Accepted Manuscript

Synthesis and structure-activity relationships of small-molecular di-basic esters, amides and carbamates as flaviviral protease inhibitors

Tom R. Sundermann, Clarissa V. Benzin, Tonko Dražić, Christian D. Klein

PII: S0223-5234(19)30434-9

DOI: https://doi.org/10.1016/j.ejmech.2019.05.025

Reference: EJMECH 11335

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 27 March 2019

Revised Date: 3 May 2019

Accepted Date: 7 May 2019

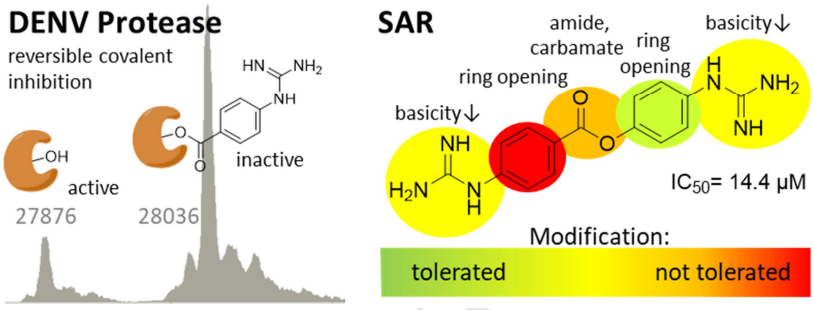
Please cite this article as: T.R. Sundermann, C.V. Benzin, T. Dražić, C.D. Klein, Synthesis and structure-activity relationships of small-molecular di-basic esters, amides and carbamates as flaviviral protease inhibitors, *European Journal of Medicinal Chemistry* (2019), doi: https://doi.org/10.1016/j.ejmech.2019.05.025.

Graphical Abstract

Synthesis and structure-activity relationships of small-molecular di-basic esters, amides and carbamates as flaviviral protease inhibitors

Tom R. Sundermann,
Clarissa V. Benzin,
Tonko Dražić,
and Christian D. Klein*

Leave this area blank for abstract info.



Highlights

- Synthesis of small dibasic molecules as dengue protease inhibitors
- SAR study of non-peptic inhibitors on dengue and West-Nile virus protease, thrombin and trypsin
- Mechanism-of-action analysis of the synthesized compounds
- Covalent-reversible inhibition mode pinpointed by mass spectrometry

ELSEVIER

European Journal of Medicinal Chemistry
journal homepage: www.elsevier.com

Synthesis and structure-activity relationships of small-molecular di-basic esters, amides and carbamates as flaviviral protease inhibitors

Tom R. Sundermann[1,2], Clarissa V. Benzin[1], Tonko Dražić[1] and Christian D. Klein[1]

[1]*Medicinal Chemistry, Institute of Pharmacy and Molecular Biotechnology IPMB, Heidelberg University, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany*
[2]*Institute of Forensic and Traffic Medicine, University Hospital Heidelberg, Voßstr. 2, 69115 Heidelberg, Germany*

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Flavivirus
Dengue
Protease
Covalent-reversible inhibition
Carbamate
Ester

ABSTRACT

Inhibitors of the flaviviral proteases, crucial serine proteases in the replication dengue and West-Nile virus, have attracted much attention over the last years. A dibasic 4-guanidinobenzoate was previously reported as inhibitor of the dengue protease with potency in the low-micromolar range. In the present study, this lead structure was modified with the intent to explore structure-activity relationships and obtain compounds with increased drug-likeness. Substitutions of the guanidine moieties, the aromatic rings, and the ester with other functionalities were evaluated. All changes were accompanied by a loss of inhibition, indicating that the 4-guanidinobenzoate scaffold is an essential element of this compound class. Further experiments indicate that the target recognition of the compounds involves the reversible formation of a covalent adduct.

1. Introduction

Flaviviruses like the dengue virus (DENV) or West-Nile virus (WNV) are an increasing global health threat [1]. On the basis of million infections per year and the absence of specific antiviral drugs or efficient vaccines, the global disease burden caused by flaviviral infections is considerable [1, 2]. In particular, the development of a treatment or prophylactic agent against DENV covering all four serotypes proves to be difficult. The viral NS2B-NS3 proteases, which are essential for the viral replication and conserved in all flaviviruses [3], represent attractive targets for the development of therapeutics against these arboviral pathogens [4]. The flaviviral serine proteases are responsible for the cleavage of the viral polyprotein and have a solvent-exposed, topologically shallow active site [4]. They possess a trypsin-like fold and are selective for substrates containing basic amino acids (arginine and lysine) at P1 and P2 [5, 6].

Substrate-mimicking compounds proved to be potent inhibitors of the viral protease but their peptidic nature poses a challenge for pharmacokinetic [7]. Over the last decade, several groups of low-molecular, non-peptidic inhibitors were identified by experimental and virtual high throughput screenings for the DENV protease [4, 8, 9]. In the research group of the authors, the inhibitory activity against the viral protease was evaluated for a set of α-ketoamides [10], arylcyanoacrylamides [11], and hydantoins [12] but none of these compound classes reached the potency of peptidic compounds.

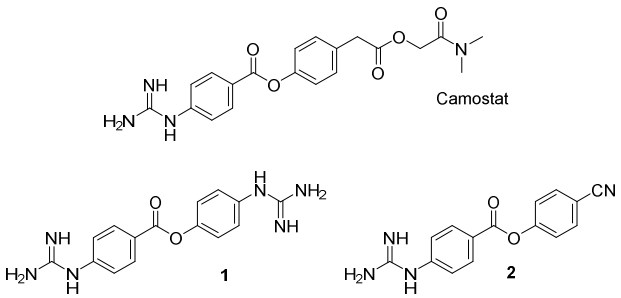


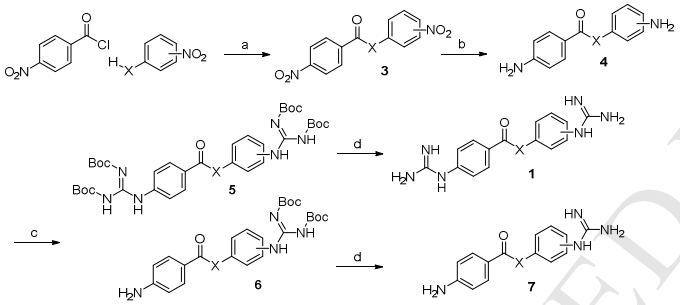
Figure 1. Camostat, a broad spectrum serine protease inhibitor, and the 4-guanidinobenzoates **1** and **2** as micromolar dengue protease inhibitors [13, 14].

Camostat, an aryl 4-guanidinobenzoate used in the treatment of pancreatitis, is a synthetic, broad-spectrum serine protease inhibitor which inhibits enzymatic activities including trypsin, thrombin, kallikrein, and plasmin [13, 15]. Structurally similar 4-guanidinobenzoates like **1** or **2** (Figure 1) were predicted by virtual screening as potential DENV protease inhibitors, and their activity in the low micromolar range could be confirmed experimentally [14]. Evaluation of their inhibitory activity in a biochemical assay revealed a low micromolar inhibition of **1** against the DENV protease [14]. The low molecular size and the proposed formation of a covalent bond between such esters and the nucleophilic serine of the protease represent a valuable starting point for further investigations. In this study, the covalent binding mode, which was shown for nafamostat with trypsin [16] and assumed for **1** with the dengue protease [14], were further

investigated and proven. In addition, a set of derivatives of **1** was synthesized and evaluated in order to illuminate structure-activity relationships (SARs) and prove the importance of covalent binding. Aryl 4-guanidinobenzoates are known for the formation of a reversible or pseudo-irreversible covalent bond [17]. While the replacement of the ester with an amide should prevent covalent interactions, the introduction of a carbamate is expected to result in a practically irreversible, or very slowly reversible covalent inhibition of the serine proteases [18]. Therefore, the introduction of an amide and carbamate group between the aromatic rings and as a substituent was performed and evaluated.

2. Chemistry

In order to validate the inhibitory activity against DENV protease, which was previously reported [14], the lead structure **1** was synthesized as a reference. The general methodology shown in Scheme 1 was used to synthesize **1** and its derivatives **1b-d**. Herein, 4-nitrobenzoyl chloride was reacted with the phenol or aniline derivatives to form esters or amides **3**. After reduction of the nitro groups, the resulting anilines **4** were guanidinylated with *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine. While the amine group of the former phenol or aniline part reacted entirely, the other amine group was nearly unreactive. Therefore, the main products of this reaction were anilines **6**, whereas the desired products **5** were formed to a lesser extent. Deprotection under acidic conditions resulted in the final dibasic products **1** and **7**.

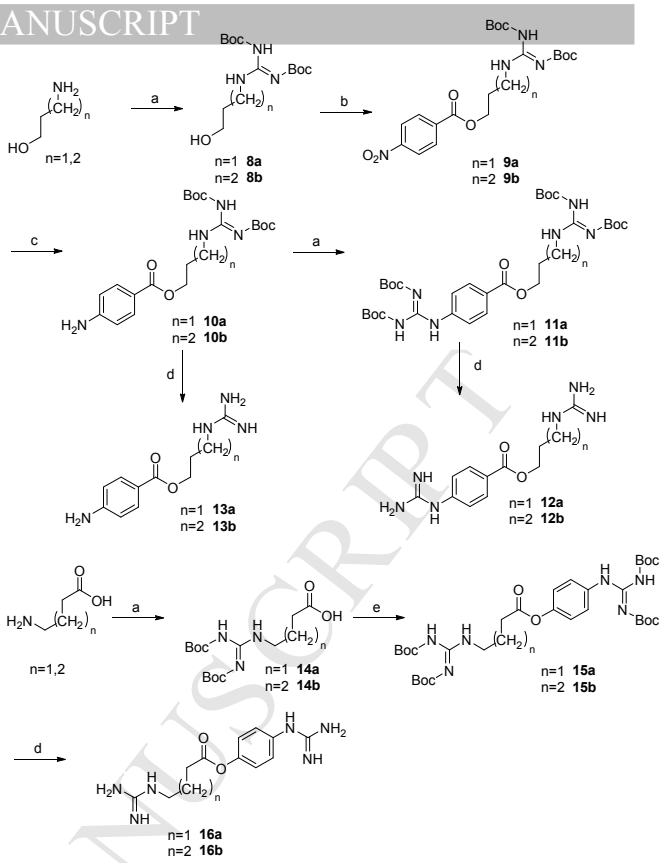


X	substitution	3	4	5	1	6	7
O	para	**3a**	**4a**	**5a**	**1**	**6a**	**7a**
O	meta	**3b**	**4b**	**5b**	**1b**	**6b**	**7b**
NH	para	**3c**	**4c**	**5c**	**1c**		
NH	meta	**3d**	**4d**	**5d**	**1d**		

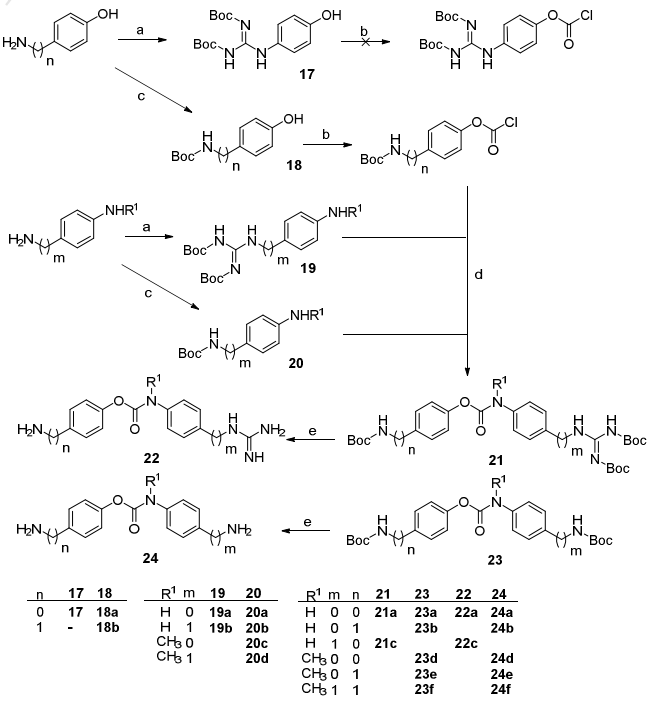
Scheme 1. Synthesis of esters **1**, **1b**, **7a** and **7b** and amides **1c-d**: a) TEA, DCM, rt, 12 h; b) H_2, Pd/C, EtOAc, rt, 16 h; c) *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine, TEA, THF; d) 50% TFA in DCM.

The syntheses of the dibasic compounds with one aromatic ring are displayed in Scheme 2. In order to replace the phenolic ring system of **1**, 3-aminopropanol and 4-aminobutanol were used instead of phenols as educts. At first, the aminoalcohols were guanidinylated to afford alcohols **8**, which were reacted with 4-nitrobenzoyl chloride to create esters **9**. After reduction of the nitro group, a part of the anilines **10** were deprotected under acidic conditions to form the dibasic compounds **13**. Guanidinylation of the anilines **10** afforded **11** and was followed by deprotection under acidic conditions to obtain the desired compound group **12**.

For the replacement of the benzoate part of **1**, 4-aminobutanoic acid and 5-aminopentanoic acid were employed as starting materials. These amines were guanidinylated to yield carboxylic acids **14**. After coupling with 4-(*N*′,*N*″-di-Boc)guanidinophenol, the esters **15** were deprotected under acidic conditions to obtain **16**.



Scheme 2. Synthesis of aliphatic derivatives: a) *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine, TEA, THF; b) 4-nitrobenzoyl chloride, TEA, DCM, rt, 12 h; c) H_2, Pd/C, EtOAc, rt, 16 h; d) 50% TFA in DCM; e) 4-(*N*′,*N*″-di-Boc)guanidinophenol, EDC HCl, DMAP, THF, rt, 16 h.



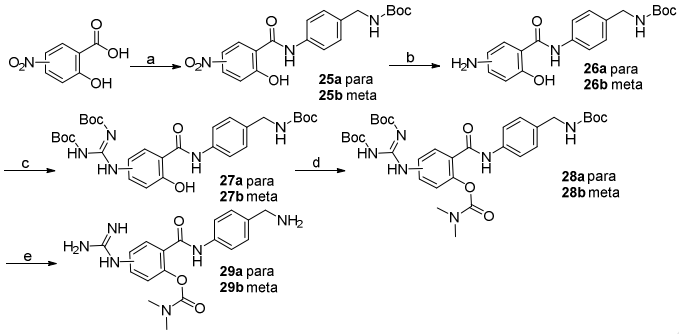
n	17	18
0	**17**	**18a**
1	-	**18b**

R^1	m	19	20
H	0	**19a**	**20a**
H	1	**19b**	**20b**
CH_3	0		**20c**
CH_3	1		**20d**

R^1	m	n	21	23	22	24
H	0	0	**21a**	**23a**	**22a**	**24a**
H	0	1		**23b**		**24b**
H	1	0	**21c**		**22c**	
CH_3	0	0		**23d**		**24d**
CH_3	0	1		**23e**		**24e**
CH_3	1	1		**23f**		**24f**

Scheme 3. Synthesis of carbamates **22a**, **22c** and **24a,b,d-f**: a) *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine, TEA, THF; b) diphosgene, DIPEA, DCM, 30 min at 0°C, then 30 min at rt; c) di-*tert*-butyl dicarbonate, TEA, DCM or THF, rt, 3 h; d) DIPEA, DCM, rt, 1-8 d; e) 50% TFA in DCM.

While the replacement of the ester with an amide was straightforward, the introduction of a carbamate proved to be challenging. The electrophilicity of the carbamate and the nucleophilicity of the basic residues can result in intra- or intermolecular reactivity. This instability limits the amount of synthetically stable compounds, and only a few of the desired compounds could be isolated. Particularly the guanidino group tended to destabilize the compounds. Therefore, we started the synthesis with aromatic amines and methylamines and proceeded later with the guanidines. The employed synthetic methodology is shown in Scheme 3. In general, the phenol building blocks **17**, **18a** and **18b** and the aniline building blocks **19a-b** and **20a-d** were synthesized separately. The terminal amino group of the educts was Boc-protected (**18a-b** and **20a-d**) or converted into a di-Boc-protected guanidino group (**17** and **19a-b**). The synthesis of the chloroformate of **17** with diphosgene and subsequent carbamate formation was not successful. After in-situ generation with diphosgene, the chloroformates **18a** and **18b** were reacted with the anilines **19a**, **19b** and **20a-d**. The obtained carbamates **21a**, **21c** and **23a,b,d-f** were deprotected under acidic conditions to afford the desired carbamates **22a**, **22c** and **24a,b,d-f**.



Scheme 4. Synthesis of carbamates **28**: a) *tert*-butyl (4-amino-benzyl)carbamate, triphenyl phosphite, toluene, reflux, 8 h; b) H_2, Pd/C, MeOH, rt, 16 h; c) *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine, TEA, THF; d) dimethylcarbamoyl chloride, TEA, DCM, rt, 24 h; e) 50% TFA in DCM.

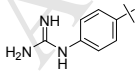
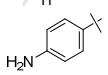
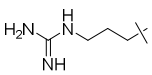
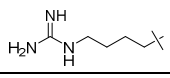
Molecular modelling indicated that the carbamate as a substituent of the aromatic ring may reach the catalytically active serine. Therefore, the introduction of an *N*,*N*-dimethyl-carbamate as a ring substituent was performed as shown in Scheme 4. At first, 3- and 4-nitrosalicylic acid were coupled with *tert*-butyl (4-aminobenzyl)carbamate to obtain amides **25a** and **25b**. After reduction of the nitro group, amines **26** were guanidinylated to form **27**. The phenol moieties were converted to the *N*,*N*-dimethylcarbamates **28**, followed by deprotection under acidic conditions to afford the desired compounds **29a** and **29b**. The synthesis of **30**, **31** and **40** is described in the supporting informations.

3. Structure-activity relationships

Camostat, **1** and the synthesized series of 19 dibasic compounds were evaluated against DENV (serotype 2) and WNV protease. The SAR explorations were focused on the necessity of the aromatic rings, of the guanidine groups and the electrophilic ester. In addition, the off-target inhibitory activity against thrombin and trypsin were evaluated for all compounds (Table 1 and 2). While Camostat showed a considerable inhibition of trypsin (IC_{50} = 3.5 nM), an inhibition of DENV or WNV protease was not detected. As expected, reference compound **1** is an excellent inhibitor of trypsin (IC_{50} = 1.35 nM) and with an IC_{50} of 14.4 µM, the described inhibition of the DENV protease in the literature [14] could be reproduced.

First, modifications of the 4-guanidinobenzoate part of **1** were evaluated (Table 1). The replacement of the aromatic ring with an aliphatic chain in compounds **16a** and **16b** significantly decreased the inhibitory activity against all proteases. While aniline **7a** was only twofold less active against trypsin, the loss of the guanidine group was less tolerated by the flaviviral proteases. Next, modifications of the 4-guanidinophenol part of **1** were explored (Table 2). While the modifications of the 4-guanidinobenzoate part (Table 1) were disadvantageous for the inhibitory activity, modifications at the phenolic part were not favored but still tolerated. The replacement of the highly basic guanidino group with other less basic residues like amino or methylamino groups or the change from para to meta position was accompanied by a loss of inhibitory activity. While compounds **12a** and **12b**, where the aromatic ring was replaced with an aliphatic chain, were more than 4000-fold less active against trypsin than **1**, they showed only a 3-fold loss of activity against DENV protease. As expected, the replacement of the ester with the – supposedly non-reactive – amide function results in a major loss of inhibitory activity.

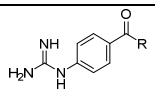
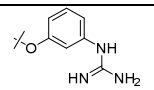
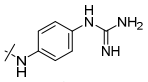
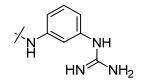
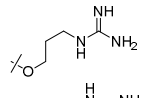
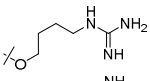
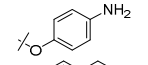
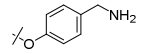
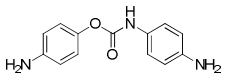
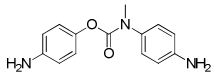
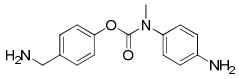
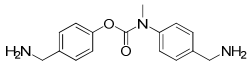
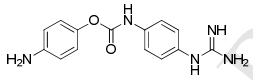
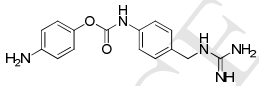
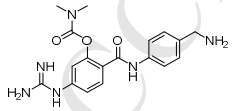
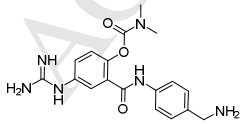
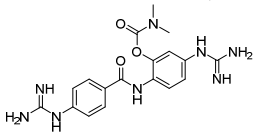
Table 1. Inhibitory activity against DENV, WNV protease, thrombin, and trypsin.

Compounds	R	Biological Data			
		DENV protease[a]	WNV protease[a]	Thrombin[b]	Trypsin[a]
		Inhibition [%] (IC_{50})			
Camostat		19.6	12.6	94.0 (0.78 µM)	100.0 (3.5 nM)
1		98.1 (14.4 µM)	29.8	99.0 (4.08 µM)	100.3 (1.35 nM)
7a		45.4	n.i.	76.7 (17.6 µM)	100.6 (2.78 nM)
16a		4.2	n.i.	13.2	18.3
16b		4.6	n.i.	72.5	65.9

[a]50 µM substrate concentration and 50 µM compound concentration.

[b]50 µM substrate concentration and 25 µM compound concentration; n.i. no inhibition.

Table 2. Inhibitory activity against DENV, WNV protease, thrombin, and trypsin.

		Biological Data			
Compounds	R	DENV protease[a]	WNV protease[a]	Thrombin[b]	Trypsin[a]
		Inhibition [%] (IC_{50})			
1b		51.3	17.5	100.0	100.1
1c		23.2	17.9	23.9	87.2
1d		36.8	n.i.	16.4	94.6 (23.9 µM)
12a		56.8	n.i.	22.8	78.9 (9.84 µM)
12b		49.0	17.1	27.3	84.0 (8.27 µM)
30		8.8	n.i.	97.1 (0.80 µM)	102.5 (28 nM)
40		40.1	36.5	92.5 (0.53 µM)	100.7 (1.56 nM)
1.	2.	3.	4.	5.	6.
Compounds	Chemical structure	DENV protease[c]	WNV protease[c]	Thrombin[d]	Trypsin[c]
		Inhibition [%] (IC_{50})			
24a		33.7	72.8	83.3	99.2
24d		29.8	68.0	72.7	95.9
24e		22.9	59.3	46.9	95.6
24f		31.3	62.9	12.2	43.9
22a		52.0	61.9	12.1	100.6 (46.3 µM)
22c		60.8	68.8	44.4	98.4
29a		74.4	73.3	12.4	43.7
29b		56.0	76.1	24.3	19.3
31		20.2	23.9	67.8	99.2

[a]50 µM substrate concentration and 50 µM compound concentration.

[b]50 µM substrate concentration and 25 µM compound concentration.

[c]50 µM substrate concentration and 1 mM compound concentration.

[d]50 µM substrate concentration and 500 µM compound concentration; n.i. no inhibition (<5%).

In contrast to the esters **1** and **1b**, amide **1d** with a meta guanidine group was superior in its inhibitory activity in comparison to the para-substituted **1c**. Under screening conditions (50 µM compound concentration), nearly all synthesized carbamates showed no or only negligible inhibition. In order to explore structure-activity relationships of the carbamates, the final compound concentration in the enzyme assay was increased 20-fold to 1 mM. Under these conditions, carbamates with less basic groups like **24a**, **24d**, **24e**, and **24f** show comparable inhibition of the tested proteases. By introducing a guanidine group as in **22a** and **22c**, the inhibitory activity against some of the tested proteases increases significantly. However, in comparison to **7a** and **30**, which inhibit trypsin in the low nanomolar range, **22a** is a relatively weak inhibitor of this protease. The introduction of a peripheral carbamate function, as in compounds **29a**, **29b** and **31**, was also detrimental for the inhibition of the flaviviral proteases.

4. Binding mode analysis

To obtain information about the binding mode of the synthesized compounds with the DENV protease, three types of experiments were carried out. At first, the time-dependent inhibition (TDI) of compounds **1**, **1c**, **7a**, **12a**, **22a** and **40** was investigated (Figure 2a). TDI was examined by varying pre-incubation times in an otherwise routine DENV protease in-vitro assay. Inhibitors that slowly form a covalent bond and inactivate their designated target should show increased potency after extended incubation times. Therefore, any time-dependent loss of initial substrate cleavage rate indicates a mechanism-based inhibition [19]. MB-53 (Bz-Arg-Lys-(O-benzyloxy)-D-Phg-NH_2), a well characterized peptidic competitive inhibitor of DENV (IC_{50} = 0.37 µM), was used as a reference [7]. In the presence of DMSO, MB-53, **1c** and **22a**, no effect is observed when changing preincubation times. For **1**, **7a** and **40** a time-dependent increase of the inhibitory activity is observed.

To further characterize the binding mode, the release of inhibitors from the enzyme-inhibitor complex was studied (Figure 2b). For this purpose, the enzyme was pre-incubated with inhibitor **1** at 100 µM to achieve 100% inhibition and then the solution was diluted with buffer. After different incubation times the substrate was added (final inhibitor concentration: 3.33 µM) and its cleavage was measured continuously. Three controls are included: 1. No inhibitor throughout to reflect full enzyme activity, 2. 100 µM inhibitor concentration throughout to achieve 100% inhibition, and 3. 3.33 µM inhibitor concentration throughout to reflect the expected amount of inhibition remaining after substrate dilution. With reversible inhibitors, the enzyme will regain its activity while irreversibly inhibited enzymes will remain inactive. As shown in Figure 3, the DENV protease which was pre-incubated with **1** remains inactive directly after dilution and then regains its activity slowly over time. The decrease of inhibition after 120 min for the 100µM graph originates from the low stability of **1** in the assay buffer but also reflects that in absence of **1** the protease recovers its activity. Therefore, the formation of a reversible covalent bond through **1** with the DENV protease is the most plausible assumption.

Another method to study the reversible-covalent or irreversible-covalent bond formation of ligands with their targets is the detection of adducts by mass spectrometry [20, 21]. In order to confirm a covalent modification of the DENV protease, an MS-based approach was elaborated (Figure 3). DENV protease was incubated with compounds **1**, **1c**, **7a** and **22a** over different time periods (5 min, 30 min, 2 h, 20 h, and 24 h) at elevated concentrations (3 µM enzyme, 100 µM inhibitor) and analyzed by ESI-MS simultaneously. While no adducts were observed for compounds **1c** and **22a** showed, an adduct formation over time was demonstrated for compounds **1** and **7a**. In comparison to an unmodified sample (m/z 27874), a mass difference of m/z 162 was observed for **1** (Figure 3a). Therefore, the 4-guanidinobenzoic acid moiety (M_r 162 g/mol) from **1** was transferred from the inhibitor to the enzyme to form the acyl-enzyme complex. With a mass difference of m/z 120, the same can be observed for the 4-aminobenzoic acid moiety (M_r 120 g/mol) from **7a** (Figure 3b). Adduct formation of compound **1** could be observed after 5 min incubation time. After 30 min, the acyl-enzyme complex represented the most abundant species. The reversibility of the covalent inhibition of compound **1** could be demonstrated by longer incubation times. After 20 h incubation, only a small amount of the acyl-enzyme complex was observed in the incubation solution. In comparison to compound **1**, the adduct formation of compound **7a** proceeded slowly. After 2 h incubation, a small amount of the acyl-enzyme complex appeared, which became more prominent after incubation overnight.

For the exclusion of unspecific binding of compounds **1** and **7a** with the protease, a mutant of the DENV protease (DENV protease S135A) with a non-functional active side (catalytic serine135 was replaced with alanine) was also tested under the same conditions (Figure 3c). Both compounds showed no adduct formation within an incubation period of 24 h.

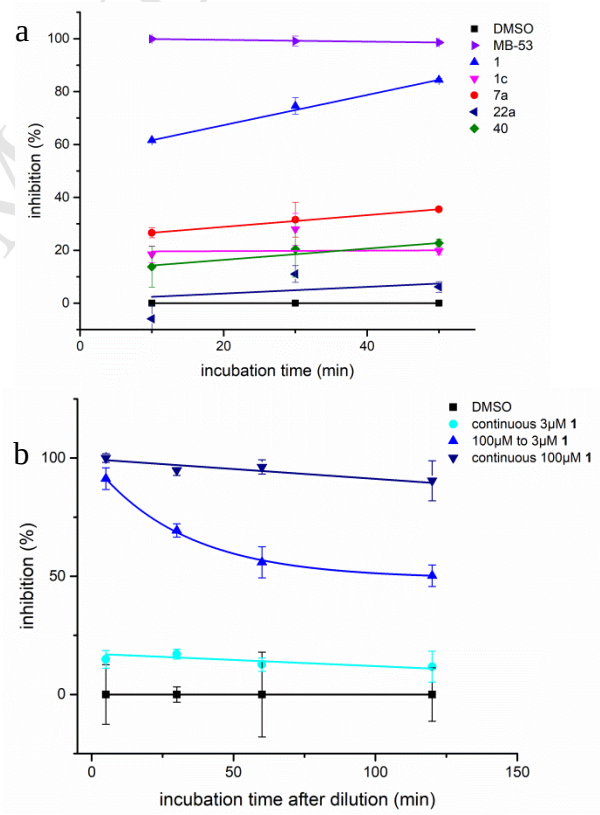


Figure 2. (a) Time dependent inhibition of the DENV protease for compounds **1**, **1c**, **7a**, and **40** at 50µM and compound **22a** at 200µM with different incubation times; (b) Dilution experiment for compound **1**.

5. Stability study

Stability and shelf-life are an important factor in drug development. Because of the combination of electrophilic and nucleophilic moieties in a single molecule, all synthesized compounds can be affected by major instabilities. In addition, electrophiles are prone to hydrolysis under basic, aqueous

conditions. Therefore, the stability of selected compounds was evaluated in different buffer systems (assay buffer at pH 7.5 and pH 9; phosphate buffer at pH 7.5, pH 9 and pH 11). **1c**, **12a**, and **30** proved to be stable under all tested conditions. Lead structure **1** proved to be unstable in the routine 50 mM TRIS assay buffer for flaviviral proteases at pH 9 ($t_{1/2}$= 16.78 min) but not in phosphate buffer of the same pH or a Tris-containing buffer of pH 7.5. At pH 11, the lead structure was also instable in phosphate buffer ($t_{1/2}$= 17.83 min). This might explain the previously observed fluctuation of the measured results (IC_{50}) of **1**. The biochemical assay of the dengue protease has an incubation time of 15 min and the compounds are prediluted in the utilized buffer.

6. Conclusion

In this study, 19 dibasic compounds inspired by **1** were synthesized and their properties and inhibitory activities against different serine proteases were evaluated. The compiled SAR study indicates that the electrophilic ester functional group, as well as the 4-guanidinobenzoate part, are essential for inhibitory activity. While the introduction of a carbamate between the aromatic rings or as a substituent was detrimental, the replacement of the phenolic part was well tolerated. The covalent interaction of **1** with dengue protease was confirmed but was affected by the low stability in basic buffers. Therefore, further lead optimization should focus on increasing the stability in buffer systems, lowering the polarity by removing the second basic residue and modification of the phenolic part to achieve better selectivity for the flaviviral proteases.

7. Experimental section

Synthetic and analytical details and information on the assays are described in the supporting information.

Dilution experiment

The DENV protease (concentration: 3.33 µM) was preincubated with compound **1** (concentration: 100 µM, from 10 mM stock solutions in DMSO) in assay-buffer (50 mM Tris-HCl pH 9, ethylene glycol (10% v/v), and 0.0016% Brij 58) at room temperature for 1 h. Afterward, 3.33 µL of the preincubation solution was diluted with 86.7 µL assay-buffer. After different incubation times, 10 µL of the FRET substrate solution (concentration: 500 µM) was added to obtain a final assay volume 100 µL per well and its cleavage was measured continuously (final concentrations: 100 nM enzyme; 3.33 µM inhibitor; 50 µM substrate). Three controls are included: 1. No inhibitor throughout to reflect full enzyme activity, 2. 100 µM inhibitor concentration throughout to achieve 100% inhibition, and 3. 3.33 µM inhibitor concentration throughout to reflect the expected amount of inhibition remaining after substrate dilution.

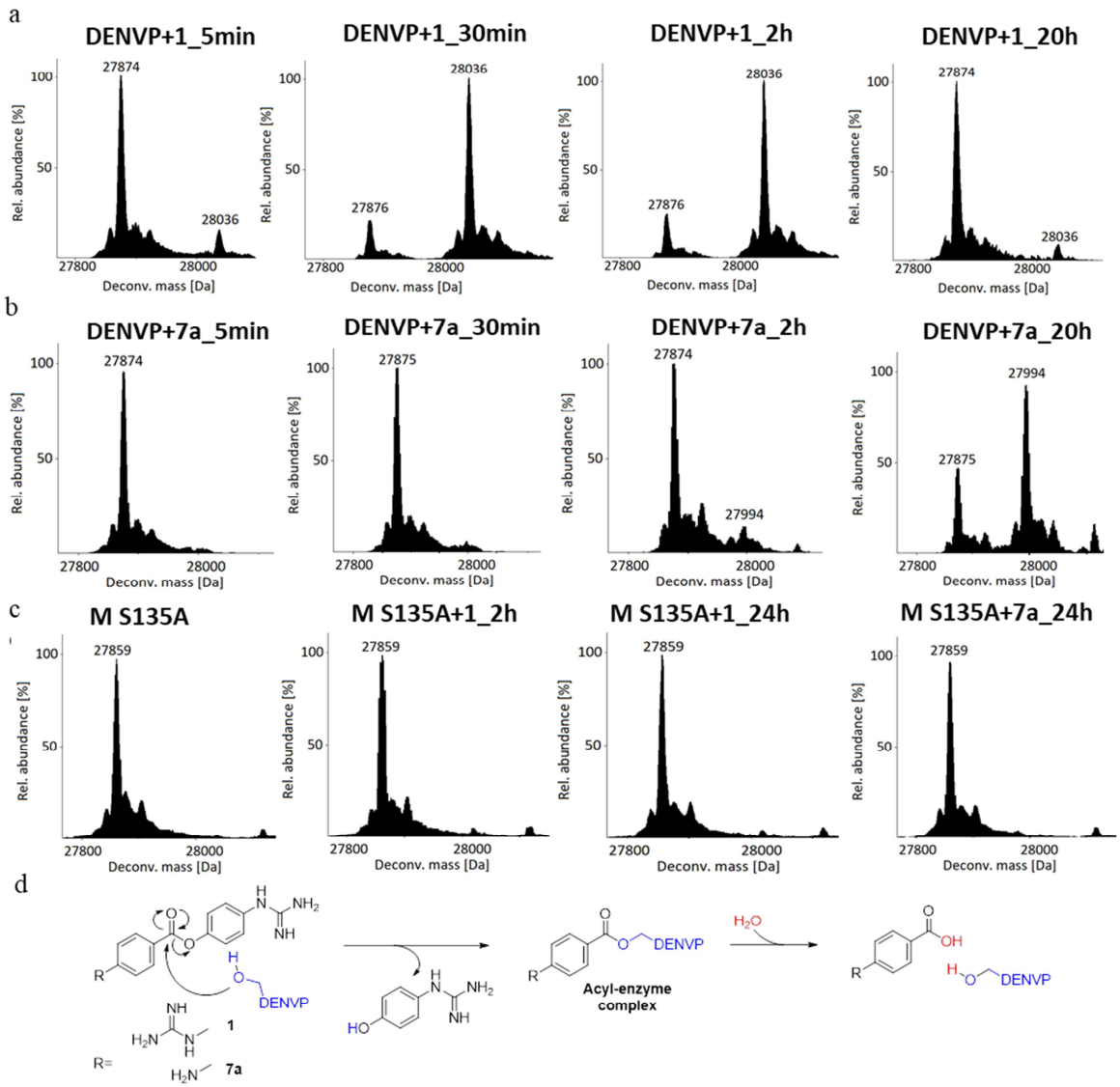


Figure 3. (a) ESI-MS of DENV protease (DENVP) with **1** after different incubation times; (b) ESI-MS of DENV protease (DENVP) with **7a** after different incubation times;(c) ESI-MS of DENVP mutant S135A (M S135A) with **1** and **7a** after different incubation times; (d) reaction of **1** with the catalytic serine of the DENV protease, followed by hydrolysis of the resulting acyl-enzyme complex.

The continuous enzymatic assays were performed in black 96 well V-bottom plates (Greiner Bio-One, Germany) on a BMG Labtech Fluostar OPTIMA Microtiter fluorescence plate reader using excitation and emission wavelengths of 320 and 405 nm, respectively. The enzymatic activity was determined as slope per second (relative fluorescence units per second) and monitored for 15 min. Percentage inhibition was calculated relative to positive controls (without the inhibitor). All experiments were performed in triplicate and averaged.

ESI-MS Analysis of Covalent Inhibitor Binding

The DENV protease (DENVP) and the DENV protease mutant (M S135A) (final concentration: 3 µM) were incubated with the test compounds (final concentration: 100 µM, from 10 mM stock solutions in DMSO) in assay-buffer (50 mM Tris-HCl pH 9, ethylene glycol (10% v/v), and 0.0016% Brij 58) at room temperature. Untreated enzymes served as controls by replacing the volume of the stock solution with pure DMSO. The incubation volume (200 µL) was stored in an HPLC vial. After chosen incubation times a volume of 20 µL was analyzed by flow injection analysis using an Agilent 1200 series HPLC device coupled to an ESI-MS instrument (micrOTOF-QII, Bruker Daltonik, Bremen) operating in positive ionization mode. Column chromatography was performed as follows: column: Reprosil-Pur ODS-3, Dr. Maisch GmbH, Germany, 3 µm, 50 x 2 mm; method: eluent A, water (+0.1% formic acid); eluent B, acetonitrile (+0.1% formic acid); flow rate, 0.3 mL/min; gradient, 10% B (0 min), 95% B (4 min), 95% B (8 min), 10% B (0 min) and 10% B (5-7 min). Instrument calibration and external mass calibration were performed with ESI Tuning mix (Fluka) calibration standard at the end of each analysis run. Mass spectra of analytes with multiple, variable charges were deconvoluted using the Maximum Entropy Deconvolution algorithm (Compass DataAnalysis Version 4.0 SP4, Bruker Daltonik).

Acknowledgements

We thank Heiko Rudy for measuring ESI high resolution mass spectra and Natascha Stefan for measuring routine protease assays. The project was sponsored by the Deutsche Forschungsgemeinschaft, KL-1356/3-2. Tonko Dražić acknowledges support by an EU Marie-Curie grant (LIsTEN 748447).

References and notes

[1] M.G. Guzman, S.B. Halstead, H. Artsob, P. Buchy, J. Farrar, D.J. Gubler, E. Hunsperger, A. Kroeger, H.S. Margolis, E. Martínez, M.B. Nathan, J.L. Pelegrino, C. Simmons, S. Yoksan, R.W. Peeling, Dengue: a continuing global threat, Nature Reviews Microbiology, 8 (2010) 7-16.

[2] S. Bhatt, P.W. Gething, O.J. Brady, J.P. Messina, A.W. Farlow, C.L. Moyes, J.M. Drake, J.S. Brownstein, A.G. Hoen, O. Sankoh, M.F. Myers, D.B. George, T. Jaenisch, G.R.W. Wint, C.P. Simmons, T.W. Scott, J.J. Farrar, S.I. Hay, The global distribution and burden of dengue, Nature, 496 (2013) 504-507.

[3] V. Boldescu, M.A.M. Behnam, N. Vasilakis, C.D. Klein, Broad-spectrum agents for flaviviral infections: dengue, Zika and beyond, Nature Reviews Drug Discovery, 16 (2017) 565-586.

[4] C. Nitsche, S. Holloway, T. Schirmeister, C.D. Klein, Biochemistry and Medicinal Chemistry of the Dengue Virus Protease, Chemical Reviews, 114 (2014) 11348-11381.

[5] L. Cregar-Hernandez, G.-S. Jiao, A.T. Johnson, A.T. Lehrer, T.A.S. Wong, S.A. Margosiak, Small Molecule Pan-dengue and West Nile Virus NS3 Protease Inhibitors, Antiviral chemistry & chemotherapy, 21 (2011) 209-218.

[6] K.-H. Lin, E.A. Nalivaika, K.L. Prachanronarong, N.K. Yilmaz, C.A. Schiffer, Dengue Protease Substrate Recognition: Binding of the Prime Side, ACS Infectious Diseases, 2 (2016) 734-743.

[7] M.A.M. Behnam, D. Graf, R. Bartenschlager, D.P. Zlotos, C.D. Klein, Discovery of Nanomolar Dengue and West Nile Virus Protease Inhibitors Containing a 4-Benzyloxyphenylglycine Residue, Journal of Medicinal Chemistry, 58 (2015) 9354-9370.

[8] A.K. Timiri, B.N. Sinha, V. Jayaprakash, Progress and prospects on DENV protease inhibitors, European Journal of Medicinal Chemistry, 117 (2016) 125-143.

[9] D. Ekonomiuk, X.-C. Su, K. Ozawa, C. Bodenreider, S.P. Lim, G. Otting, D. Huang, A. Caflisch, Flaviviral Protease Inhibitors Identified by Fragment-Based Library Docking into a Structure Generated by Molecular Dynamics, Journal of Medicinal Chemistry, 52 (2009) 4860-4868.

[10] C. Steuer, C. Gege, W. Fischl, K.H. Heinonen, R. Bartenschlager, C.D. Klein, Synthesis and biological evaluation of α-ketoamides as inhibitors of the Dengue virus protease with antiviral activity in cell-culture, Bioorganic & Medicinal Chemistry, 19 (2011) 4067-4074.

[11] C. Nitsche, C. Steuer, C.D. Klein, Arylcyanoacrylamides as inhibitors of the Dengue and West Nile virus proteases, Bioorganic & Medicinal Chemistry, 19 (2011) 7318-7337.

[12] T. Mendgen, C. Steuer, C.D. Klein, Privileged Scaffolds or Promiscuous Binders: A Comparative Study on Rhodanines and Related Heterocycles in Medicinal Chemistry, Journal of Medicinal Chemistry, 55 (2012) 743-753.

[13] G. Bönner, H. Babst, W. Kaufmann, In vivo effects of camostat mesilate on plasma kallikrein, plasma kininase II and renal kallikrein of man, Arzneimittelforschung, 37 (1987) 535-537.

[14] T. Knehans, A. Schüller, D.N. Doan, K. Nacro, J. Hill, P. Güntert, M.S. Madhusudhan, T. Weil, S.G. Vasudevan, Structure-guided fragment-based in silico drug design of dengue protease inhibitors, Journal of Computer-Aided Molecular Design, 25 (2011) 263-274.

[15] M. Hosoya, S. Matsuyama, M. Baba, H. Suzuki, S. Shigeta, Effects of protease inhibitors on replication of various myxoviruses, Antimicrobial Agents and Chemotherapy, 36 (1992) 1432-1436.

[16] M.K. Ramjee, I.M.J. Henderson, S.B. McLoughlin, A. Padova, The Kinetic and Structural Characterization of the Reaction of Nafamostat with Bovine Pancreatic Trypsin, Thrombosis Research, 98 (2000) 559-569.

[17] S.A. Beyler, L.J.D. Zaneveld, Antifertility activity of systemically administered proteinase (acrosin) inhibitors, Contraception, 26 (1982) 137-146.

[18] M. Bartolini, V. Cavrini, V. Andrisano, Characterization of reversible and pseudo-irreversible acetylcholinesterase inhibitors by means of an immobilized enzyme reactor, Journal of Chromatography A, 1144 (2007) 102-110.

[19] R.A. Bauer, Covalent inhibitors in drug discovery: from accidental discoveries to avoided liabilities and designed therapies, Drug Discovery Today, 20 (2015) 1061-1073.

[20] X. Koh-Stenta, J. Joy, S.F. Wang, P.Z. Kwek, J.L.K. Wee, K.F. Wan, S. Gayen, A.S. Chen, C. Kang, M.A. Lee, A. Poulsen, S.G. Vasudevan, J. Hill, K. Nacro, Identification of covalent active site inhibitors of dengue virus protease, Drug Design, Development and Therapy, 9 (2015) 6389-6399.

[21] C. Jöst, C. Nitsche, T. Scholz, L. Roux, C.D. Klein, Promiscuity and Selectivity in Covalent Enzyme Inhibition: A Systematic Study of Electrophilic Fragments, Journal of Medicinal Chemistry, 57 (2014) 7590-7599.

Supplementary Material

Supplementary material is provided as a separate electronic file.