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# Influence of azide incorporation on binding affinity by small papain inhibitors

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ABSTRACT

In order to develop affinity-based biosensor platforms, appropriate ligands with a functional handle for immobilization onto a biosensor surface are required. To this end, a library of papain inhibitors was designed and synthesized, containing different azide linkers for subsequent immobilization by 'click' chemistry, in this particular case by copper-free, strain-promoted azide–alkyne cycloaddition (SPAAC). Furthermore, a molecular docking study was performed to obtain a better insight as to at which position such azide handles could be tolerated without affecting binding affinity. Although the azide moiety is small, in some cases its introduction strongly influenced the binding affinity. For one class of inhibitors a swapped binding mode was proposed to explain the results. In addition, a specific site for linker introduction was identified, which did not significantly affect the binding affinity.



## 1. Introduction

Biosensors, analytical devices to detect the presence of a specific biomolecule in a natural sample, have become an essential tool in medicinal, forensic and environmental analysis.[1,2] In general, a biosensor consists of two main components, namely a recognition element and a transducer element: the recognition element ensures detection of the target structure (the analyte) by a binding event, while the transducer element signals the binding to a device for read-out. To obtain a sensitive and selective biosensor, it is important to construct a recognition element that provides a stable interface for interaction between the target biomolecules and the immobilized binding partners (ligands), which are packed with optimal density. Conceptually, immobilization of a ligand to the surface of a biosensor requires the prior installment of a suitable linking moiety or handle. Although typically small, it must be taken into consideration that such a handle may compromise binding of the ligand to the analyte, for example due to steric bulk or unspecific hydrophobic interactions. Hence, a judicious choice of the handle, as well as the site of attachment on the ligand is required. The interface should furthermore be insensitive to non-specific binding interactions. Commonly established tools for immobilization include non-covalent streptavidin–biotin interaction, covalent attachment by amide coupling or so-called 'click' chemistry.[3] One powerful example of the latter approach is the strain-promoted azide–alkyne cycloaddition (SPAAC), that is, reaction between an azide and a strained alkyne, typically a cyclooctyne.[4–9] Although the linker is generally small, it can significantly compromise the requisite binding of the ligand to the analyte. This again requires a deliberate choice for a specific linker, as well as the site of attachment.

To investigate the influence of the incorporation of the azide moiety, a biosensor for exotic fruit allergen detection was used as a model system. We chose to work with papain, a cysteine protease present in papaya fruit, which has been studied previously in our group for enzymatic amide bond formation.[10–12] Furthermore, papain is a good model protein for exotic fruit allergens, since its active site shows great homology with the allergens found in, for example, kiwi and pineapple. Thus, a set of potential new inhibitors (**3**–**5**) for papain was designed and synthesized based on compounds which are known to inhibit the enzyme (Fig. 1, compounds **1** and **2**).[13]

The cysteine proteases of the papain superfamily display relatively broad substrate specificity. As a result, designing inhibitors that are highly selective for papain has proven to be a challenging task.[14] To date, a variety of inhibitors have been developed with

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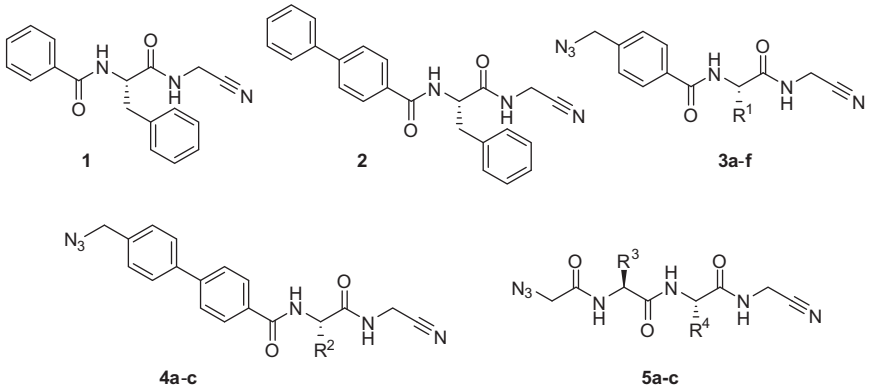


**Figure 1.** Peptidonitriles **1** and **2** are known papain inhibitors.[13] Compound **3**: **a** $R^1$ = iBu (Leu), **b** $R^1$ = *n*-Bu (Nle), **c** $R^1$ = Bn (Phe), **d** $R^1$ = $CH_2C_6H_4OH$ (Tyr), **e** $R^1$ = $(CH_2)_2Ph$ (*h*-Phe), **f** $R^1$ = $(CH_2)_2C_6H_4OH$ (*h*-Tyr); **4**: **a** $R^2$ = *n*-Bu (Leu), **b** $R^2$ = Bn (Phe), **c** $R^2$ = $CH_2C_6H_4OH$ (Tyr); **5**: **a** $R^3$ = iBu (Leu), $R^4$ = iBu (Leu); **b** $R^3$ = iBu (Leu), $R^4$ = Bn (Phe); **c** $R^3$ = Bn (Phe), $R^4$ = Bn (Phe).

different binding functionalities,[13,15–18] but for this research in particular nitrile-containing peptides were designed for interaction in the active site of papain. Crucial in the design was to preserve the inhibition properties by introducing the azide linker in a position where the binding to papain was not disturbed.

The designed inhibitors were synthesized and tested in an enzymatic assay. Furthermore, the binding mode was analyzed using molecular docking studies. The predicted binding modes of the inhibitors were reviewed, which led to a better understanding of the structure activity relationships.

## 2. Results and discussion

### 2.1. Synthesis

Synthesis of the ligands started with the incorporation of a nitrile functional group by coupling of aminoacetonitrile to the *N*-Boc-protected amino acids **6a–f** with the mixed anhydride method (Scheme 1). Thus, isobutyl chloroformate was added to the *N*-Boc-protected amino acid in the presence of freshly distilled triethylamine and THF as solvent, leading to compounds **7a–f** in moderate to good yields. Subsequent Boc deprotection was performed with TFA in THF/water (1:1 v/v) to afford compounds **8a–f** in (near) quantitative yields.

The next step involved the incorporation of an azido-containing benzoyl or 4-phenylbenzoyl moiety at the N-terminus. The required 4-azidomethylbenzoic acid (**9**) was readily obtained by nucleophilic substitution of 4-bromomethylbenzoic acid, using a literature procedure (not depicted).[19] The synthesis of biphenyl derivative **10**, however, proved to be more troublesome (Scheme 2). Synthesis in this case started with a palladium-catalyzed Suzuki coupling of 4-bromobenzyl alcohol (**11**) with boronic acid derivative **12**. Although a yield of only 24% was attained, sufficient material was obtained to proceed so that no further optimization was performed. The Suzuki coupling was followed by azide substitution of the hydroxyl group via the corresponding mesylate, leading to biphenyl ester **14** in 98% yield. Subsequent saponification provided the desired 4-[4-(azidomethyl)phenyl]benzoic acid derivative **10** in quantitative yield. Coupling of 4-azidomethylbenzoic acid (**9**) to compounds **8a–f** was performed under standard amide coupling conditions using EDC in the presence of HOBt (Scheme 1), providing the desired benzoylated compounds **3a–f** in moderate to good yields. Introduction of biphenyl derivative **10** was performed under the same conditions, however, products **4a**, **c** and **d** were obtained in low yields due to incomplete conversion and solubility issues during workup and purification. Nevertheless, sufficient material was obtained to perform binding studies with papain.

The synthesis of compounds **5a–c** commenced with peptide coupling of nitriles **8a** and **c** with Boc-protected leucine (**6a**) or phenylalanine (**6c**) to obtain compounds **15a–c** in moderate yield (Scheme 3). Boc deprotection was performed as described above, yielding compounds **16a–c** in quantitative yield. In this case, the azide moiety was incorporated by acylation of nitriles **16a–c** with azidoacetic acid, yielding azides **5a–c** in acceptable yields.

In the anticipated final situation as in a biosensor, where the ligands are immobilized on a surface, the azide will no longer exist as such, but will be converted into a triazole moiety. We therefore also evaluated the influence of such a transformation on binding affinity by synthesis of compounds **17** and **18**, involving SPAAC modification with two different strained cyclooctynes, namely BCN[8] and DIBAC.[5] Both triazoles **17**[9] and **18** were obtained in good yield starting from nitrile **3c** by stirring in MeOH in the presence of the cyclooctyne (Scheme 4). Due to the asymmetry in the cyclooctyne, **18** was obtained as an unseparable mixture of 1:1 triazole regioisomers.

### 2.2. Enzymatic evaluation

After successful synthesis of the library, dipeptide nitriles **3a–f**, **4a**, **c**, **d** and **5a–c** were evaluated for their papain affinity and the influence of the azide on the inhibitory properties. Our library was evaluated based on $IC_{50}$ values obtained from a competitive UV assay.

Schechter and Berger introduced comprehensive nomenclature to describe the interaction between a peptide (P), the enzyme's substrate, and the enzyme recognition subsites (S).[20] This nomenclature is exemplified in Figure 2 for leupeptin, a tripeptide natural ligand for papain featuring an aldehyde function at the C-terminal arginine.[21] The $S_1$ pocket of papain is poorly defined as becomes clear from Figure 2b (arginine side-chain on the far right) and the arginine from leupeptin is solvent-exposed. The $S_2$ pocket is a hydrophobic pocket defined by Val133, Ala160 and Pro68 and the $S_3$ binding pocket is formed by two aromatic groups, Tyr61 and Tyr67.

#### 2.2.1. Benzoyl ligands

The $IC_{50}$ value for benzoyl ligand **1** was 0.36 ± 0.06 μM, which remained nearly the same for the azidomethylated derivative ligand **3c** (Table 1, entries 1 and 5). As expected for benzoyl compound **3d**, replacing phenylalanine by tyrosine had little influence on the $IC_{50}$ value either (Table 1, entries 5 and 6). The homophenylalanine- and homotyrosine-derived ligands **3e** and **3f**, showed somewhat reduced binding affinity (Table 1, entries 7 and 8), as a potential indication that the side chains of homophenylalanine