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# Synthesis and biological evaluation of α-ketoamides as inhibitors of the Dengue virus protease with antiviral activity in cell-culture

Christian Steuer [a], Christian Gege [a], Wolfgang Fischl [b], Karl H. Heinonen [a], Ralf Bartenschlager [b], Christian D. Klein [a,*]

[a] Medizinische Chemie, Universität Heidelberg, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany
[b] Molekulare Virologie, Department of Infectious Diseases, Molecular Virology, University Heidelberg, Im Neuenheimer Feld 345, 69120 Heidelberg, Germany

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ABSTRACT

The development of small molecule inhibitors of the viral protease is of considerable interest for the treatment of emergent flaviviral diseases such as Dengue or West Nile fever. Until today little progress has been made in finding drug-like compounds that inhibit the protease and provide a starting point for lead optimization. We describe here the initial steps of a drug discovery effort that focused on the styryl pharmacophore, combined with a ketoamide function to serve as electrophilic trap for the catalytic serine. This resulted in a fragment-like lead compound with reasonable target affinity and good ligand efficiency, which was extensively modified to explore structure–activity relationships. Selected compounds were cross-tested against the West Nile virus protease and thrombin, indicating that selectivity for one or more flaviviral proteases can be achieved. Finally, the antiviral activity of several protease inhibitors was confirmed in a cell-culture model of Dengue virus replication. The SAR presented here may serve as starting point for further drug discovery efforts with the aim of targeting flaviviral proteases.



## 1. Introduction

The Dengue virus (DenV) belongs to the family Flaviviridae and is closely related to the West Nile virus (WNV), the Yellow Fever virus (YFV) and the Hepatitis C virus (HCV). Dengue viruses are transmitted by mosquitos of the species *Aedes aegypti* and cause clinical symptoms ranging from mild fever to Dengue hemorrhagic fever (DHS) and Dengue shock syndrome (DSS). DenV can be subgrouped into four highly homologous but antigenically distinct serotypes (DenV 1–4). Immunity against one serotype does not protect against an infection by another but may enhance disease severity if re-infection with another serotype occurs.[1] At the moment an estimated 2.5 billion people live in regions at high risk for epidemic transmission. Because of global warming and international travel, the virus currently spreads into the temperate zones. Accordingly, there is a considerable interest in developing effective vaccines or chemotherapeutics against DenV and other closely related flaviviruses like WNV. Until today neither vaccination nor a targeted drug therapy is available for treatment of Dengue infections. DenV consists of a single stranded and positive sensed RNA genome of approximately 11 kb. The genome encodes for a polyprotein with three structural proteins (C, prM, E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Host proteases (furin, signalase) as well as the two-component viral protease NS2B–NS3 (the 'DenV protease') process the polyprotein.[2] The correct processing of the precursor is essential for the viral life-cycle.[3,4] Therefore, the viral protease is an interesting target for the development of DenV therapeutics.

NS3 is a multifunctional protein in which the N-terminal domain (NS3pro, 184 residues) represents the protease. The C-terminal part comprises the enzymatic functions of a helicase, a nucleoside triphosphatase and RNA 5′-triphosphatase.[3,5] The protease is a serine endoprotease with the catalytic triad His51-Asp75-Ser135. In common with mammalian thrombin, flaviviral NS2B–NS3 requires basic residues at the P1 position of the substrate. Basic residues in the P2 position greatly increase substrate-binding. The P1′ position is preferably glycine or serine, whereas the other C-terminal positions of the substrate are not relevant for substrate recognition.[6] The protease displays a chymotrypsin fold consisting of two β-barrels each formed by six β-strands.[7] For optimal catalytic activity the protease requires the hydrophilic core domain of the NS2B protein as a cofactor.[8] The cofactor provides a single β-strand to the N-terminal β-barrel and stabilizes this domain by covering hydrophobic residues of NS3pro.[9]

Targeting the flaviviral proteases of DenV, WNV or YFV appears to be similarly difficult for medicinal chemistry as the drug discovery of inhibitors against the HCV NS3–NS4A protease. We were unable to identify any reports of small-molecular, drug-like inhibitors of DenV protease that entered the clinical development phases.

* Corresponding author. Tel.: +49 6221 544875; fax: +49 6221 546430.
*E-mail address:* c.klein@uni-heidelberg.de (C.D. Klein).

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In the other published efforts aimed at flaviviral proteases, the substrate-binding region is usually addressed by a tetrameric (or larger) peptide chain that resembles the non-prime residues of the natural substrate, combined with various electrophilic traps such as aldehydes and fluoroketones, to address the catalytic serine residue.[10,11] Due to the basic nature of the non-prime amino acids of the substrate (Arg, Lys), and the considerable size of the resulting peptide, these inhibitors are not drug-like and probably not cell-permeant. A noteworthy exception is the work of Bodenreider et al.,[12] who described and characterized a set of drug-like inhibitors with molecular masses in the 500–600 g/mol range and $K_D$ values in the lower micromolar range that were probably identified by high-throughput screening. Further development or cell-culture studies with these compounds, however, have not been reported.

The drug discovery effort described here was initiated with the screening of small-molecular aldehydes. The rationale behind this was that we required a reference inhibitor (without the requirement of drug-likeness) to assess the optimized screening procedure described previously.[13] Aldehydes can interact with the nucleophilic serine of serine proteases in a covalent–reversible manner by forming hemiketals. In order to generate compounds with increased drug-likeness, we decided to replace the aldehyde group with a more selective electrophilic trap—the α-ketoamide function—and thus to create a SAR dataset of α-ketoamides and α-ketoamide derivatives. For the serine protease from HCV, which is related to DenV protease, there are currently several inhibitors in clinical trials that employ the α-ketoamide group as electrophilic trap (Telaprevir, Boceprevir).[14,15] This moiety is common in pharmaceuticals like serine or cysteine protease inhibitors[16–20] and can be found in several bioactive natural products such as the immunosuppressant drugs FK-506 and rapamycin.[21] The binding towards serine proteases is based on the formation of a hemiketal or hemithioketal with the –OH or –SH group of serine or cysteine protease, respectively.[22]

In contrast, β,γ-unsaturated α-ketoamides are found only in a few number of pharmaceuticals with biological activities such as antibacterials[23,24] and in one natural product.[25] They are also used as chemical intermediates for the synthesis of 5-aryl-3-carboxamide-4,5-dihydro-(1*H*)-pyrazoles.[26] Therefore, the work presented here, which also includes a new synthetic route towards this compound class, is also relevant in the light of synthetic organic chemistry.

## 2. Results and discussion

### 2.1. Chemistry

For the synthesis of the targeted 4-substituted 2-oxo-but-3-enoic acid amides, several approaches were pursued as outlined in Figure 1. Route A started with an aldol condensation of aromatic aldehydes with pyruvic acid in the presence of potassium hydroxide (1.5 equiv),[27] followed by peptidic coupling of the intermediate with various amines and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt) as powerful coupling reagent.[28] The key compound **1** was crystallized and showed *trans* geometry in X-ray diffraction structure analysis (data not shown). Since with some aldehydes (e.g., with 2-pyridyl aldehyde) only a sluggish aldol condensation was observed, we investigated the direct coupling of an aldehyde with *N*-*tert*-butyl pyruvic amide[29] or *N*-benzyl pyruvic amide[30] using copper triflate as catalyst as recently described for the coupling of pyruvic esters (Route B).[31] The reaction times could be reduced by the use of microwave irradiation. Particularly good yields could be obtained from electron-rich aldehydes. Aldol condensation of pyruvic amide under basic conditions using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as catalyst resulted in no improvements. Passerini reaction (Route C) was performed as described by Denmark and Fan[32] and gave moderate yields. The hydroxyamides were subjected to Dess–Martin oxidation as described by Speicher et al.[33] This two-step method was chosen for the α-ketoamides that could not be obtained by Route A and B (7–9 in Table 1).

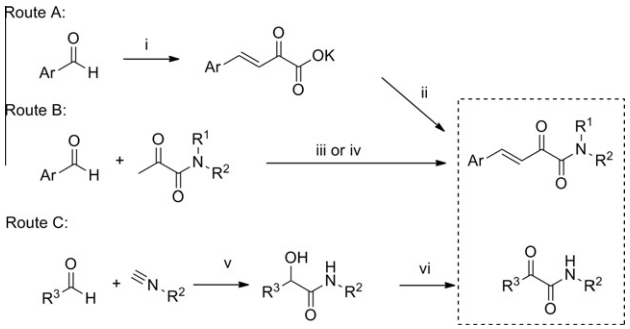



**Figure 1.** Reagents and conditions: (i) pyruvic acid, KOH (1.5 equiv), MeOH; (ii) $HNR^1R^2$, HATU, HOAt, $NEt_3$, DMF, 0 °C–rt; (iii) $Cu(OTf)_2$, $CH(OMe)_3$, $CH_2Cl_2$, microwave, 100 °C; (iv) DBU, $CH_2Cl_2$, rt; (v) $SiCl_4$, $CH_2Cl_2$, −78 °C, 4 h, then $NaHCO_3$; (vi) Dess–Martin periodinane, $CH_2Cl_2$, rt.

With the goal of exploring the aryl moiety, we kept the β,γ-unsaturated α-keto *tert*-butyl amide moiety constant. Inhibitors listed in Table 2 were synthesized as depicted in Figure 1 with the following exceptions: phenol **20** was synthesized from compound **15** via O-demethylation using boron tribromide. Compound **21** was synthesized by reducing nitro derivative **26** with excess tin chloride in refluxing ethanol. Derivative **34** was obtained by acetylation of amine **21** with $Ac_2O$ in pyridine. Finally, nitrile 35 was prepared from bromide **25** using zinc(II) cyanide and $Pd(PPh_3)_4$ as catalyst. For details see the data in the Supplementary data.

For the exploration of the substituent on the amide nitrogen, the phenyl moiety was kept constant. The inhibitors listed in Table 3 were synthesized as depicted in Figure 1 with the exception of compound **46**, which was synthesized from alcohol **44** by acetylation with $Ac_2O$ in pyridine.

**Table 1**
Influence of modifications at the core structure of lead compound **1** on the inhibition of DenV NS2B–NS3 serine protease. Inhibitor concentration: 50 μM

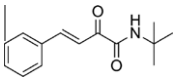
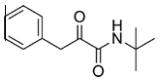
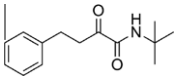
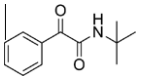
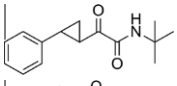
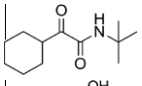
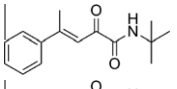
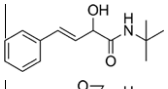
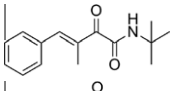
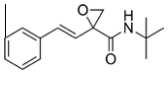
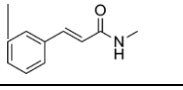
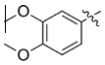
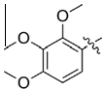
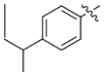
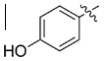
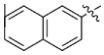
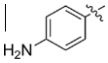
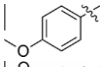
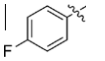
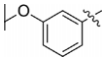
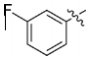
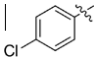
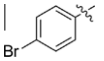
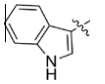
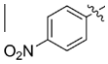
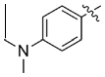
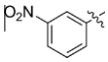
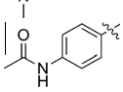
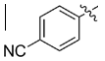
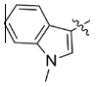
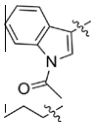
| # | Structure | % inhib. (sd%) | # | Structure | % inhib. (sd%) |
|---|---|---|---|---|---|
| **1** |  | 26.3 (3.2) | **7** |  | 20.4 (1.6) |
| **2** |  | 0 (4.3) | **8** |  | 6.9 (0.7) |
| **3** |  | 7.4 (4.7) | **9** |  | 21.5 (1.6) |
| **4** |  | 19.6 3.1 | **10** |  | 8.2 (3.2) |
| **5** |  | 25.6 (3.4) | **11** |  | 12.6 (1.4) |
| **6** |  | 15.2 (3.0) | | | |

**Table 2**
Inhibitory activities of unsaturated α-ketoamides against DenV NS2B–NS3 serine protease: Variation of the γ-aryl substituent. Inhibitor concentration: 50 μM

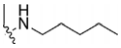
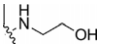
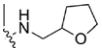
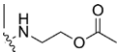
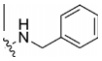
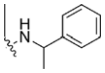
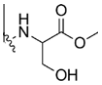
| # | | % inhib. (sd%) | # | | % inhib. (sd%) |
|---|---|---|---|---|---|
| **1** |  | 26.3 (3.2) | **18** |  | 36.1 (6.8) |
| **12** |  | 27.6 (5.2) | **19** |  | 27.8 (0.4) |
| **13** |  | 25.5 (1.2) | **20** |  | 37.6 (2.4) |
| **14** |  | 8.9 (2.3) | **21** |  | 40.6 (0.9) |
| **15** |  | 16.7 (2.8) | **22** |  | 23.9 (3.5) |
| **16** |  | 17.8 (2.1) | **23** |  | 16.8 (7.5) |
| **17** |  | 23.3 (2.8) | **24** |  | 15.9 (5.2) |
| **25** |  | 15.1 (1.3) | **32** |  | 39.1 (0.7) |
| **26** |  | 12.7 (2.2) | **33** |  | 13.8 (1.2) |
| **27** |  | 9.6 (1.1) | **34** |  | 32.9 (2.6) |
| **28** |  | 12.9 (1.7) | **35** |  | 13.5 (3.4) |
| **29** |  | 18.4 (3.0) | **36** |  | 31.3 (2.7) |
| **30** |  | 23.3 (2.0) | **37** |  | 29.8 (3.7) |
| **31** |  | 27.7 (4.0) | **38** |  | 4.4 (6.3) |

To investigate the relevance of the central β,γ-unsaturated α-keto moiety, the analogs shown in Table 1 were synthesized. The introduction of the cyclopropyl moiety in compound **1** was accomplished by the method of Corey and Chaykovsky[34] using trimethylsulfoxonium iodide and sodium hydride as base to give derivative **3**. By reaction of compound **1** with trimethylsulfonium iodide and potassium *tert*-butylate as base,[34] oxirane **11** was obtained. Compound **6** can be prepared as previously described.[35] The other derivatives were prepared by applying the Passerini reaction (Fig. 1, Route C) to the corresponding aldehyde and, with the exception of alcohol **10**, with subsequent oxidation using Dess–Martin periodinane.

## 2.2. General considerations: lead-likeness, absence of promiscuous inhibition, ligand efficiency

It has not escaped our attention that, in comparison to other drug development projects, the compounds described here do not have a high affinity towards the target enzyme. In the absence of any other pursuable, small-molecular leads—either from literature or our own screening process—we nevertheless decided to study this compound class in more detail, in order to evaluate the potential for further development.

**Table 3**
Inhibitory activities of unsaturated α-ketoamides against DenV NS2B–NS3 serine protease: Variation of the substituent at the amide nitrogen. Inhibitor concentration: 50 μM

| # | R | % inhib. (sd%) | # | R | % inhib. (sd%) |
|---|---|---|---|---|---|
| **1** |  | 26.3 (3.2) | **43** |  | 25.9 (1.4) |
| **39** |  | 23.8 (6.3) | **44** |  | 26.1 (1.3) |
| **40** |  | 26.9 (0.9) | **45** |  | 28.9 (2.6) |
| **41** |  | 31.2 (3.6) | **46** |  | 35.8 (6.1) |
| **42** |  | 14.5 (4.4) | **47** |  | 14.6 (2.1) |
| **48** |  | 0 (4.9) | **51** |  | 35.1 (3.0) |
| **49** |  | 29.9 (2.7) | **52** |  | 29.0 (1.8) |
| **50** |  | 29.4 (4.2) | **53** |  | 30.8 (1.5) |

The occurrence of unspecific-/promiscuous inhibitors is a widespread phenomenon in drug discovery. Therefore many tools have been developed to eliminate such candidates in early stages of this process.[12] We were well aware of the dangers of promiscuous binding and took various measures to exclude this risk before advancing the project. These measures included: Analysis of compound solutions by dynamic light scattering; testing against numerous other enzymes including metalloproteases, ligases and other serine proteases; Analysis of the Hill slope of dose–response curves; application of orthogonal assay procedures such as a HPLC-based assay and the tryptophane-fluorimetric assay described by Bodenreider et al.[12] None of these procedures gave any hint whatsoever on a promiscuous binding mode of the compounds. Furthermore, kinetic analyzes of selected compounds demonstrated a competitive binding mode.

The ligand efficiency (LE)[36,37] as measure of affinity per atom (or per molecular weight unit) of compd **1** is 16.6 Perola[37] determined ligand efficiencies of typical lead compounds to be generally above 12.4. Ligand efficiencies of the optimized compounds shown here, such as **18** and **32**, are in the range of 14–15, which is a typical value for clinical candidates and established drugs.

We therefore conclude that the inhibitors presented here exploit the potential interaction sites near the catalytic serine of Dengue protease with high effectivity. These analogs represent valuable 'serine anchors' which may be expanded to yield high-affinity inhibitors. Finally, the cell-culture data shown below clearly indicate that these chemotypes have the potential to be used in further preclinical studies on Dengue therapy.

## 2.3. Structure–activity relationships

In analogy to the α-ketoamide inhibitors of other serine proteases, we expected a nucleophilic attack by the catalytic serine at the ketoamide α-carbonyl group (1,2-addition), resulting in a covalent-reversible binding mode. A Michael (1,4) addition of the serine at the γ-position appears unlikely, although 1,4-addition to the nucleophilic hydroxyl group of threonine proteases (e.g., in the proteasome) has been described.[38] To elucidate the binding mode

and to explore possible alternatives for the potentially problematic acrylic moiety, a number of variants to the unsaturated α-ketoamide core structure were generated. These variants are presented in Table 1.

The saturated analog **2** has no effect on the DenV protease. Reduction of the linker length between the electrophilic trap and the phenyl-/cyclohexane moiety results in a decreased activity of the compounds (compds. **7**–**9**). Interestingly, reduction of the distance between the aryl moiety and the α-keto functionality is tolerated as depicted with compound **7**. However, a further reduction of the distance is not tolerated (compds **8** and **9**). Replacement of the double bond with a cyclopropyl group—to explore the relevance of steric and conformational effects—resulted in marginal activity. The necessity of the α-keto functionality is also exemplified by the α-hydroxy derivative **10** and the α-epoxy derivative **11**, both showing reduced inhibitory activity. Methylation is completely tolerated at the β-position (compd **5**): Further work may aim at substituents in this position to activate the electrophilic trap and to reach the S2 subsite of the DenV protease. Methylation at the γ-position leads to a marginally less active compound (compd **4**). This indicates that the binding does not depend on the 'Michael' reactivity in the γ-position but rather on the reversible formation of a hemiketal at the ketoamide carbon. The acrylamide **6** was only marginally active. Compound **11** was synthesized to explore the potential of an epoxide electrophile, but appears less promising. To summarize, work on the central moiety confirmed that the unsaturated ketoamide structure is the essential pharmacophore.

With respect to the shallow binding area,[39] the unsaturated moiety and the resulting planarity and loss of conformational freedom is apparently essential for the molecular recognition of this compound class. The SAR data also indicates that the β-position may be well suited for the extension of the compounds towards the S2 and further binding regions.

Variation of the γ-aryl moiety (Table 2) revealed the following picture: While electron donating residues (e.g., methoxy, pyridyl) promote inhibitory activity, electron withdrawing residues (e.g., nitro, cyano, fluoro) diminish activity in most cases.

As shown with derivative **14**, incorporation of hydrophobic bulk is not tolerated. However, the branched isopropyl group **13** is still accepted by the target. Substitution of the aryl moiety with less hydrophobic hydrogen-bond-acceptor functions are tolerated to some extent, as shown in methoxy derivatives **15**–**17**. No clear preference upon the site of substitution is detectable, also in consideration of the three nitro isomers **26** to **28** or the pyridyl derivatives **29** to **31**. In the dimethoxy derivative **18**, the increased electron donating potential results in high inhibition, but this positive trend can not be maintained in the trimethoxy derivative **19**, probably due to steric constraints. The substituent in 4-position is preferably a hydrogen bond donor: Phenol **20** has a significantly higher activity than the methoxy analog **15**. The relevance of a hydrogen bond donor substituent on the aromatic moiety also becomes evident in the comparison of the 4-amino and the 4-dimethylamino analogs **21** and **33**. In these cases the activity is abolished by methylation, while the electronic effects on the α-ketoamide moiety should be negligible. A similar interpretation is possible for the detrimental effect of methylation (**36**) and acetylation (**37**) on the activity of the 3-indole **32**, which is among the most potent compounds of the dataset. The binding of the 3-indole moiety into the S1 subsite is in remarkable agreement with the results of Yin et al., whose second most active inhibitor was a tetrapeptide aldehyde with tryptophane in the S1 position that achieved a $K_i$ of 7.5 μM.[40]

Table 3 shows the effects of various substituents on the ketoamide nitrogen. We reasoned that the γ-aryl moiety is probably located in or near the S1 pocket of the DenV protease, while the α-ketoamide nitrogen substituents are oriented towards the S1′ pocket. Many substituents are accepted in this pocket—bulky *tert*-butyl residues as well as smaller alkyl groups, for example, methyl, propyl or cyclopropyl, which can additionally be substituted with heteroatoms. While the size could be extended to longer alkyl chains, for example, the pentyl analog **43**, 'branched' bulk like cyclohexyl (compd **42**) is less tolerated. In addition, hydrophobic aromatic systems like benzyl or 1-methylbenzyl should be avoided, whereas more hydrophilic residues like pyridyl (compds **49** and **50**) are able to compensate that effect. Based on the assumption that the substituents on the α-ketoamide nitrogen occupy the S1′-pocket, we decided to explore the potential of moieties that resemble serine, which is a preferred P1′ residue in DenV protease substrates. In fact, remarkable activity was observed with the serine analog **51**, but also with the similar compounds **44** and **46**. Alkylation of the amide nitrogen does not have significant influence on the activity (cf. **40** vs **52** and **53**).

### 2.4. Selectivity against other serine proteases

Selectivity data for a subgroup of compounds against the Dengue and West Nile virus proteases and thrombin—as a mammalian serine protease with similar substrate recognition requirements—are presented in Table 4. The lead compound **1** has limited selectivity under these conditions. Compound **5** is remarkable because a very minor structural variation leads to selectivity against DenV

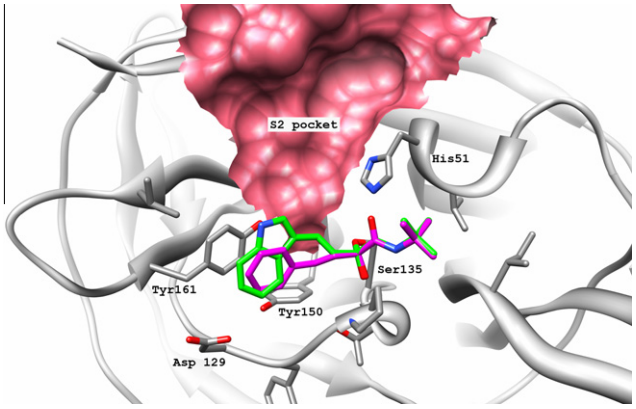



**Figure 2.** Compounds **1** (magenta) and **32** (green) were covalently docked to the oxygen of the Ser135 Important residues of the S1 pocket as well as residues of the catalytic triad (Ser135, His51) are shown as sticks. The S2 pocket is represented as surface (magenta). The picture was generated with CHIMERA.[41]

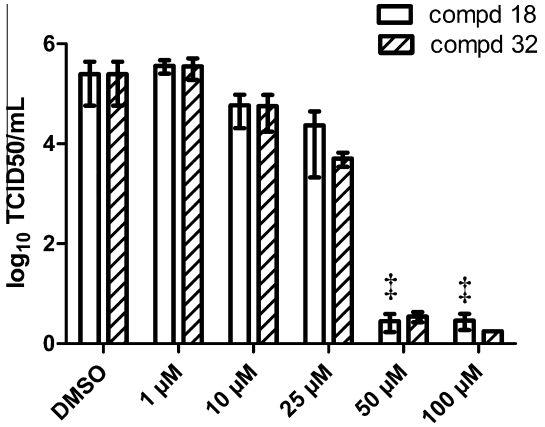
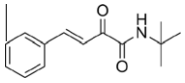
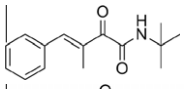
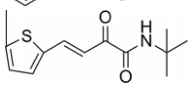
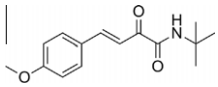
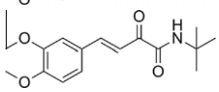
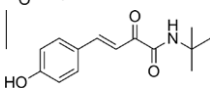
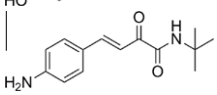
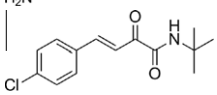
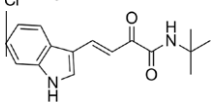
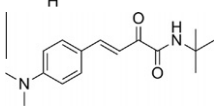
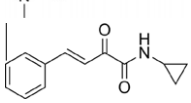
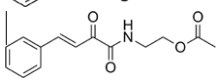
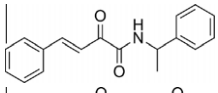
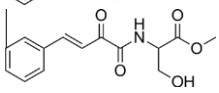



**Figure 3.** Compounds **18** and **32** inhibit DenV replication in cell-culture. Error bars represent standard deviation from the mean of three independent experiments. ‡Indicates cytotoxic activity of compd **18** at 50 and 100 μM. No cytotoxicity was observed for compd **32** at any of the tested concentrations.

**Table 4**
Activity of selected compounds against DenV NS2B–NS3, WNV NS2B–NS3 and thrombin. Inhibitor concentration: 50 μM for DenV and WNV protease, 25 μM for thrombin.

| # | Structure | DenV % inhib. (sd%) | WNV % inhib. (sd%) | Thrombin % inhib. (sd%) |
|---|---|---|---|---|
| **1** |  | 26.3 (3.2) | 20.0 (6.0) | 19.6 (3.4) |
| **5** |  | 25.6 (3.4) | 0 (2.9) | 5.7 (2.4) |
| **11** |  | 27.6 (5.2) | 20.3 (1.7) | 12.1 (3.7) |
| **15** |  | 16.7 (2.8) | 27.3 (5.1) | 14.2 (5.3) |
| **18** |  | 36.1 (6.8) | 31.0 (0.5) | 22.5 (2.1) |
| **20** |  | 37.6 (2.4) | 31.4 (4.6) | 17.3 (3.7) |
| **21** |  | 40.6 (0.9) | 39.5 (4.5) | 21.1 (2.2) |
| **24** |  | 15.9 (5.2) | 6.3 (5.6) | 6.7 (4.4) |
| **32** |  | 39.1 (0.7) | 40.6 (0.7) | 16.9 (1.9) |
| **33** |  | 13.8 (1.2) | 33.9 (1.1) | 28.9 (3.7) |
| **41** |  | 31.2 (3.6) | 20.0 (6.7) | 17.1 (5.2) |
| **46** |  | 35.8 (6.1) | 29.8 (2.5) | 16.3 (1.8) |
| **48** |  | 0 (4.9) | 12.2 (3.2) | 5.1 (1.0) |
| **51** |  | 35.1 (3.0) | 27.5 (3.2) | 12.6 (2.2) |

protease. Selectivity is slightly improved in compounds **32**, **46**, and **51**, which show higher activity against the flaviviral proteases than against thrombin. Hydrogen-bond-donors in the aromatic moiety (**20**, **21**, **32**) improve the activity and selectivity for flaviviral proteases in comparison to thrombin. Variations at the amide nitrogen, in particular those that mimic the preferred P1′ residues glycine and serine (**46**, but especially **51**), lead to improved selectivity profiles. In summary, selectivity against flaviviral proteases can be achieved by several, minor structural variations which may be combined to yield an optimized structural scaffold.

## 2.5. Molecular modeling

To rationalize the SAR and guide further syntheses, we performed a covalent docking of selected compounds into the active site of DenV protease. The search space is limited because of the defined, covalent bond between the ketoamide moiety and the serine sidechain. The results are shown in Figure 2. The aromatic moieties of the compounds, shown here for **1** and **32**, occupy the S1 binding region. Hydrophilic substituents on the aromatic moieties, which have been shown to increase activity, probably interact with the Tyr150 and Asp129 sidechains at the bottom of the S1 pocket. With respect to further modifications targeting the S2 pocket, it appears most promising to extend the analogs either at the γ position of the ketoamide sidechain or at the *ortho* position on the aromatic ring.

## 2.6. Antiviral activity in cell-culture

Compounds **2**, **18** and **32** were initially screened for antiviral activity in a reporter-gen cell-culture assay of Dengue replication (cf. Fig. 3), with **18** and **32** showing activity in that assay. As a negative control, compound **2**, which is inactive against the protease, was found to be inactive in cell-culture (data not shown). Therefore, antiviral activity of this class of compounds appears to be linked to protease inhibition. To further characterize the active compounds, we treated DenV-2 (wild-type) infected Huh-7 cells

with different concentrations of compounds **18** and **32** immediately after infection and determined infectivity titers 24 h later (cf. Fig. 3). Furthermore, cytotoxicity of all compounds was determined in Huh-7-Fluc cells. Compound **32** was not cytotoxic in all tested concentrations (up to 100 μM), as determined by visual inspection of the cells as well as by measuring luciferase expression as a marker of cell viability. Importantly, for compound **32** we determined an inhibitory effect on DenV replication in a dose-dependent manner, achieving a more than 1000-fold reduction of virus titers at non-cytotoxic concentrations (cf. Fig. 3).

## 3. Conclusion

In summary, novel inhibitors for DenV protease were identified and the SAR around the β,γ-unsaturated α-ketoamide pharmacophore was investigated. With minor modification, selectivity towards related proteases such as West Nile virus protease and thrombin could be achieved. Although the derivatives showed only moderate affinity towards DenV protease in the enzymatic assay, compound **32** inhibits DenV replication in a cell-culture assay in a dose-dependent manner, achieving a more than 1000-fold reduction of virus titers at non-cytotoxic concentrations. In view of the minimal size of the compounds described here and their remarkable activity against Dengue virus replication in cell-culture they appear as valuable starting points for the exploration of further structural variations to discover and develop novel inhibitors of flaviviral proteases. Future work should, under the perspectives of potency and selectivity, aim at modifications on the β-position and on the amide nitrogen, while retaining the indole moiety. Given the antiviral activity and lack of cytotoxicity in cell-culture, it is also tempting to pursue a medium-scale screening effort in a cell-culture based assay of virus replication.

## 4. Experimental section

### 4.1. Chemical methods

All chemicals were obtained from Sigma–Aldrich (Germany), Bachem, (Germany) and Acros (Belgium) and were of analytical grade. No further purification steps were performed unless indicated. Passerini and Dess–Martin reactions were performed in dried glassware and under a nitrogen atmosphere. All reactions were performed with dried solvents (molecular sieve). $^1$H NMR spectra were recorded on a Varian (300 MHz) instrument at 300 K in $CDCl_3$, or acetone-$d_6$. Chemical shifts are reported in $\delta$ values (ppm) and the residuals of non-deuterated solvents were used as internal standard ($CDCl_3$: $\delta$ 7.26 ppm and acetone: $\delta$ 2.05 ppm). Signals are described as s, d, t, dd, m, and br for singlet, doublet, triplet, double-doublet, multiplet, and broad, respectively. Mass spectra (EI) were measured on a Finnigan MAT 8200 instrument. Chemical names follow IUPAC nomenclature. Column chromatography was performed using silica gel cartridges (KP-Sil) and UV monitoring, and the reaction progress was determined by thin layer chromatography analyzes on Merck silica gel plastic plates $60F_{254}$. Compounds were visualized by UV and/or treatment with a solution of $KMnO_4$ (2.5 g) and $Na_2CO_3$ (12.5 g) in water (250 mL) and (optionally) heating.

Synthetic and analytical details for the target compounds are given in the Supplementary data. Three representative chemical syntheses are described below:

#### 4.1.1. (*E*)-*N*-(*tert*-Butyl)-2-oxo-4-(pyridin-3-yl)but-3-enamide (30)

To a solution of 3-pyridine carboxaldehyde (2.05 g, 19.1 mmol) and pyruvic acid (1.70 g, 19.3 mmol) in methanol (10 mL) was added under ice cooling a solution of potassium hydroxide (1.6 g, 29 mmol) in methanol (20 mL). Precipitation occured during addition of the base and the mixture was allowed to reach room temperature for 1 h. The mixture was allowed to stand at 8 °C for three days and then the precipitate was filtered and washed with few cold MeOH and then diethyl ether. The faint yellow crystals (2.81 g, 68%) were used without further purification. To a suspension of the intermediate above (254 mg, 1.18 mmol), HATU (450 mg, 1.18 mmol), HOAt (160 mg, 1.18 mmol) and *tert*-butyl amine hydrochloride (137 mg, 1.25 mmol) in dry DMF (5 mL) was added triethyl amine (200 μL, 1.4 mmol) under ice cooling and the suspension was temporarily sonicated. The mixture was stirred overnight at room temperature and then concentrated. The remaining residue was suspended in 5% aqueous citric acid and extracted with $CHCl_3$ and the solution was dried and purified by flash chromatography using cyclohexane–ethyl acetate 1:1 to 0:1 as eluent to afford the title compound (251 mg, 92%) as yellow crystals. NMR $^1$H $\delta$ (300 MHz, acetone, 293 K): 8.93 (d, 1H, $J$ = 2.4 Hz), 8.65 (dd, 1H, $J$ = 4.8, $J$ = 1.7 Hz), 8.25–8.21 (m, 1H), 7.48 (dd, 1H, $J$ = 4.8, $J$ = 7.8 Hz), 7.36 (br s, 1H), 1.43 (s, 9H). NMR $^{13}$C $\delta$ (75 MHz, acetone, 293 K): 188.0 (CO), 162.7 (CO), 153.5 (CH), 152.6 (CH), 144.8 (CH), 136.5 (CH), 132.2 (C), 125.8 (CH), 122.7 (CH), 52.8 (C), 29.5 (3 × $CH_3$). MS (EI): $C_{13}H_{16}N_2O_2$; Mw: 232.1; MS 232.1 $M^+$.

#### 4.1.2. (*E*)-*N*-(*tert*-Butyl)-2-oxo-4-(3,4,5-trimethoxyphenyl)but-3-enamide (19)

A solution of 3,4,5-trimethoxy benzaldehyde (128 mg, 652 μmol), $Cu(OTf)_2$ (24 mg, 66 μmol), trimethyl orthoformate (78 μL, 71 μmol) and *N*-*tert*-butyl pyruvic amide[29] (113 mg, 778 μmol) in dry $CH_2Cl_2$ (2 mL) was degassed under Argon and heated under microwave irradiation to 100 °C for 10 min. The brown solution was absorbed on silica and purified by flash chromatography using cyclohexane–ethyl acetate 1:0 to 3:1 as eluent to afford the product (77 mg, 37%) as yellow crystals. NMR $^1$H $\delta$ (300 MHz, acetone, 293 K): 7.79 (d, 1H, $J$ = 15.9 Hz), 7.61 (d, 1H, $J$ = 15.9 Hz), 7.31 (br s, 1H), 7.14 (s, 1H), 3.92 (s, 6H), 3.79 (s, 3H), 1.43 (s, 9H). NMR $^{13}$C $\delta$ (75 MHz, acetone, 293 K): 188.1 (CO), 163.0 (CO), 155.7 (C), 148.9 (CH), 143.2 (C), 143.2 (C), 131.9, (C), 119.9 (CH), 108.4 (CH), 61.7 ($CH_3$), 57.6 ($CH_3$), 52.7 (C), 29.5 (CH3). MS (EI): $C_{17}H_{23}NO_5$; Mw: 321.2; MS 321.2 $M^+$.

#### 4.1.3. (*E*)-*N*-(*tert*-Butyl)-4-(4-chlorophenyl)-2-oxobut-3-enamide (24)

A mixture of (*E*)-3-(4-chlorophenyl)acrylaldehyde (1 mmol), *tert*-butyl isocyanide (1.2 equiv) and $SiCl_4$ (1.1 equiv) in $CH_2Cl_2$ (1 mL) was stirred for 4 h at −78 °C. The reaction was stopped by the addition of cold aq $NaHCO_3$. After stirring for 2 h, the mixture was filtered through Celite®. The filtrate was extracted with $CH_2Cl_2$ (4 × 20 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The product was used without any further purification.

A mixture of α-hydroxyamide (1 mmol) and Dess–Martin periodinane (1.15 equiv) in $CH_2Cl_2$ (3 mL) was stirred for 2 h at room temperature. The reaction was stopped by addition of aq $NaHCO_3$. The organic phase was separated and the aqueous phase was extracted with diethyl ether (3 × 20 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography eluting with cyclohexane–ethyl acetate to give 199 mg of the desired compound as a yellow solid, yield 75%. NMR $^1$H $\delta$ (300 MHz, acetone, 293 K): 7.82 (d, 1H, $J$ = 16.2 Hz), 7.69 (d, 1H, $J$ = 16.3 Hz), 7.83–7.80 (d, 2H), 7.53–7.48 (d, 2H), 7.33 (br s, 1H), 1.42 (s, 9H). MS (EI): $C_{14}H_{16}NO_2Cl$: Mw: 265.1; MS: 265.1 $M^+$.

The internally quenched DenV NS2B–NS3 protease substrate Abz-NleKRRS-3-($NO_2$)Y was synthesized by solid-phase synthesis

on Rink amide resin according to the Fmoc-protocol. It was purified by preparative HPLC using an ÄKTA Purifier, GE Germany, with a RP-18 chromatography column (Lobar®, Merck, Germany). The mobile phase consisted of MeOH/0.1% TFA and $H_2O$/0.1% TFA following a gradient of 20–100% MeOH in water with a flow rate of 1.5 mL/min. The internally quenched substrate for the WNV assay (Abz-GLKRGG-3-($NO_2$)Y) was synthesized and purified as described above. The purity of both substrates was assessed by HPLC and found to be higher than 95%. The identity was confirmed by MALDI-ToF-MS.

### 4.2. Biological methods

Expression and purification of DenV protease: The Dengue protease NS2B–NS3 gene, with the hydrophilic cofactor NS2B connected to the protease domain NS3pro via a flexible glycine linker, was synthesized by Geneart. The expression plasmid pET28a (Novagen) with the inserted gene was used to transform *Escherichia coli* BL 21 λ (DE3) cells. Overnight cultures of the transformed cells were grown at 37 °C in standard LB-medium containing 50 μg/mL kanamycin. After the OD of 0.6–0.8 at 600 nm was reached, the expression was induced by addition of IPTG to a final concentration of 1 mM. The cells were grown at 30 °C for a further 4 h and then collected by centrifugation at 4500 g. The pellet was resuspended in buffer A (Lysis buffer: 50 mM Tris–HCl pH 7.9, 100 mM NaCl, 5% glycerol and 5 mM imidazol) and passed through a cell disruptor (One Shot, Constant Systems). Afterward the solution was centrifuged at 18,500 g and 4 °C for 40 min. The supernatant was then purified by $Ni^{2+}$-affinity chromatography. The protein was eluted by increasing the imidazol concentration from 5 to 250 mM. Stocks of purified protein were stored at −70 °C in 100 mM Tris–HCl pH 7.9, 50 mM NaCl and 50% glycerol. The expression and purification protocol for the WNV protease was similar to the one given for DenV (cf. Supplementary data for details).

#### 4.2.1. Flourimetric DenV protease assay

The DenV protease assay was performed as described previously.[13] In short, continuous enzymatic assays were performed on a BMG Labtech Fluostar OPTIMA microtiter fluorescence plate reader using black 96 well V-bottom plates from Greiner. The excitation wavelength was 320 nm and the emission was monitored at 405 nm. The inhibitor concentration was 50 μM. The inhibitors were preincubated for 15 min with the enzyme. Afterward, the reaction was initiated by the addition of the substrate to a final concentration of 50 μM. The activity of the enzyme was determined as the initial slope per second and monitored for 15 min. Experiments were performed in triplicate ($n = 3$) and the experimental values were averaged.

#### 4.2.2. Flourimetric West Nile virus protease assay

The WNV protease assay was performed in analogy to the DenV protease assay. Final concentration of the enzyme was 150 nM. The reaction was initiated by addition of the substrate to a final concentration of 50 μM.

#### 4.2.3. Thrombin assay

The thrombin assay was performed as a continuous fluorimetric assay on a BMG Labtech Fluostar OPTIMA microtiter fluorescence plate reader. The excitation wavelength was 355 nm and the emission wavelength was 460 nm. The protease was assayed against the substrate Boc-Val-Pro-Arg-AMC, (Bachem, Germany). The final concentrations of the enzyme and substrate were 10 nM and 50 μM, respectively. The inhibitors were preincubated with the enzyme for 15 min at a concentration of 25 μM. The cleavage reaction was initiated by addition of the substrate. The assay buffer consisted of 50 mM Tris–HCL pH 7.5, 150 mM NaCl and 0.05% Tween 20.[42] The activity of the enzyme was determined as the initial slope per second and monitored for 10 min.

#### 4.2.4. Tryptophane quenching assay

This was performed as described by Bodenreider et al.[12]

Dynamic light scattering: Inhibitors were typically diluted from 10 mM stocks in DMSO with filtered 50 mM $KP_i$ buffer, pH 7, to a final concentration of 100 μM. All measurements were performed at room temperature using a Zetasizer 3000 HS (Malvern Instruments GmbH, Herrenberg, Germany). The laser power and integration times were comparable for all experiments. The scattering angle was 173°.

#### 4.2.5. Docking

All calculations were performed on an Intel(R) Core(TM)2 Quad CPU Q9450 @ 2.66 GHz running open SuSE 11.0 while using GOLD v5.0 and its graphical interface Hermes 1.4.[43] As there is no catalytic active crystal structure of the Dengue NS2B–NS3 protease a homology model was applied.[44] Prior to the docking the embedded ligand was extracted and hydrogens were added except to the oxygen of the Ser135 where the covalent bond is formed later using the in Hermes implemented functions. A covalent docking to the oxygen of Ser135 was performed using GOLD v5.0 with a search radius of 8 Å and a given substructure common to all inhibitors. Automatic cavity detection was switched on. Furthermore, the GoldScore fitness function and default parameters for the genetic algorithm were applied. During the creation of the covalent bond between the ligand and the Ser135 a chiral hemiacetal was formed. For each compound both chiral conformers were use and 10 different solutions were calculated and ranked according to their GoldScore. The conformer with the better score was used for visualization.

Antiviral activity in cell-culture: Huh-7 cells were infected with DenV 2 at an MOI of 1 TCID50 per cell. Four hours after infection, cell-culture supernatants were supplemented with the test compounds at the indicated concentrations. Control wells were treated with DMSO alone at a final concentration of 1% (diluent control). Infectious titers were determined 24 h after infection by a limiting dilution assay.[45]

#### 4.2.6. Cytotoxicity

Huh-7-Fluc cells were treated with the compounds at the indicated concentrations and incubated for 48 h at 37 °C, 5% $CO_2$. Subsequently, cells were lysed and assayed for luciferase activity as a surrogate for cell viability.

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.015.

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