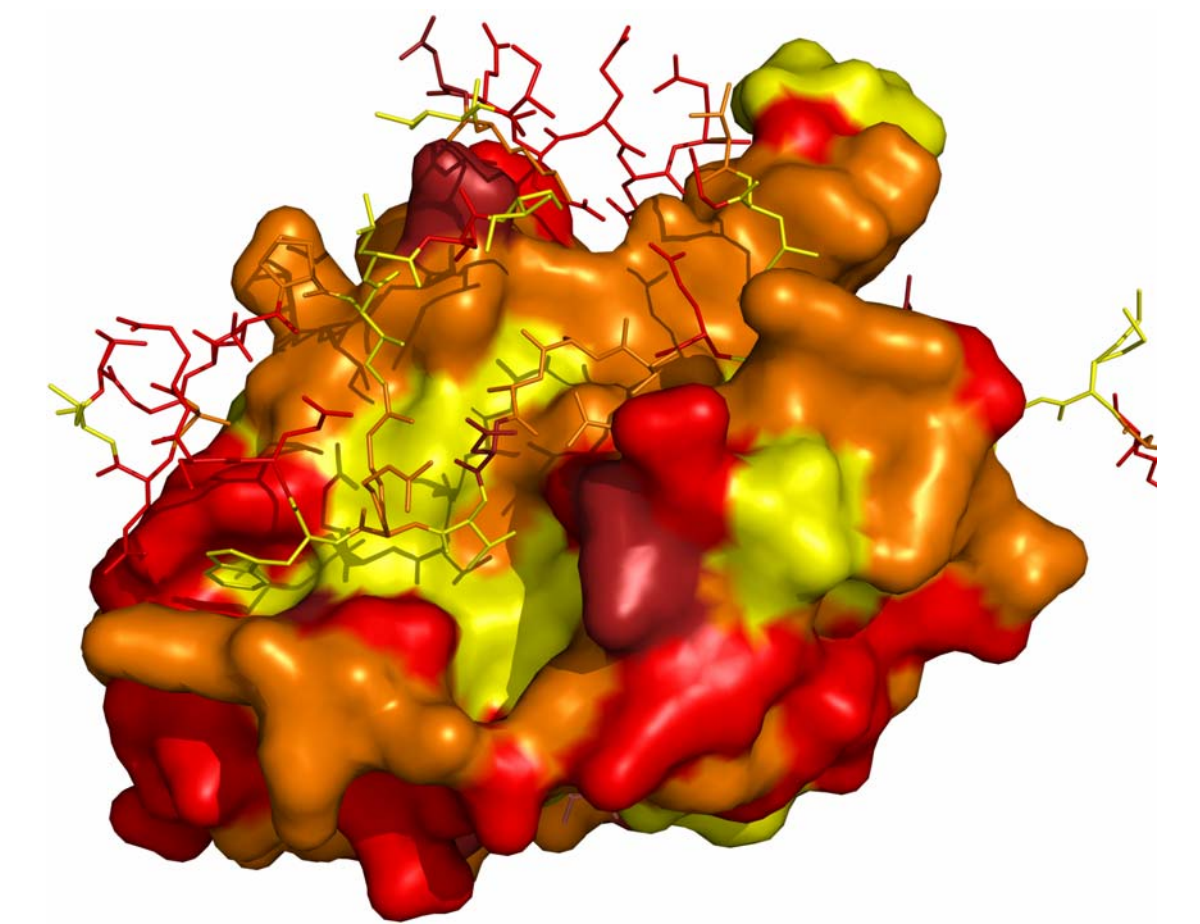




# Identification of Lead Compounds for Dengue NS3 Protease Inhibitors

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## Introduction

The Dengue (DEN) virus belongs to the genus *Flaviviridae* and is closely related to the Hepaci viruses (e.g. Hepatitis C virus). Recently, outbreaks of Dengue diseases as well as the spread of DEN virus and other flaviviruses like West Nile virus into the northern hemisphere have shown the necessity of the development of new antiviral inhibitors. Currently, there are no antiviral therapies available. The DEN virus is an enveloped virus containing a single stranded, positive sense 11 kb RNA genome. The genome encodes for a polyprotein precursor, which consists of three structural proteins (C, capsid; prM, membrane; E, envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The polyprotein precursor is processed by host proteases, signalase and furin and also by the viral protease. The virus encoded trypsin-like serine protease lies within the N-terminal end of the trifunctional NS3 protein. For full activity, the protease domain needs its cofactor, the 40 amino acid central domain of the NS2B protein.<sup>[1]</sup> Therefore the protease is also called NS2B-NS3. Because of its critical part in the viral life cycle, the NS2B-NS3 is an interesting target for drug development. For inhibitor development it is essential to have a stable and powerful assay system. In the following we present the optimized assay settings and also first results of the Endotherm and our in-house library screening.

## Optimization of Assay Conditions

Various fluorogenic and chromogenic assays have been developed for DEN protease. Some substrates contain a cleavable, fluorogenic or chromogenic moiety such as aminomethylcoumarin (AMC) or *p*-nitroaniline (pNA)<sup>[2]</sup> in the P 1' position of the cleavage sequence. In this work, we used an internally quenched anthranilamide/nitrotyrosine substrate<sup>[3]</sup> extending from position P4 to P1' and corresponding to the preferred cleavage site of the NS2B-NS3 protease.

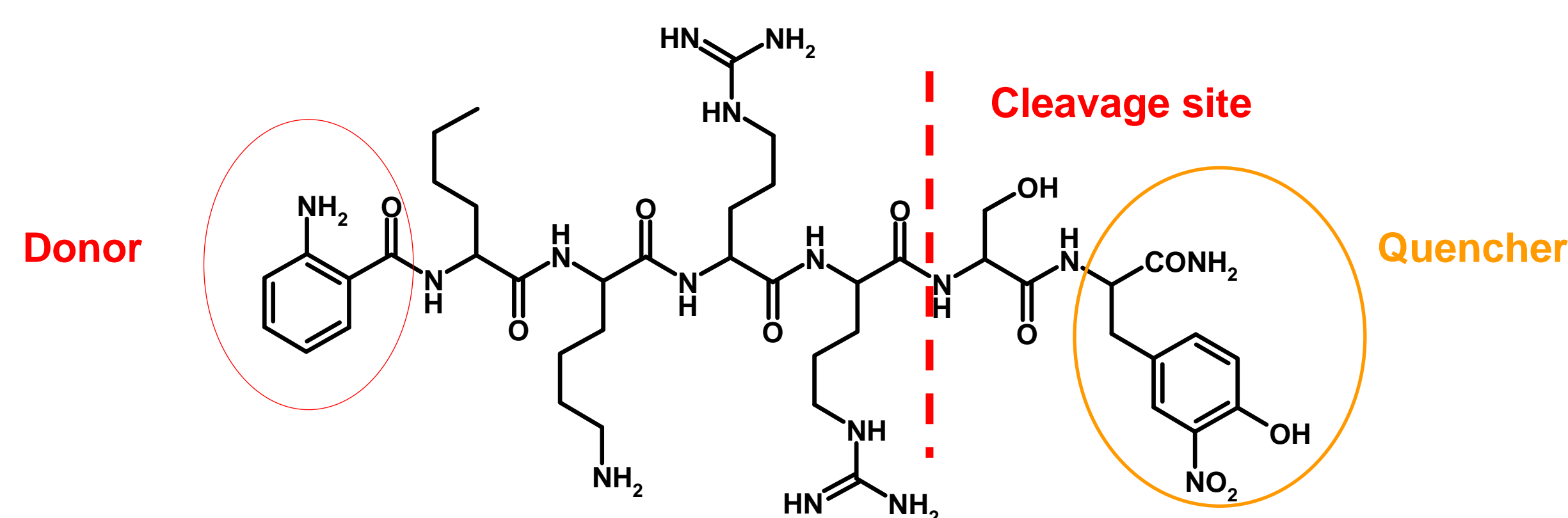
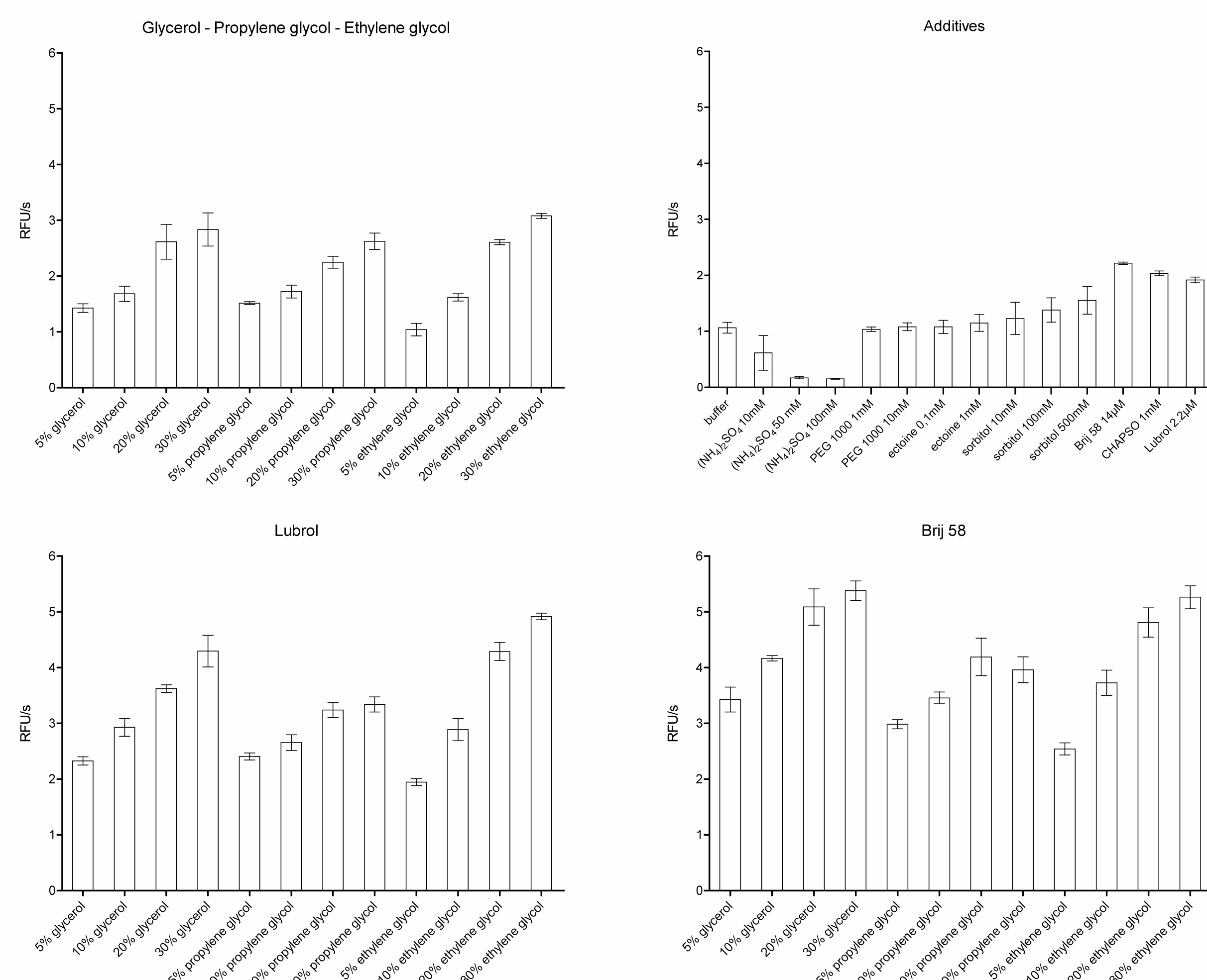


Fig. 1: Internally quenched peptide substrate of the dengue protease was synthesized by solid phase synthesis according to the Fmoc protocol. Aminobenzoic acid acts as donor group and 3- nitrotyrosine as quencher group. After cleavage an increasing fluorescence signal is detected at 420 nm.

Glycerol is an essential component of all published DEN protease assays and additionally ionic detergents as well as non-ionic detergents are often added to the assay mixture. However, glycerol increases the viscosity and can therefore cause inhomogeneous mixing of the assay solution. In consideration of a possible inhibitor with probably basic residues, it is conceivable that this inhibitor interacts not only with negative charges in the P1 and P2 pocket but also with the ionic groups of the detergent. Therefore we focused our search on other glycerol-like additives as well as non-ionic detergents to ensure a high activity of the DEN protease. For further investigations we chose the combination of 10% ethylene glycol and 14  $\mu$ M Brij 58 because this mixture ensures a high activity of the protease similar to the standard procedures with 20% glycerol and enjoys the advantage of easier handling. With the corresponding Z'-factor of 0.84, this assay setup is well suited for identifying inhibitors of the NS3 protease.



Tab. 1: In combination with the polyalcohol, Brij 58 and Lubrol are used in a concentration of 14  $\mu$ M and 2.2  $\mu$ M respectively.

## Promiscuous Inhibition

We also studied the potential of the new assay conditions to prevent promiscuous inhibition in comparison to the settings with glycerol. Curcumin and nordihydroguaiaretic acid were employed as exemplary promiscuous inhibitors. Promiscuous inhibitors act via an unspecific inhibition mode<sup>[4]</sup> and result in false-positive screening hits.

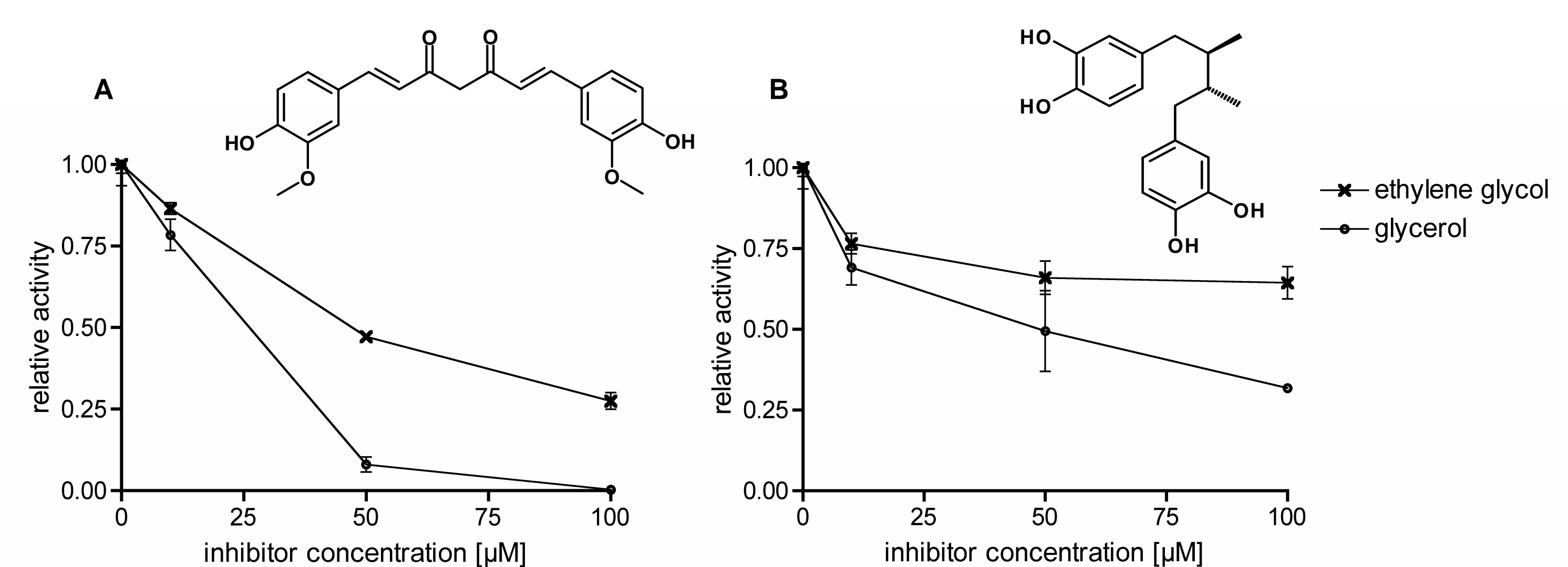
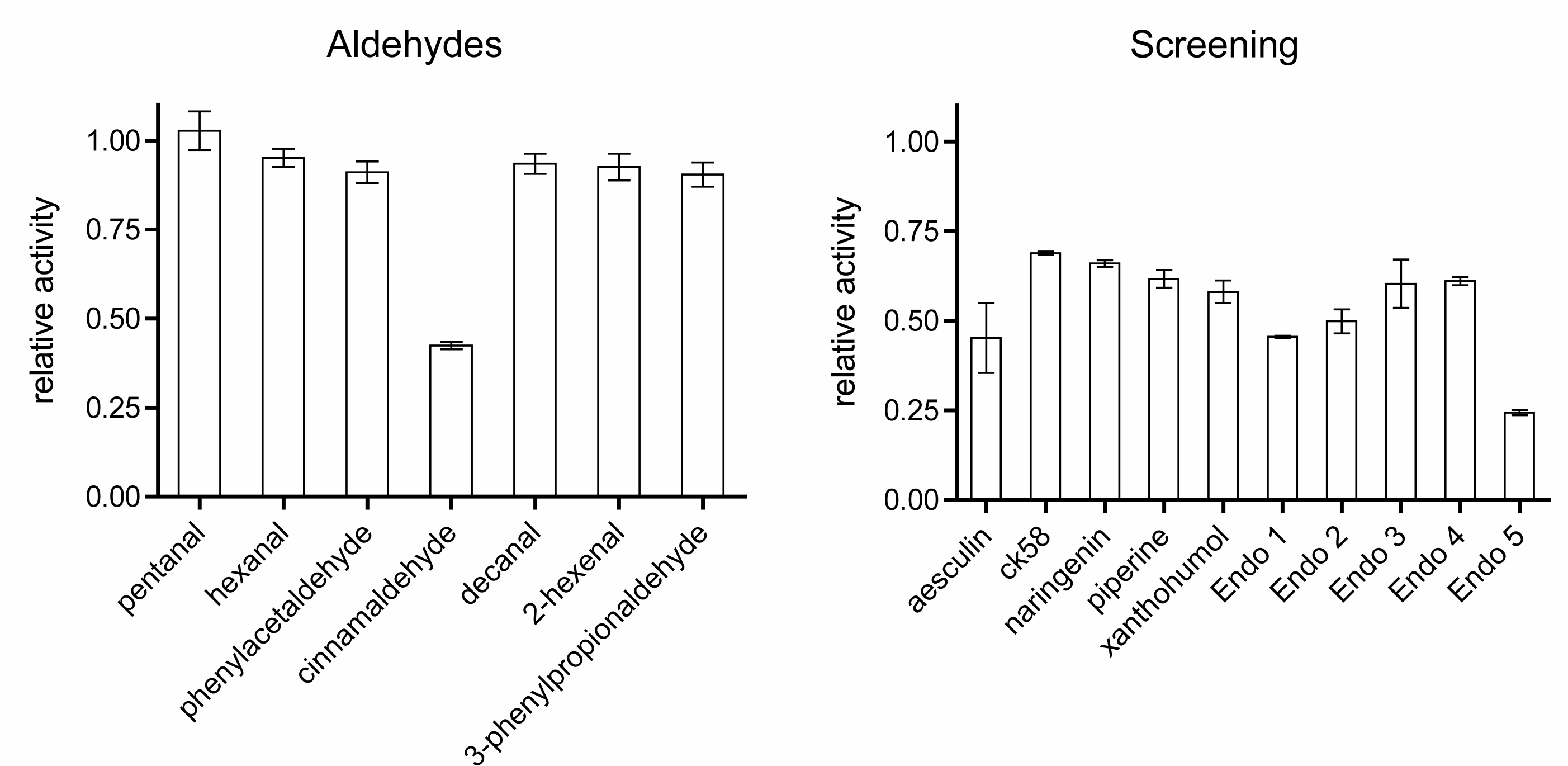


Fig. 2: Exemplary used promiscuous inhibitors; A) curcumin, B) nordihydroguaiaretic acid. The enzyme was preincubated for 15 min with the inhibitors. The assay was started by addition of the substrate.

## Aldehydes and library screening

Aldehydes and the activated serine of the catalytic triad form a hemiacetal which is an analog of the transition state for substrate hydrolysis. Therefore aldehydes are useful to determine the structural prerequisites of the P1 pocket of the NS3.



Tab. 2: All aldehydes are tested in a concentration of 100  $\mu$ M. The compounds of the library are tested in a concentration of 50  $\mu$ M. The enzyme was preincubated for 15 min with the inhibitors. The assay was started by addition of the substrate.

In order to find new potent inhibitors for the DEN protease the libraries of the Klein group and Endotherm GmbH, containing together about 500 highly diverse small molecules, were screened for activity against the protease.

All compounds were dissolved in DMSO and pre-added as duplicates in a 96-well plate to a final assay concentration of 50  $\mu$ M. Every measurement was performed twice to minimize fluctuations. In contrast to the assay conditions described above the library screening was performed without ethylene glycol. After comparing the initial velocities to an untreated control concentration studies of potent hits were conducted. This led to the identification of the (to our knowledge) most potent small-molecule inhibitor of DEN protease known to date ( $K_i = 12 \mu$ M).

## References

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