v formic acid. The infusion flow was adjusted to 5.0 μL/min and the electrospray source was operated in positive mode, applying 4.5 kV to the electrospray capillary.

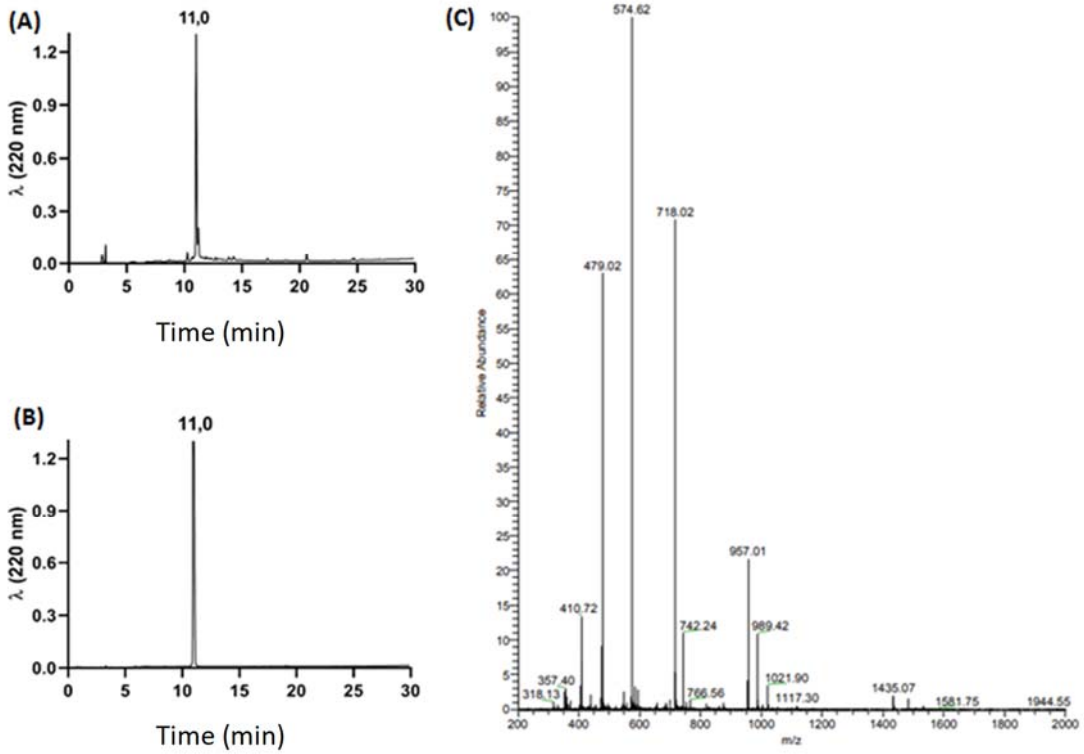


**Figure S4.** Chromatogram of the crude (a) and purified (kkyryhlkpf)$_2$K (D-dimer) peptide (b) - analytical reverse phase Phenomenex Jupiter C18 column (150 x 4.6 mm), packed with spherical 5 mm particle and 300 Å pore size, using a linear gradient of 5–95% (v/v) of solvent B for 30 min, at a flow rate of 1.0 mL/min and UV detection at 220 nm. Mass spectrum of the peptide (c) - Thermo LCQ-fleet mass spectrometer, with ESI-IT-MS configuration. Direct infusion of the sample solutions was carried out at a concentration of about 10 ppm in acetonitrile/water containing 0.1% v/v formic acid. The infusion flow was adjusted to 5.0 μL/min and the electrospray source was operated in positive mode, applying 4.5 kV to the electrospray capillary.

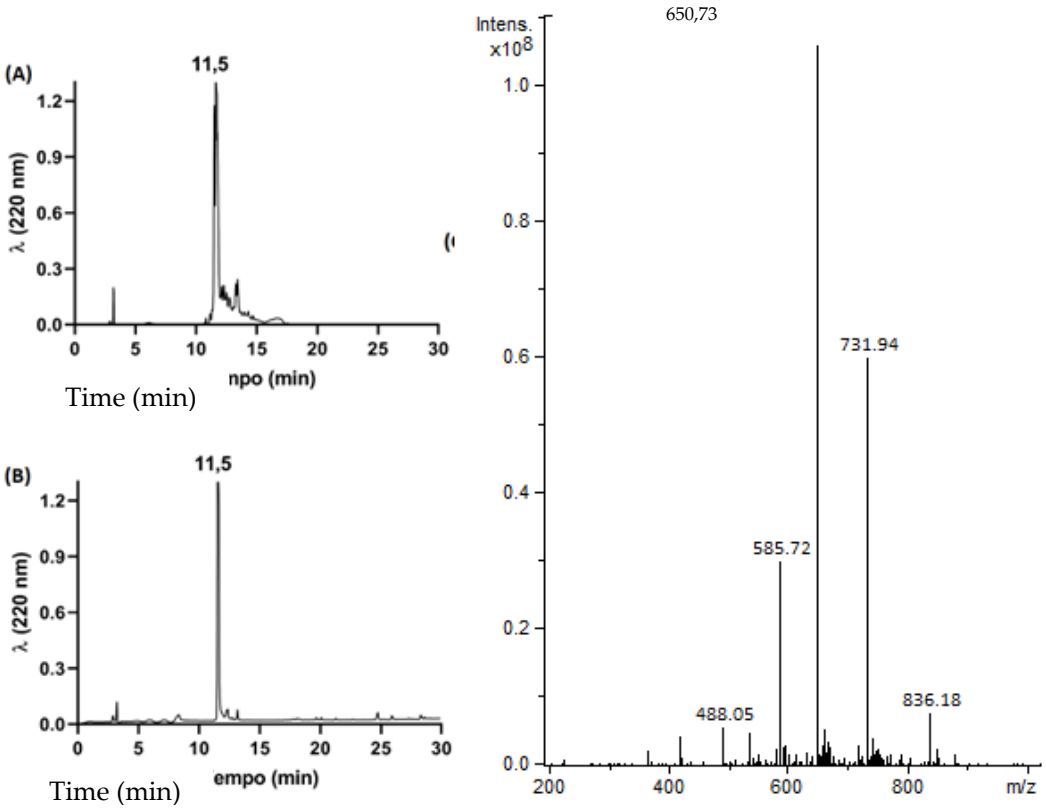


**Figure S5.** Chromatogram of the crude (a) and purified (KKYRYHLKPF)$_4$K$_2$K (L-tetramer) peptide (b) - analytical reverse phase Phenomenex Jupiter C18 column (150 x 4.6 mm), packed with spherical 5 mm particle and 300 Å pore size, using a linear gradient of 5–95% (v/v) of solvent B for 30 min, at a flow rate of 1.0 mL/min and UV detection at 220 nm. Mass spectrum of the peptide (c) - Thermo LCQ-fleet mass spectrometer, with ESI-IT-MS configuration. Direct infusion of the sample solutions was carried out at a concentration of about 10 ppm in acetonitrile/water containing 0.1% v/v formic acid. The infusion flow was adjusted to 5.0 μL/min and the electrospray source was operated in positive mode, applying 4.5 kV to the electrospray capillary.

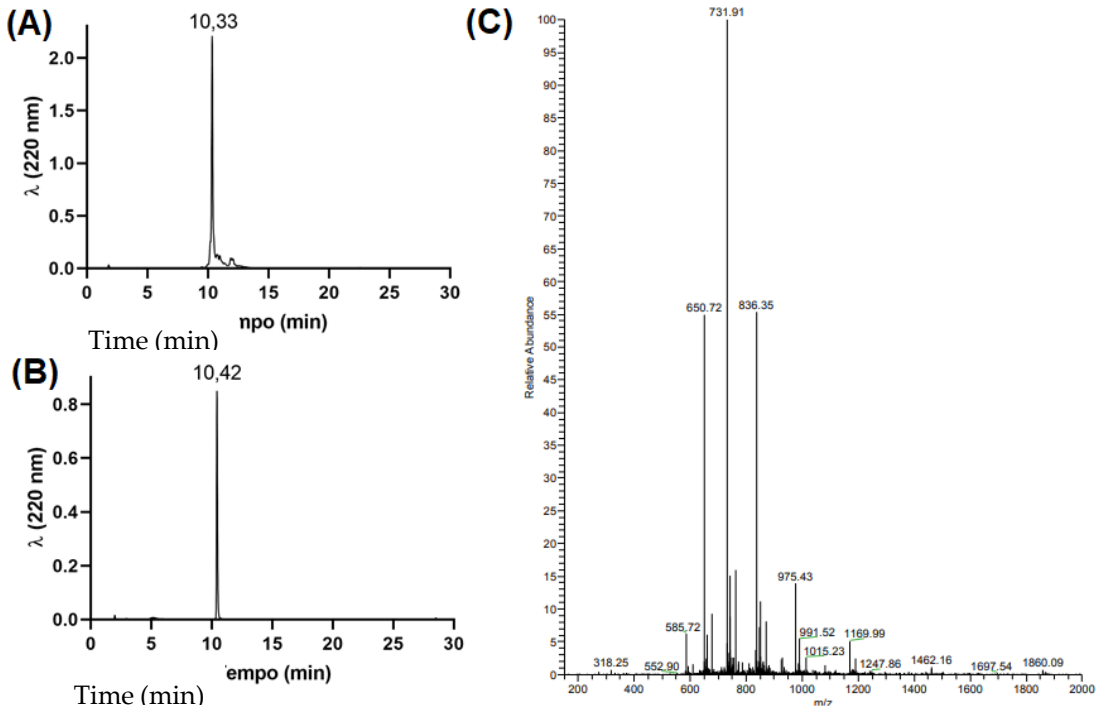


**Figure S6.** Chromatogram of the crude (a) and purified (kkyryhlkpf)$_4$K$_2$K (D-tetramer) peptide (b) - analytical reverse phase Phenomenex Jupiter C18 column (150 x 4.6 mm), packed with spherical 5 mm particle and 300 Å pore size, using a linear gradient of 5–95% (v/v) of solvent B for 30 min, at a flow rate of 1.0 mL/min and UV detection at 220 nm. Mass spectrum of the peptide (c) - Thermo LCQ-fleet mass spectrometer, with ESI-IT-MS configuration. Direct infusion of the sample solutions was carried out at a concentration of about 10 ppm in acetonitrile/water containing 0.1% v/v formic acid. The infusion flow was adjusted to 5.0 μL/min and the electrospray source was operated in positive mode, applying 4.5 kV to the electrospray capillary.

**Table S1.** Molecular weight and mass/charge ratio of the synthetic peptides.

| **eptide** | **MW g/mol** | **mass/charge ratio** | | |
|---|---|---|---|---|
| | | **Peak 1** | **Peak 2** | **Peak 3** |
| L-monomer | 1378.7 | 690.4/2 | 460.6/3 | 345.7/4 |
| D-monomer | 1378.7 | 690.4/2 | 460.6/3 | 345.7/4 |
| L-dimer | 2868.5 | 574.7/5 | 479.1/6 | 410.8/7 |
| D-dimer | 2868,5 | 718,1/4 | 574.7/5 | 479.1/6 |
| L-tetramer | 5848.1 | 732.0/8 | 650.8/9 | 585.8/10 |
| D-tetramer | 5848.1 | 836.4/7 | 732.0/8 | 650.8/9 |

**PL$^{pro}$ Inhibition Assays**

Our findings indicated that the analogs showed comparable inhibition profiles [2] and $IC_{50}$ values. The inhibition profiles of L-dimer, D-dimer and L-tetramer was published

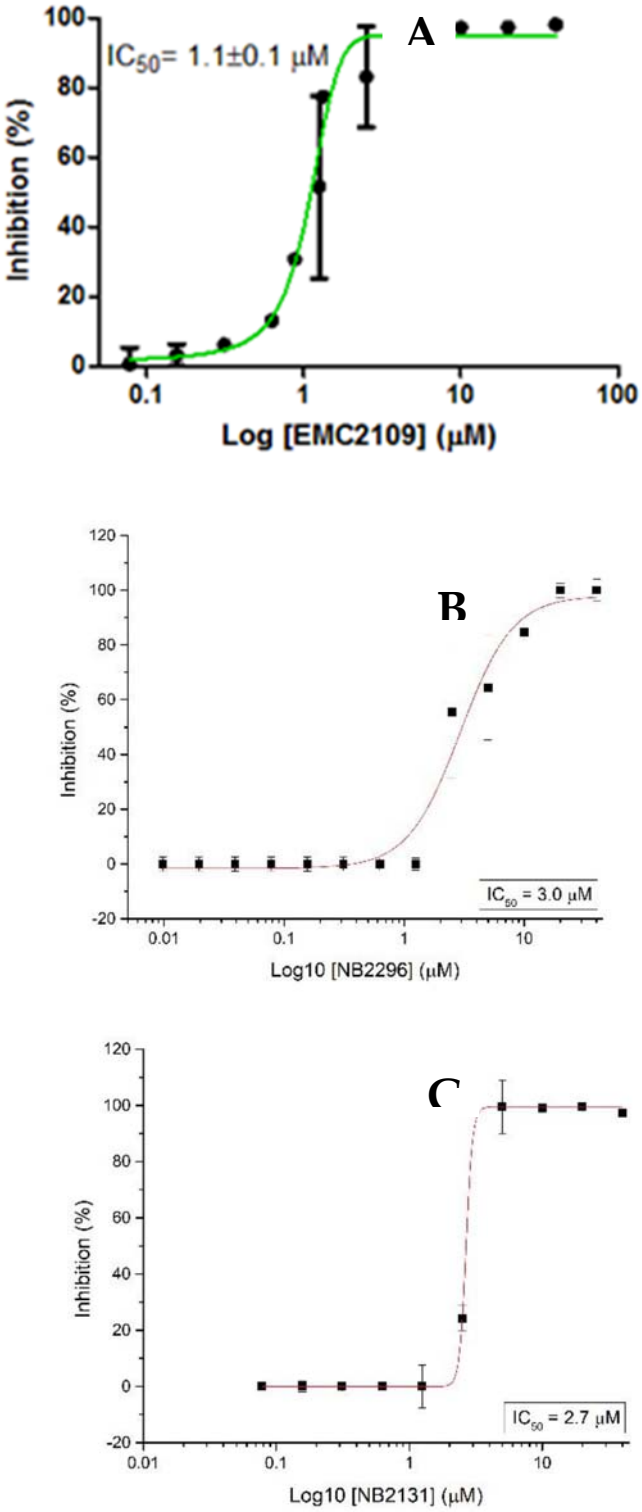


**Figure S7.** Representative concentration–response inhibition curves against PL$^{pro}$ from SARS-CoV-2 for L-monomer (A), D-monomer and D-tetramer. Two independent experiments were done.

1. Santos-Filho, N. A., Righetto, G. M., Pereira, M. R., Piccoli, J. P., Almeida, L. M. T., Leal, T. C., Camargo, I. L. B. C., Cilli, E. M. Effect of C-terminal and N-terminal dimerization and alanine scanning on antibacterial activity of the analogs of the peptide p-BthTX-I. *Peptide Science*, **2022**, *114:e24243*, 1-10. DOI: 10.1002/pep2.24243.
2. Freire, M. C.; Noske, G. D.; Bitencourt, N. V.; Sanches, P. R..; Santos-Filho, N. A.; Gawriljuk, V. O.; Souza E. P.; Nogueira, V. H. R.; Godoy, M. O.; Nakamura, A. M.; Fernandes, R. S.; Godoy, A. S.; Juliano, M. A.; Peres, B. M.; Barbosa, C. G.; Moraes, C. B.; Freitas-Junior, L. H. G.; Cilli, E. M.; Guido, R. V. C.; Oliva, G. Non-Toxic Dimeric Peptides Derived from the Bothropstoxin-I Are Potent SARS-CoV-2 and Papain-like Protease Inhibitors. *Molecules,* **2021**, *26(16*), 4896.