SUPPORTING INFORMATION

Phenylalanine and Phenylglycine Analogs as Arginine Mimetics in Dengue Protease Inhibitors

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Contents

1. Aprotinin Competition Assay.

Table S1. Inhibitory activity of compound **47** against DENV protease, WNV protease, thrombin and trypsin.

No.	**DENV** **IC_{50} [µM][a]**	**Hill** **Slope**	**WNV** **[%][b]**	**THR** **[%][c]**	**TRY** **[%][d]**
47	1.8 ± 0.12	0.91	38.2	25.2	15.1

All measurements were carried out in triplicate. Standard deviations were ≤ 10 %

[a] IC_{50} value against DENV NS2B-NS3pro protease serotype 2 (substrate concentration: 50 µM)

[b] Percent inhibition of the West Nile virus (WNV) NS2B-NS3pro protease (cmpd.: 50 µM; substrate: 50 µM, K_m: 212 µM; enzyme: 150 nM)

[c] Percent inhibition of thrombin (cmpd.: 25 µM; substrate: 50 µM, K_m: 16 µM; enzyme: 10 nM)

[d] Percent inhibition of trypsin (cmpd.: 50 µM; substrate: 50 µM, enzyme: 1 nM)

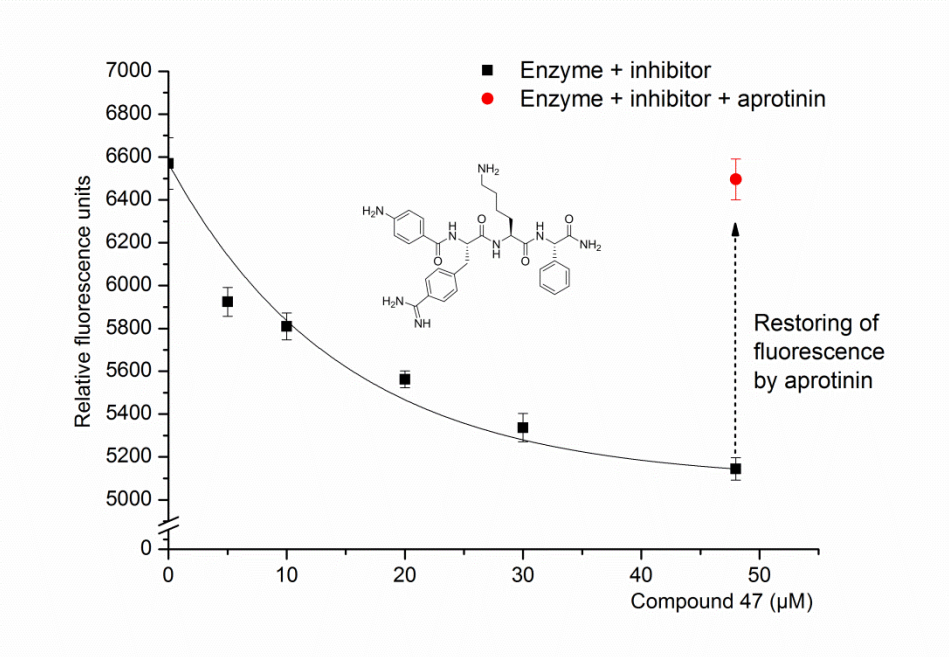

Figure S1. Results of aprotinin competition assay with compound **47**. The quenching of the dengue virus protease autofluorescence by inhibitor **47** is monitored at 320 nm. The solid line is the fit by Origin with equation of model *ExpDecay*. The symbol (●) shows the fluorescence intensity after the addition of aprotinin.

2. Permeability Data in the Parallel Artificial Membrane Permeability Assay (PAMPA).

All compounds tested in PAMPA showed a P_e of 0.0.[1] For the references caffeine and phenytoin a P_e of 6.92 and 4.20 was found, respectively.

Table S2. Permeability Data of Selected Compounds.

No	**C_{Acc} [µM][a]**	**C_{Don} [µM][b]**	**R [%][c]**
32a	0; below detectable limit	217.4 ± 3.0	n.d.
32b	0; below detectable limit	215.0 ± 8.2	n.d.
33a	0; below detectable limit	211.9 ± 1.9	n.d.
33b	0; below detectable limit	200.9 ± 2.7	n.d.
34a	0; below detectable limit	200.0 ± 3.1	n.d.
34b	0; below detectable limit	184.6 ± 5.5	7.7
37	0; below detectable limit	193.3 ± 3.0	3.4
39	0; below detectable limit	204.5 ± 1.1	n.d.
40	0; below detectable limit	195.7 ± 1.1	2.1
42a	0; below detectable limit	188.9 ± 10.9	5.6
42b	0; below detectable limit	197.2 ± 0.4	1.4
43	0; below detectable limit	186.9 ± 2.1	6.5
45a	0; below detectable limit	199.9 ± 0.9	0.1
45b	0; below detectable limit	192.4 ± 2.0	3.8
46	0; below detectable limit	196.4 ± 1.2	1.8

n.d. = not determined

[a] Concentration of compound detected in acceptor plate ($t_{incubation}$ = 5 h)

[b] Concentration of compound detected in donor plate ($t_{incubation}$ = 5 h)

[c] Mass retention of compound calculated according to the literature.[1]

3. Synthesis and Analytical Data of Peptide Hybrids.

Procedure A: Synthesis of Peptide Hybrids

N-terminal peptide hybrids were synthesized as described previously with some modifications.[2-3] The peptide sequence was synthesized analogously to the Fmoc protocol using Rink amide resin. Rink amide resin was swelled in DCM overnight. The resin was washed with DMF and Fmoc was removed by 25 % piperidine solution for 10 minutes. The procedure was repeated for another five minutes. After Fmoc removal the resin was washed wit DMF, DCM and DMF. Subsequently, amino acid (3.0 equiv) and HATU (3.0 equiv) were dissolved in 500 µl DMF and DIPEA (5.0 equiv) was added. The reaction mixture was added to the resin and shaken for at least one hour. After coupling the resin was again washed with DMF, DCM and DMF. The procedure was repeated for all amino acids. Caps were coupled to the peptide substituted resin by adding a mixture of cap (3.0 equiv), HATU (3.0 equiv) and DIPEA (5.0 equiv). The benzoyl protected amino acids (2.0 equiv) were coupled with HATU (2.0 equiv) and diisopropylethylamine (2.2 equiv) to the dipeptide sequence. The reaction mixture was stirred for three hours. The resin was washed with DMF, DCM and DEE. Then the resin was dried overnight and the peptide was cleaved by the standard procedure (92.5 % TFA, 5.0 % TIPS and 2.5 % water) for 2.5 hours. After precipitation in cold diethyl ether the peptides were purified. Purification was performed by preparative RP-HPLC on an ÄKTApurifier, GE Healthcare (Germany) with an RP-18 column (Rephrospher, Dr. Maisch GmbH, Germany, C18-DE, 5 µm, 30 x 16 mm and 120 x 16 mm). All peptides were freeze-dried and characterized by HR-ESI and ^{1}H NMR for selected compounds. The purity was determined by analytical HPLC on a Jasco HPLC system with UV detector (method A) and an Agilent 1200 HPLC system with MWD detector combined with a Bruker micrOTOF-Q II instrument (method B). As RP-18 column ReproSil-Pur-ODS-3, Dr. Maisch GmbH, Germany, 5 µm, 50 x 2 mm was used. The

conditions for method A were: eluent A: water (+ 0.1 % TFA), eluent B: acetonitrile (+ 0.1 % TFA), flow rate: 1 ml/min, gradient: 1 % B (0.2 min), 100 % B (3.5 min), 100 % B (4.5 min), 1 % B (4.6 min), 1 % B (5 min). The purity of compound **30a**, **31a** and **32a** were determined by method B. The conditions for method B were: eluent A: water (+ 0.1 % formic acid), eluent B: acetonitrile (+ 0.1 % formic acid), flow rate 0.3 ml/min, gradient: 5 % B (1 min), 95 % B (6 min), 95 % B (10 min), 5 % B (10.1 min), 5 % B (12 min). All evaluated peptide hybrids were obtained with a purity of at least 95 % unless indicated otherwise.

No.	**Molecular Formula**	**$[M+H]^+$ m/z calcd**	**$[M+H]^+$ m/z found**	**Retention time [min]**	**HPLC purity [%]**
29a	$C_{29}H_{34}N_6O_4$	531.2714	531.2704	1.63	≥ 95 %
29b	$C_{29}H_{34}N_6O_4$	531.2714	531.2715	1.63	≥ 95 %
30a	$C_{30}H_{36}N_6O_4$	545.2870	545.2863	0.81	≥ 95 %
30b	$C_{30}H_{36}N_6O_4$	545.2871	545.2874	1.77	≥ 95 %
31a	$C_{31}H_{34}N_6O_4$	555.2714	555.2719	0.55	≥ 95 %
31b	$C_{31}H_{34}N_6O_4$	555.2714	557.2756	2.07	≥ 95 %
32a	$C_{31}H_{38}N_6O_4$	559.3027	559.3014	1.76	≥ 95 %
32b	$C_{31}H_{38}N_6O_4$	559.3027	559.3011	1.72	≥ 95 %
34a	$C_{31}H_{38}N_8O_4$	587.3089	587.3079	1.45	≥ 95 %
34b	$C_{31}H_{38}N_8O_4$	587.3089	587.3087	1.82	≥ 95 %
39	$C_{32}H_{39}N_7O_4$	586.3136	586.3129	1.79	≥ 95 %
40	$C_{32}H_{38}N_6O_5$	587.2976	587.2983	1.99	≥ 95 %
41	$C_{28}H_{37}F_3N_8O_4$	607.2963	607.2958	1.88	≥ 95 %
43	$C_{32}H_{37}F_3N_8O_4$	655.2962	655.2951	1.96	≥ 95 %
44	$C_{29}H_{42}N_8O_4$	567.3402	567.3408	1.77	90 %
46	$C_{33}H_{42}N_8O_4$	615.3402	615.3399	1.71	≥ 95 %

29b: ^{1}H NMR (500 MHz, D_2O): δ = 7.53-7.66 (m, 5 H), 7.40-7.53 (m, 8 H), 7.34 (t, J=8.50 Hz, 1 H), 5.36 (s, 1 H), 4.71-4.74 (m, 1 H), 4.34 (d, J=7.83 Hz, 1 H), 3.16 (d, J=7.95 Hz, 2 H) 2.90-2.96 (m, 2 H) 1.61-1.86 (m, 5 H) 1.33-1.42 (m, 2 H) ppm.

40: ^{1}H NMR (500 MHz, D_2O): δ = 7.39-7.68 (m, 7 H), 7.32-7.38 (m, 3 H), 7.15-7.28 (m, 3 H), 5.37 (d, J=16.63 Hz, 1 H), 4.69-4.72 (m, 1 H), 4.60-4.65 (m, 1 H), 4.34 - 4.40 (m, 1 H), 3.02-3.24 (m, 2 H), 2.79-2.96 (m, 2 H), 2.13 (d, J=9.90 Hz, 2 H) 1.30-1.86 (m, 6 H) 0.91-1.08 (m, 2 H) ppm. Minor impurities detected.

34a: ^{1}H NMR (500 MHz, D_2O): δ = 7.69 (d, J=7.58 Hz, 2 H), 7.58-7.63 (m, 1 H), 7.31-7.52 (m, 9 H), 7.21 (d, J=8.19 Hz, 2 H), 5.34 (s, 1 H), 4.33-4.38 (m, 1 H), 3.17 (d, J=7.83 Hz, 2 H), 2.84 (t, J=7.52 Hz, 2 H), 1.51-1.73 (m, 5 H), 1.19-1.37 (m, 3 H) ppm.

Procedure B: Synthesis of Benzamidine-containing Peptides

Purification was performed by preparative RP-HPLC as described in procedure A. All peptides were freeze-dried and characterized by HR-ESI and ^{1}H NMR for selected compounds. The purity was determined by analytical HPLC with method A. All evaluated peptide hybrids were obtained with a purity of at least 95 % unless indicated otherwise.

No.	**Molecular Formula**	**$[M+H]^+$ m/z calcd**	**$[M+H]^+$ m/z found**	**Retention time [min]**	**HPLC purity [%]**
33a	$C_{31}H_{37}N_7O_4$	572.2980	572.2972	1.68	≥ 95 %
33b	$C_{31}H_{37}N_7O_4$	572.2980	594.2784	1.77	≥ 95 %
35	$C_{30}H_{35}N_7O_4$	558.2823	558.2815	1.70	≥ 95 %
42a	$C_{32}H_{36}F_3N_7O_4$	640.2854	640.2837	1.95	≥ 95 %
42b	$C_{32}H_{36}F_3N_7O_4$	640.2854	640.2840	1.95	≥ 95 %
45a	$C_{33}H_{41}N_7O_4$	600.3293	600.3304	1.82	≥ 95 %
45b	$C_{33}H_{41}N_7O_4$	600.3293	600.3290	1.87	≥ 95 %
47	$C_{31}H_{38}N_8O_4$	587.3089	587.3065	1.65	≥ 95 %

33a: ^{1}H NMR (500 MHz, D_2O): δ = 7.57-7.68 (m, 3 H), 7.32-7.53 (m, 11 H), 5.37 (s, 1 H), 4.78-4.81 (m, 1 H), 4.42 (m, 1 H), 3.21 (d, J=7.70 Hz, 2 H), 2.87-2.97 (m, 2 H), 1.60-1.87 (m, 5 H), 1.31-1.49 (m, 3 H) ppm.

42a: ^{1}H NMR (500 MHz, D_2O): δ = 7.74-7.84 (m, 4 H), 7.33-7.50 (m, 9 H), 5.36 (s, 1 H), 4.79-4.82 (m, 1 H), 4.37-4.44 (m, 1 H), 3.21 (d, J=7.70 Hz, 2 H), 2.94 (t, J=7.52 Hz, 2 H), 1.60-1.86 (m, 5 H), 1.32-1.49 (m, 3 H) ppm.

42b: ^{1}H NMR (500 MHz, D_2O): δ = 7.74-7.83 (m, 4 H), 7.60-7.66 (m, 2 H), 7.49-7.58 (m, 2 H) 7.36-7.47 (m, 5 H), 5.29 - 5.39 (m, 1 H), 4.80-4.85 (m, 1 H), 4.29 - 4.37 (m, 1 H), 3.18-3.33 (m, 2 H), 2.94 (m, J=7.50 Hz, 1 H), 2.84 (t, J=7.58 Hz, 1 H), 1.52-1.85 (m, 5 H), 1.18-1.46 (m, 3 H) ppm.

Procedure C: Synthesis of 37, Guanidinophenylglycine-Containing Peptide

Purification was performed by preparative RP-HPLC as described in procedure A. All peptides were freeze-dried and characterized by HR-ESI and ^{1}H NMR. The purity was determined by analytical HPLC with method A. Compound **37** was obtained with a purity of at least 95 %.

No.	**Molecular Formula**	**$[M+H]^+$ m/z calcd**	**$[M+H]^+$ m/z found**	**Retention time [min]**	**HPLC purity [%]**
37	$C_{30}H_{36}N_8O_4$	573.2932	573.2925	1.78	≥ 95 %

^{1}H-NMR (500 MHz, D_2O): δ = 1.20-1.90 (m, 6 H), 2.76-3.01 (m, 2 H), 4.41 (m, 1 H), 5.32 (d, *J* = 6.2 Hz, 1 H), 5.62 (d, *J* = 6.2 Hz, 1 H), 7.29-7.67 (m, 12 H), 7.75 (m, 2 H) ppm. Minor impurities detected.

4. Synthesis and Analytical Data of Precursors.

(3-Nitro)-L-phenylalanine methyl ester hydrochloride (1). Synthesized according to procedure D with some modifications. A cold solution of thionly chloride (3.65 ml, 50 mmol) in methanol (40 ml) was dropped into a solution of (3-nitro)-L-phenylalanine (2.0 g, 9.5 mmol) in methanol (40 ml) at 0 °C and the mixture was stirred for four hours at room temperature before the solvent was evaporated. Pale brown solid (Yield: 2.5 g, quant.). ^{1}H-NMR (300 MHz, acetone-d_6): δ = 3.69 (dd, *J* = 14.3, 3.7 Hz, 1 H), 3.82 (s, 3 H), 3.96 (dd, *J* = 14.2, 10.4 Hz, 1 H), 5.35 (dd, *J* = 10.4, 4.3 Hz, 1 H), 7.63 (t, *J* = 7.9 Hz, 1 H), 8.13 (m, 2 H), 8.44 (m, 1 H) ppm; HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{10}H_{13}N_2O_4$: 225.0870, found: 225.0873.

***N*-Benzoyl-(3-boc-amino)-L-phenylalanine methyl ester (4).** A solution of crude **3** (110 mg, 0.33 mmol), di-*tert*-butyl dicarbonate (360 mg, 1.65 mmol) and DIPEA (85 µl, 0.5 mmol) in

dichloromethane (10 ml) was stirred overnight at room temperature. The solvent was evaporated and the residue was purified by flash chromatography (cyclohexane/ethyl acetate) to obtain **4** as colorless solid (Yield: 85 mg, 65%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.52 (s, 9 H), 3.24 (m, 2 H), 3.80 (s, 3 H), 5.10 (m, 1 H), 6.44 (m, 1 H), 6.62 (m, 1 H), 6.82 (m, 1 H), 7.18 (m, 2 H), 7.34-754 (m, 4 H), 7.78 (m, 2 H) ppm; HRMS (ESI): *m/z* $[M+Na]^+$ calcd for $C_{22}H_{26}N_2NaO_5$: 421.1734, found: 421.1737.

***N*-Benzoyl-(3-bis-boc-guanidino)-L-phenylalanine methyl ester (6).** A mixture of crude **3** (170 mg, 0.5 mmol), bis-boc-pyrazole-1-carboxamidine (235 mg, 0.75 mmol), DMAP (13 mg, 0.1 mmol) and DIPEA (85 µl, 0.5 mmol) in methanol (20 ml) was stirred 48 hours at room temperature. The solvent was evaporated and the residue was purified by flash chromatography to obtain **6** as colorless solid (Yield: 210 mg, 78%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.48 (s, 9 H), 1.54 (s, 9 H), 3.27 (dd, *J* = 5.5, 2.9 Hz, 2 H), 3.80 (s, 3 H), 5.08 (m, 1 H), 6.64 (d, *J* = 7.5 Hz, 1H), 6.90 (m, 1 H), 7.26 (m, 1 H), 7.39-7.57 (m, 5 H), 7.75 (m, 2 H), 10.4 (s, 1 H), 11.6 (s, 1 H) ppm; HRMS (ESI): *m/z* $[M+Na]^+$ calcd for $C_{28}H_{36}N_4NaO_7$: 563.2476, found: 563.2483.

(3-Cyano)-L-phenylalanine methyl ester hydrochloride (8b). Synthesized according to procedure D. White solid (Yield: 1012 mg, quant). ^{1}H NMR (300 MHz, D_2O): δ = 7.70-7.75 (m, 1 H), 7.66 (s, 1 H), 7.51-7.62 (m, 2 H), 4.41-4.49 (m, 1 H), 3.34-3.43 (m, 1 H), 3.24-3.31 (m, 1 H) ppm; HRMS (ESI) *m/z* $[M + H]^+$ calcd: 205.0971 found: 205.0971.

***N*-Benzoyl-(3-boc-aminomethyl)-L-phenylalanine methyl ester (11b).** Synthesized according to procedure G. White oil (Yield: 362 mg, 90 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.73 (d, J=6.90 Hz, 2 H), 7.40-7.56 (m, 3 H), 7.15-7.26 (m, 2 H), 7.01-7.09 (m, 2 H), 6.56 (d, J=7.49 Hz, 1 H), 5.04-5.14 (m, 1 H), 4.77 (br. s, 2 H), 4.27 (d, J=5.72 Hz, 2 H), 3.78 (s, 3 H), 3.18-3.34 (m, 2 H), 1.46 (s, 9 H) ppm; HRMS (ESI) *m/z* $[M + Na]^+$ calcd: 435.1890, found: 435.1894.

***N*-Benzoyl-(4-nitro)-L-phenylalanine methyl ester (18).** Synthesized according to procedure E. White-yellowish solid (Yield: 650 mg, quant.). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.12-8.21 (m, 2 H), 7.70-7.79

(m, 2 H), 7.42-7.60 (m, 3 H), 7.29-7.37 (m, 2 H), 6.67 (d, J=6.90 Hz, 1 H), 5.14 (dt, J=7.05, 5.65 Hz, 1 H), 3.81 (s, 3 H), 3.27-3.52 (m, 2 H) ppm; HRMS (ESI) *m/z* $[M + H]^+$ calcd: 329.1132, found: 329.1132.

Fmoc-(3-cyano)-L-phenylalanine. To a solution of (3-cyano)-L-phenylalanine in acetonitrile/ water (1:1) was added diisopropylethylamine (370 µl, 2.10 mmol) and Fmoc-OSu (0.32 g, 0.95 mmol). The reaction mixture was stirred for two hours at room temperature and then acidified with 1 N HCl. The aqueous phase was extracted with dichloromethane, dried with magnesium sulfate and the solvent was evaporated under reduced pressure to afford a colorless oil. The oil was crystallized from methylene chloride-hexane to afford the desired product as a white solid. White solid (Yield: 428 mg, quant.). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.79 (d, J=7.34 Hz, 2 H), 7.57 (d, J=7.05 Hz, 3 H), 7.29 - 7.49 (m, 7 H), 5.25 (d, J=7.05 Hz, 1 H), 4.65 - 4.77 (m, 1 H), 4.35 - 4.58 (m, 2 H), 4.21 (t, J=6.46 Hz, 1 H), 3.07 - 3.33 (m, 2 H) ppm; HRMS (ESI) *m/z* $[M + Na]^+$ calcd: 435.1315, found: 435.1318.

Synthesis of ethylacetimidate hydrochloride.[4] A mixture of acetonitrile (5 ml, 95.74 mmol) and ethanol (5 ml, 85.74 mmol) was saturated with hydrogen chloride (g). A precipitate was formed after five minutes. The remaining solvents were evaporated under reduced pressure and the resulting product was crystallized from cold diethyl ether to afford the desired product as a white solid. (Yield: 422 mg, 4 %) ^{1}H NMR (300 MHz, $CDCl_3$): δ = 12.46 (br. s., 1 H), 11.52 (br. s., 1 H), 4.64 (q, J=6.95 Hz, 2 H), 2.48 (s, 3 H), 1.50 (t, J=6.97 Hz, 3 H) ppm.

5. References.

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