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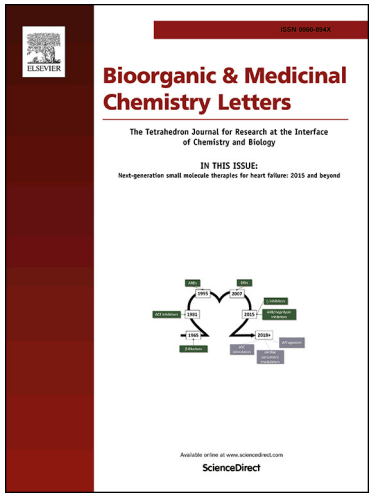

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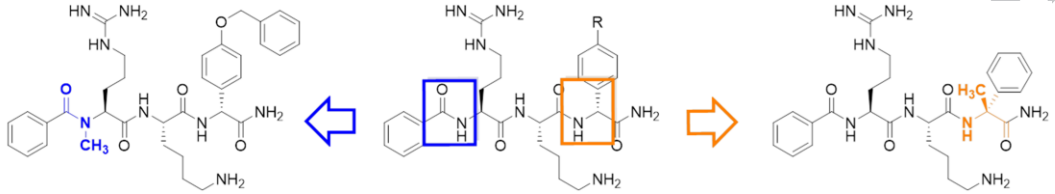

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Backbone modifications in peptidic inhibitors of flaviviral proteases

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ABSTRACT

The NS2B NS3 protease is a promising target for the development of drugs against dengue virus (DENV), West Nile virus (WNV) and related flaviviruses. We report the systematic variation of the peptide backbone of the two lead compounds Bz-Arg-Lys-D-Phg-NH_2 and Bz-Arg-Lys-D-Phg(OBn)-NH_2. While inhibitory activity against WNV protease was generally decreased, the inhibitory potency against DENV protease could be conserved and increased in several peptidomimetics, particularly in those containing a (*N*Me)arginine fragment or an *N*-terminal α-keto amide. Methylation at the α-position of the *C*-terminal phenylglycine led to a 6-fold higher potency against DENV protease. Peptidomimetics with modified backbone showed increased resistance against hydrolytic attack by trypsin and α-chymotrypsin.

Dengue and West Nile fever are viral infections caused by the respective members of the genus *flavivirus*, which are common in tropical and subtropical areas and are transmitted by mosquitos.[1] Most infections with dengue (DENV) or West Nile (WNV) viruses cause mild symptoms and are self-limiting, but severe cases may be fatal. Up to now, only one vaccine against dengue fever (*Dengvaxia*®) is commercially available. Unfortunately, recent data indicate that it might cause unforeseen adverse effects.[2] Although WNV is the most widespread of all mosquito-borne flaviviruses, a vaccine for human use against this pathogen is not available.[3] Therefore, the development of specific antiviral drugs is an important, but as-yet unachieved aim. Drugs with broad-spectral activity would be particularly useful because a reliable and timely diagnosis and differentiation between the various pathogenic flaviviruses is difficult to achieve. The flaviviral proteases show a high degree of similarity and are crucial for the replication of the pathogens, and are therefore potential targets for the development of broad-spectral antiflaviviral drugs. In HIV and HCV treatment, protease inhibitors such as atazanavir or simeprevir are used widely and with considerable success, highlighting the potential of this mode of action.[4]

The flaviviral DENV and WNV proteases are serine proteases with a trypsin-like fold. The gene for the protease is located within the nonstructural (NS) NS3 region, and the enzyme, together with NS2B as a cofactor, cleaves the viral polyprotein in order to enable the formation of offspring virions. Several classes of inhibitors of flaviviral proteases have been described.[1] Our group developed the peptidic inhibitors Bz-Arg-Lys-D-Phg-NH_2 (**MB-230**) and Bz-Arg-Lys-D-Phg(OBn)-NH_2 (**MB-53**), the latter showing *in vitro* inhibition of both DENV and WNV protease in the nanomolar range.[5 6] The substrate-mimetics **MB-230** and **MB-53** (shown in Figure 1) were inspired by the recognition pattern of the viral enzymes, which have a preference for basic sidechains like arginine or lysine.[7 8]

Based on these lead compounds, modifications of the side chains[9] and of the *N*-terminus[10] have already been explored and the scope of modifications tolerated at the *C*-terminus is currently under investigation. In the present communication, we report the

Abbreviations: DENV, dengue virus; HCV, hepatitis C virus; Dphg, diphenylglycine; HIV, human immunodeficiency virus; Narg, *N*-substituted arginine; Nlys, *N*-substituted lysine; Norn, *N*-substituted ornithine; Nphg, *N*-substituted phenylglycine; NS, nonstructural; Phg, phenylglycine; PK, pharmacokinetics; SAR, structure-activity-relationship; SPPS, solid phase peptide synthesis; THR, thrombin; TRY, trypsin; WNV, West Nile virus.

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Table 1. Influence of peptide bond modifications on the inhibitory activity against the DENV and WNV proteases and the off-targets thrombin and trypsin.[a]

Compound number	Bz-Ψ_1Arg-Ψ_2Lys-Ψ_3D-Phg-NH_2			DENV protease		WNV protease		THR	TRY
	Ψ_1	Ψ_2	Ψ_3	%[b]	IC_{50}[c]	%[d]	IC_{50}[e]	%[f]	%[g]
MB-230	amide	amide	amide	99.3[6]	3.8[6]	99.0[6]	3.8[6]	n.i.	12.5
17	amide	amide	**CONMe**	33.0	n.d.	36.7	n.d.	n.i.	n.i.
18	amide	**CONMe**	amide	36.3	n.d.	4.3	n.d.	n.i.	n.i.
19	**CONMe**	amide	amide	93.6	3.6	48.4	n.d.	n.i.	n.i.
20	**CH(CF_3)NH**	amide	amide	(*R*) 43.7 (*S*) 32.1	n.d. n.d.	24.6 6.0	n.d. n.d.	n.i. 5.7	n.i. n.i.
21	**COCONH**	amide	amide	94.0	4.1	43.2	n.d.	n.i.	33.5

[a]All measurements were carried out in triplicate; n.i. = no inhibition (≤ 5%), n.d. = not determined.

[b]Percent inhibition [%] of the DENV NS2B-NS3 protease serotype 2 (enzyme: 100 nM, inhibitor: 50 µM, substrate: 50 µM, K_m = 105 µM).

[c]IC_{50} [µM] values against DENV NS2B-NS3 protease serotype 2 (conditions as above).

[d]Percent inhibition [%] of WNV NS2B-NS3 protease (enzyme: 150 nM, inhibitor: 50 µM, substrate: 50 µM, K_m = 212 µM).

[e]IC_{50} [µM] values against WNV NS2B-NS3 protease (conditions as above).

[f]Percent inhibition [%] of thrombin (enzyme: 10 nM, inhibitor: 25 µM, substrate: 50 µM, K_m = 16 µM).

[g]Percent inhibition [%] of trypsin (enzyme: 1 nM, inhibitor: 50 µM, substrate: 50 µM, K_m = 11 µM).

influence of backbone modifications on the pharmacological properties of this inhibitor class. Aside from increasing the inhibitory activities, an important aim was to improve other parameters such as metabolic stability.

A starting point in the development of peptidomimetics is typically an *N*-methyl scan, where the nitrogen of each peptide bond is methylated. This modification influences the *cis–trans* equilibrium, but more importantly removes the H-bond donor ability.[11] A similar modification is to attach the sidechain to the amine instead of the C_α-position. The resulting peptoids (also called *N*-substituted glycines[12]) are achiral, which would facilitate chemical synthesis, particularly on a larger scale. Methylation at the C_α-position of an amino acid prevents racemization,[13] which is a particular risk for the phenylglycine-containing protease inhibitors that we reported before,[14] and in terms of steric demands it is complementary to *N*-methylated derivatives.[11] The influence of the H-bond acceptor ability of the peptide backbone can be explored by substitution of the carbonyl moiety, e.g. with trifluoroethylamines.[15 16] Incorporation of β^3-amino acids makes the backbone more flexible and potentially enhances the metabolic stability.[17] Electrophilic α-keto amides in the backbone have the potential to form a covalent bond with the nucleophilic oxygen of Ser135 in the catalytic triad of the proteases.[18] The structural modifications of the backbone presented in this work are summarized in Figure **1**. In general, the peptidomimetics were synthesised via SPPS employing Fmoc-protected amino acid derivatives and suitable coupling reagents and conditions to prevent racemization of phenylglycine.[14] Ornithine was used as starting material for the synthesis of arginine derivatives and was transformed to arginine at a later stage via guanylation. The main explorations of the backbone SAR were made with **MB-230** as lead compound because of easier synthetic accessibility. Modifications with a beneficial effect were also applied to **MB-53**.

Fmoc-D-(*N*Me)phenylglycine (**1**) was synthesised by protecting the free *N*-methylated amino acid[19] with FmocOSu. Similar, D/L-(αEt)phenylglycine, β-D-homophenylglycine and diphenylglycine were Fmoc-protected to yield the starting materials **2**, **3,** and **4** for SPPS.

Trifluoroethylamines are accessible via reductive amination (Scheme 1): [20] the condensation reaction of ornithine methyl ester and 2,2,2-trifluoroacetophenone, followed by asymmetric

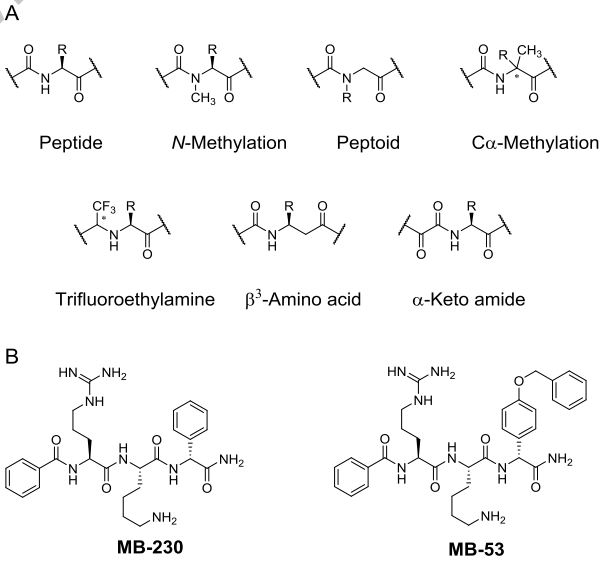


Figure 1. A: Overview of the amide bond surrogates and backbone modifications explored in this work. B: Lead compounds **MB-230** and **MB-53**.[6]

hydrogenation, yielded the diastereomers of **5** with ≥ 90% *de* (determined by ^{19}F-NMR). Cleavage of the Cbz-group yielded the free amines **6** and conversion of the ornithine sidechain furnished the desired arginine building blocks **7**.

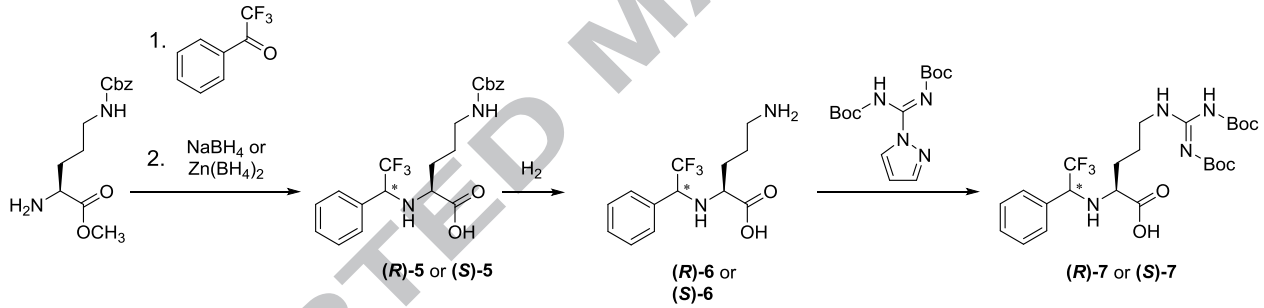
The submonomer method was used to synthesize peptomers during SPPS with 2-bromoglycine as building block.[12] The sidechain is introduced through nucleophilic replacement of the halogen with an appropriate amine to yield the peptoid moiety (Scheme 2). This strategy was only successful for the synthesis of Nlys derivative **8**. Incorporation of the Narg moiety to yield **9** was not possible via this route since bis(*boc*)guanidine propylamine underwent intramolecular cyclization.[21] Instead, the Norn derivative was synthesised during SPPS and its sidechain was

guanylated to yield **9** after capping and cleavage.[22] *N*-Phenylglycine (Nphg) was unreactive under SPPS coupling conditions, so the coupling to lysine and β-homolysine was performed in solution to yield the dipeptides **10** and **11**, which were further modified to building blocks **12** and **13** (Scheme 3).

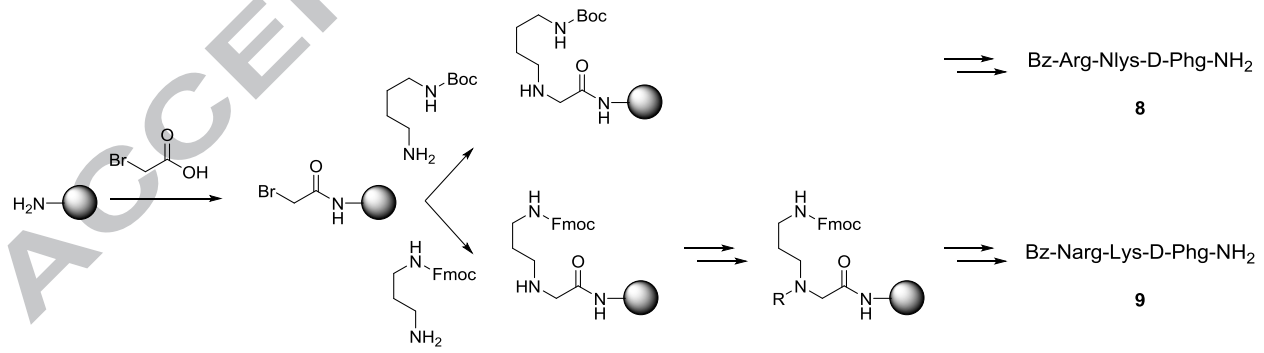
Table 2. Influence of the incorporation of peptoids, β-amino acids and α-methylated amino acids on the inhibitory activity against DENV and WNV proteases and the off-targets thrombin and trypsin.[a] **MB-230**, shown at the top of the table, is the lead compound with a D-configured phenylglycine residue. For reference purposes, the activity of its diastereomer **MB-13** (L-Phg) is also provided.

Compound number	Bz-X-Y-Z-NH_2			DENV protease		WNV protease		THR	TRY
	X	Y	Z	%[b]	IC_{50}[c]	%[d]	IC_{50}[e]	%[f]	%[g]
MB-230	Arg	Lys	D-Phg	99.3[6]	3.8[6]	99.0[6]	3.8[6]	n.i.	12.5
MB-13[9]	Arg	Lys	L-Phg	95.0	3.3	39.3	n.d.	n.i.	n.i.
9	**Narg**	Lys	D-Phg	32.7	n.d.	12.4	n.d.	n.i.	n.i.
8	Arg	**Nlys**	D-Phg	4.4	n.d.	18.4	n.d.	n.i.	10.6
22	Arg	Lys	**Nphg**	28.9	n.d.	19.8	n.d.	n.i.	n.i.
23	Arg	**β-Lys**	**Nphg**	22.7	n.d.	17.7	n.d.	n.i.	n.i.
24	Arg	**β-Lys**	D-Phg	31.8	n.d.	14.6	n.d.	n.i.	n.i.
25	Arg	Lys	**β-L-Phg**	17.6	n.d.	12.6	n.d.	n.i.	7.5
16	Arg	Lys	**D/L-(αMe)Phg**	97.8	1.3	59.8	24.3	9.1	n.i.
(*R*)-16	Arg	Lys	**D-(αMe)Phg**	99.1	0.6	82.5	14.3	6.5	11.8
(*S*)-16	Arg	Lys	**L-(αMe)Phg**	81.7	9.5	47.4	n.d.	n.i.	20.7
26	Arg	Lys	**Dphg**	20.6	n.d.	33.3	n.d.	n.i.	45.5
27	Arg	Lys	**D/L-(αEt)Phg**	35.2	n.d.	12.1	n.d.	n.i.	8.7

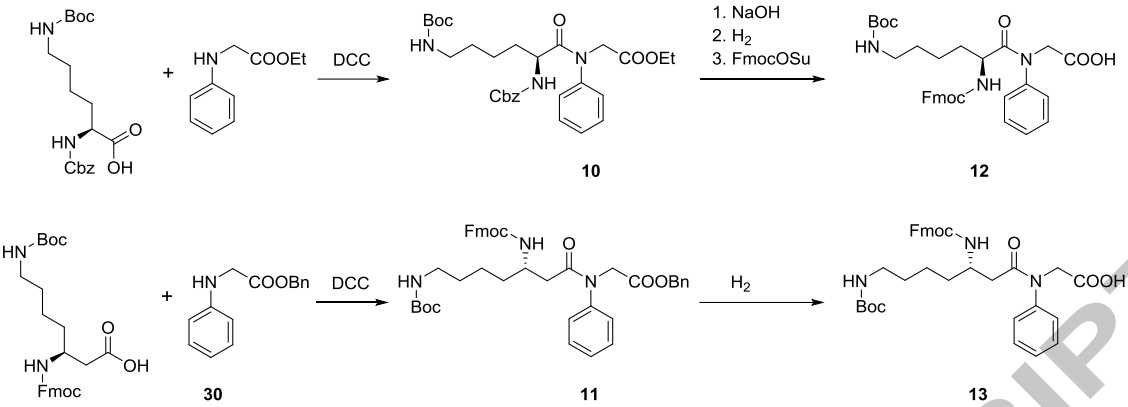
[a—g]see Table 1.



Scheme 1. Synthesis of trifluoromethyl-substituted building blocks **(*R*)-7** and **(*S*)-7**, respectively.



Scheme 2. Synthesis of peptomers **8** and **9** using the submonomer-method during solid phase peptide synthesis.



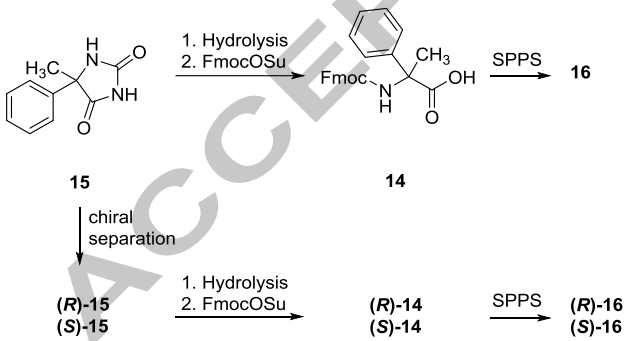
Scheme 3. Synthesis of the Nphg-building blocks **12** and **13**.

Table 3. Inhibitory activity of fragment merged derivatives against the DENV and WNV proteases and the off-targets thrombin and trypsin.[a]

Compound number	Bz-X-Lys-Z-NH_2		DENV protease		WNV protease		THR	TRY
	X	Z	%[b]	IC_{50}[c]	%[d]	IC_{50}[e]	%[f]	%[g]
(*R*)-16	Arg	**D-(αMe)Phg**	99.1	0.6	82.5	14.3	6.5	11.8
19	**(*N*Me)Arg**	D-Phg	93.6	3.6	48.4	n.d.	n.i.	n.i.
(*R*)-28	**(*N*Me)Arg**	**D-(αMe)Phg**	93.9	3.0	50.8	n.d.	n.i.	10.9
MB-53	Arg	D-Phg(OBn)	100.1[6]	0.4[6]	101.2[6]	0.8[6]	7.8	18.4
29	**(*N*Me)Arg**	D-Phg(OBn)	97.8	1.6	92.0	7.1	5.5	n.i.

[a—g]see Table 1.

The synthesis of racemic Fmoc-(αMe)phenylglycine (**14**) started from 5-methyl-5-phenylhydantoin (**15**), which was hydrolyzed[23] and Fmoc-protected. The hydantoin enantiomers (***R*)-15** and (***S*)-15** could be separated prior to hydrolysis by crystallization with a chiral reagent as described previously[24] and protected with FmocOSu to obtain Fmoc-D-(αMe)phenylglycine (***R*)-14** and its L-isomer (***S*)-14** (Scheme 4). The high purity of the resulting diastereomeric peptides was confirmed by ^{1}H-NMR: the shift in the C_α-proton signals allows differentiation of the two epimers (see Supporting Information).



Scheme 4. Synthesis of racemic Fmoc-(αMe)phenylglycine **14** and of the pure isomers **(*R*)-14** and **(*S*)-14**.

Peptidomimetics of **MB-230** with amide bond surrogates were synthesized and tested (Table 1). For DENV protease, incorporation of an *N*-methylated amide moiety in position 3 (**17**) or 2 (**18**) led to a dramatic loss of activity, but the same modification was well tolerated for the arginine in position 1 (**19**). Substitution of the amide with trifluoroethylamine led to inactive compounds (***R*)-20** and (***S*)-20**, which may be caused by steric hindrance or by the loss of the H-bond acceptor ability of the carbonyl moiety. The α-keto amide **21** was equipotent to the lead compound. WNV protease proved to be much more sensitive to changes in the backbone of the inhibitor. Every modification led to an inactive compound, including the *N*-methylated arginine derivative **19**, which was relatively potent at DENV protease. The α-keto amide **21** showed some inhibitory activity against trypsin at a concentration of 50 µM. The other compounds inhibited neither thrombin nor trypsin, indicating selectivity for flaviviral proteases.

Peptoids, β-amino acids and α-methylated amino acids were also incorporated into the lead compound **MB-230** (Table 2). Although the *N*-methylation of arginine was well tolerated, the corresponding Narg peptomer **9** showed no significant inhibition of DENV protease. For the Nlys analogue **8**, as well as the Nphg-derivative **22** the inhibitory activity was also lost, which was not surprising in consideration of the negative outcomes of the *N*-methyl scan. β-Lysine and Nphg were combined in **23** to restore the distance between the sidechains as well as the orientation of the aromatic ring, but without success, probably because of the absent H-bond donor moiety. The incorporation of β-lysine alone in **24** was also found to have a detrimental effect. Surprisingly, even the β-D-phenylglycine derivative **25** was inactive, indicating that the *C*-terminus is very sensitive to subtle changes. However, an epimeric mixture of D/L-(αMe)phenylglycine derivative **16** was slightly more active than the lead compound **MB-230**. As for **MB-230** and its L-isomer **MB-13**, **(*S*)-16** was less active than **(*R*)-16**, which showed inhibitory activity in the submicromolar range (IC_{50} = 0.6 µM) against DENV. The scope of possible substitution patterns at the α-position of the phenylglycine was then explored. The nonchiral diphenylglycine derivative **26** was inactive, and extension of the methyl to an ethyl substituent in racemic **27** led to complete loss of activity. Against WNV protease, only the D-(αMe)phenylglycine derivative **(*R*)-16** showed moderate inhibitory activity with an IC_{50} of 11.5 µM. **(*S*)-16** showed no inhibition, similar to the L-Phg epimer **MB-13** of the lead compound. None of the compounds, except **26**, showed noteworthy off-target inhibition.

Fragment merging of the most potent modifications, namely (*N*Me)arginine and D-(αMe)phenylglycine, as well as

incorporation of (*N*Me)arginine in the 4-benzyloxy-phenylglycine analogue **MB-53**, was performed (Table 3). Surprisingly, for (*N*Me)arginine derivatives the α-methylation of phenylglycine had no effect, resulting in identical activities of **19** and **(*R*)-28** against both DENV and WNV protease. Introduction of an (*N*Me)arginine residue had no influence on the negligible off-target inhibition of the (αMe)phenylglycine derivatives. In contrast, the *N*-methylated analogue **29** had significantly lower activity than the lead compound **MB-53**: for DENV protease, the activity was four times lower, and against WNV protease it was one order of magnitude less active. The 4-benzyloxy-phenylglycine lead compound **MB-53** showed some inhibitory activity against trypsin at a concentration of 50 µM, which could be suppressed through the *N*-methylation in **29**.

Metabolic stability is an important factor in the development of new drugs. We therefore studied the *in vitro* metabolic stability in the presence of rat liver microsomes and in the presence of pancreatic enzymes. We included H-Tyr-D-Arg-Phe-Phe-NH_2 (**32**) as a peptidic reference.

Enzymatic degradation has been observed for other derivatives of the lead compounds in the presence of hepatic phase I metabolic enzymes. [6 9] **MB-230**, **MB-53**, **(*R*)-16, 19, 29** and **32** together with the control substance testosterone were incubated for 120 min at 37°C with rat liver microsomes. Samples were taken at several time points and analyzed using HPLC with UV detection. The lead compounds and the peptidomimetics were stable under these conditions, while testosterone had a half-life time of 37 min and **32** was slowly digested.

Next, metabolic stability in the presence of pancreatic enzymes was determined in analogy to literature conditions.[25] **MB-230**, **(*R*)-16, 19** and **32** were incubated for 60 min at 37°C with trypsin or α-chymotrypsin and aliquots were taken. Rapid degradation of the reference **32** by α-chymotrypsin (10 µM) was observed as reported in the literature.[25] Lead compound **MB-230** was stable for several minutes ($t_{1/2}$ = 11 min), while the backbone-modified peptidomimetics (in particular the α-Me-Phg analog **(*R*)-16**) were significantly more resistant against enzymatic hydrolysis (cf. the SI for details). A reduction of the enzyme concentration and temperature was required to obtain quantifiable results for the hydrolytic stability against trypsin. Notably, the reference **32** was stable against trypsin hydrolysis under all tested conditions, which is in contrast to the literature. The data indicate a clear increase of resistance of the backbone-modified peptidomimetics **(*R*)-16** and **19** against hydrolytic attack by trypsin in comparison to the lead compounds **MB-53** and **MB-230** (cf. SI for details).

In conclusion, we demonstrate that the activity of peptidic flaviviral protease inhibitors is quite susceptible even to minor changes in the backbone, especially for WNV protease, which was less tolerant to changes than DENV protease. Although most of the modifications were detrimental for the activity against both enzymes, the amide bond between the *N*-terminal benzoyl cap and arginine tolerated some changes, e.g. incorporation of (*N*Me)arginine. Methylation at the C_α-position of D-phenylglycine increased the inhibitory activity (IC_{50} = 0.6 µM against DENV), but other α-substituted phenylglycine derivatives were not active. The α-methylation of D-phenylglycine has clear potential to be further explored towards analogues of the previously reported, highly potent 4-benzyloxy-phenylglycine-containing peptidomimetics. α-Methylation also brings the additional value of increased stereochemical stability during synthesis and formulation. Thus far, fragment merging of (*N*Me)arginine with 4-benzyloxy-phenylglycine and (αMe)phenylglycine building blocks yielded relatively potent compounds, but the inhibitory activity was not cumulative. In comparison to the lead compounds, the degradation of the peptidomimetics by pancreatic enzymes was significantly reduced.

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Supplementary Material

Supplementary data (Synthesis and analysis of amino acid derivatives and peptidomimetics including HRMS, NMR and HPLC data, as well as IC_{50} spectra and metabolism data) associated with this article can be found in the online version.