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Discovery of Phenyl Alanine Derived Ketoamides Carrying Benzoyl Residues as Novel Calpain Inhibitors

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Abstract—Novel calpain inhibitors derived from phenyl alanine aldehydes or ketoamides carrying a benzoyl residue were prepared and evaluated for their biological potency. A brief structure–activity relationship elucidated the importance of *ortho*-substitutents in the benzoyl moiety. The most potent derivative, the ketoamide **19c**, exhibited a K_i of 6 nM and represents a novel class of reversible, highly potent and non-peptidic calpain inhibitors.

Calpains represent a class of intracellular cysteine proteases whose numbers have rapidly grown in recent years.[1] The well-known calpain I or μ-calpain is ubiquitously found in man and its physiological role is attributed to many intracellular processes such as the degradation of both cytoskeleton proteins and signal transduction proteins.[2] Excessive activation of calpains contributes to serious cellular damage or even cell death[3] and calpains are thought to be involved in the progress of a number of diseases.[4] Indeed, inhibition of calpain, in particular of μ-calpain, has revealed beneficial effects in experimental models, for example, on stroke,[5] myocardial infarction,[6] brain trauma,[7] multiple sclerosis[8] and muscular dystrophy.[9] For years, considerable efforts have been focused on calpain inhibitors as a novel therapeutic principle.[10]

A number of reversible and irreversible calpain inhibitors have been reported. Most of them are derived from peptides, but meanwhile nonpeptidic calpain inhibitors have been discovered.[11] However, no calpain inhibitors are reported to show pharmacodynamic and pharmacokinetic properties, which are required to prove the principle in animals and man. Their use is generally limited by poor selectivity, poor metabolic stability, low cellular penetration, poor kinetics or, depending on the envisaged therapeutic indication, low oral availability and low water solubility.[12]

Our aim was to identify new scaffolds for reversible calpain inhibitors in order to overcome the inadequate pharmacokinetic properties. Since most reported reversible calpain inhibitors are transiton state mimics and were derived from aldehydes and ketons, we also decided to use these moieties as a warhead in the envisaged inhibitors. Recently, we reported an approach demonstrating that substituted naphthoyl piperidines in the P^2/P^3 region which are derived from ketoamides as warheads are potent calpain inhibitors.[13] Proceeding these efforts we discovered phenyl alanine derivatives **2** carrying benzoyl residues as novel calpain inhibitors (see Fig. 1). The present paper focuses on the importance of *ortho*-substitution at the benzoyl residue moiety and the discovery of the stilbene moiety as substituent at the phenyl alanine scaffold such as **19c**.

The syntheses of the compounds are outlined in Figures 2 and 3.

The benzoic acids (e.g., **5**, **8**, and **13**) were either purchased or prepared according to the following routes, also shown in Figure 2. Alkylation of the phenols **4** with chloromethyl derivatives **3** gave the phenolic ethers **5**. The Heck reaction was used to prepare the stilbene and tolane derivatives. For example, the styrol **7** was added to *o*-bromo-benzoic carboxylate **6** in the presence of $Pd[(Ph_3P)_2Cl]_2$ at 100 °C to obtain a good yield of the ester **8a**. *O*-alkylated benzoic acid **13** was prepared from 4,4-dimethyl-2-phenyl-2-oxazolidine **9** in 3 steps. Compound **9** was deprotonated by *n*-BuLi at −78 °C and 2-naphthaldehyde **10** was added to produce the alcohol **11**. Hydrolysis of the oxazolidine **11** by HCl generated

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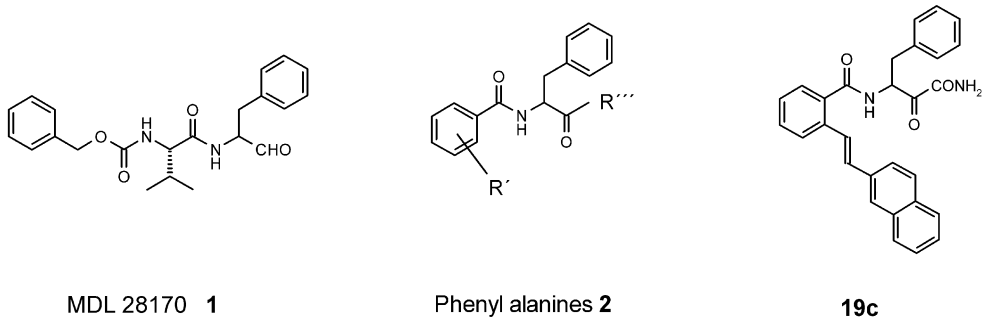


Figure 1.

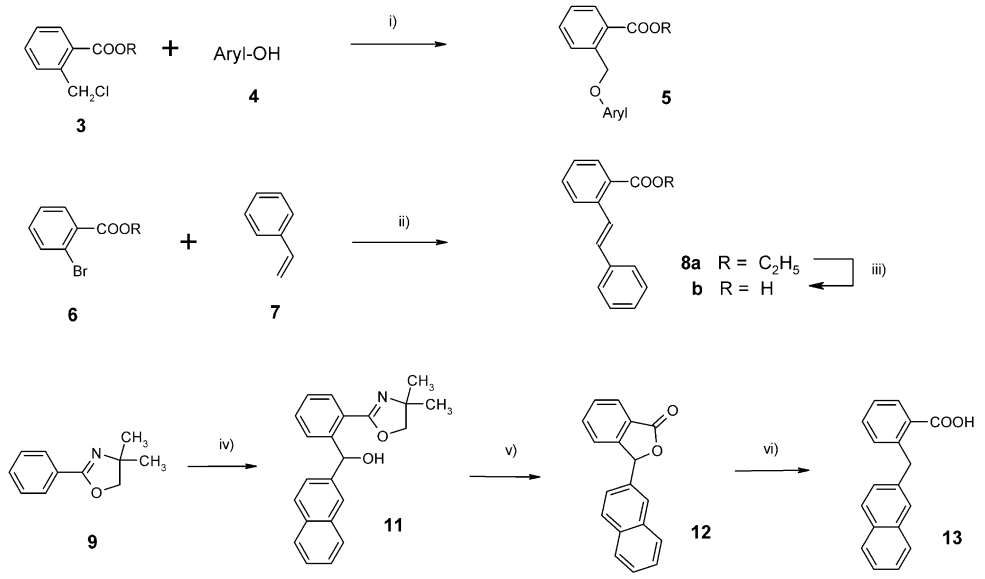


Figure 2. Routes for synthesis of the benzene carboxylates used as center building block. (i) DMF, K_2CO_3, 2. NaOH; (ii) $PdCl_2$, Ph_3P, DMF; (iii) NaOH; (iv) (1) *n*-Buli, −78 °C, (2) 2-naphthyl-carbaldehyde 10; (v) aq HCl, 80 °C; (vi) H_2, Pd/$BaSO_4$.

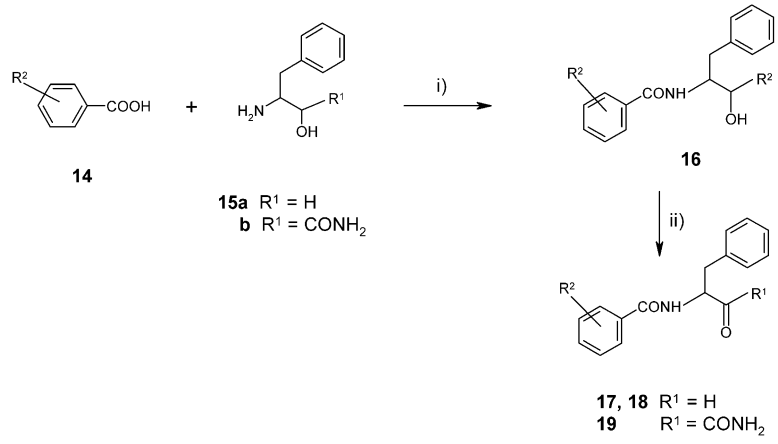


Figure 3. Routes for the preparation of aldehydes **17** and **18** as well as ketoamides **19**. (i) EDC, HOBT, rt; (ii) DMSO, py×SO_3, rt.

the carboxylate which immediately formed the lactone **12**. After that, **12** was hydrogenated in the presence of Pd/$BaSO_4$ to provide the desired carboxylate **13**. In a final step, all esters were hydrolyzed by diluted NaOH or KOH to give the carboxylates **14**.

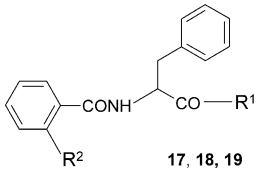
All carboxylates were coupled to either (2*S*)-2-amino-3-phenyl-1-propanol **15a** or to 3-amino-2-hydroxy-4-phenyl-butyramide **15b**[14] by convenient methods (e.g., EDC, HOBT) to obtain the amides **16**. Finally, these amides **16**, which carry the alcohol moiety, were oxi-

dized by DMSO/py·SO_3 at ambient temperature to give either the aldehydes **17** and **18** or the ketoamides **19**.

The biological activities of the prepared compounds **17**, **18** and **19** were evaluated in a common enzyme assay using human μ-calpain isolated from erythrocytes and Suc-Leu-Tyr-AMC as the fluorogenic substrate.[15] The inhibition of cathepsin B and cathepsin L was tested in corresponding assays using commercially available enzymes.[16] The results are summarized in Tables 1 and 2.

MDL 28170 **1** represents a small-peptidic aldehyde-derived calpain inhibitor and is widely used as a tool compound. According to the reported structure–activity relationship the Z-Val moiety contributes considerably to the inhibitory potency.[17a] The replacement of this moiety with a benzoyl residue resulted in a more than 90-fold drop of affinity and the K_i is roughly 1 μM (see **17**). Recently it was reported that halogen substitution at this benzoyl residue may be favorable.[17b] Therefore, we decided to employ the phenyl ring as the spacer carrying substituents selected to interact with the P^2/P^3 region of the enzyme. Superimposition of MDL 28170 **1** and **17** by molecular modeling revealed that a substitution of **17** by benzoyl residues might be favorable in the *ortho*-position. Indeed, the *ortho*-benzophenone **18a** displayed calpain inhibition ($K_i = 0.23$ μM) but this was only slightly superior to **17**.

Table 1. Synthesized compounds and their results in a common enzyme inhibition assay using purified human calpain and Suc-Leu-Tyr-AMC as the substrate



17, **18**, **19**

	R^1	R^2	Calpain K_i/μM
17	H	H	1.08
18a	H	Phenyl-CO	0.23
18b	H	Phenyl	0.09
18c	H	Phenyl-CH_2	0.37
18d	H	Phenyl-CH_2CH_2	0.28
18e	H	2-Naphthyl-CH_2	0.20
18f	H	Phenyl-O	0.05
18g	H	Phenyl-OCH_2	0.45
18h	H	2-Naphthyl-CH_2O	0.05
18i	H	E-Phenyl-CH = CH	0.14
18j	H	Phenyl-C≡C	0.45
18k	H	E-(3,4-MeO)$_2$-phenyl-CH = CH	0.04
18l	H	E-2-Naphthyl-CH = CH	0.015
19a	$CONH_2$	Phenyl	0.04
19b	$CONH_2$	E-(3,4-MeO)$_2$-phenyl-CH = CH	0.08
19c	$CONH_2$	E-2-Naphthyl-CH = CH	0.006

To verify the relevance of the carbonyl group within the bridge as the hydrogen bond acceptor we looked for the corresponding methylene derivative **18c** and, surprisingly, **18c** retained calpain inhibition ($K_i = 0.37$ μM). To elucidate this unexpected result we addressed additional efforts on variations of both the bridge and the distal aromatic ring.

The biphenyl **18b** demonstrates the importance of a distal aromatic ring. Merely a phenyl ring in the *ortho*-position at the benzoyl moiety (**18b**) resulted in a 10-fold increase in calpain inhibition (compared to **17**) and **18b** had a K_i of 90 nM. Incorporation of a methylene bridge (**18c**, **18e**) or an ethylene bridge (**18d**) into the biphenyl moiety slightly diminished the inhibition potency. Insertion of a heteroatom, such as an oxygen atom, into the bridge produced controversial effects in potency. The biphenylether **18f** showed a K_i of 50 nM and represented one of the most potent inhibitors in the present series. On the other hand, prolongation of the bridge with a methylene group (see **18g**, **18h**), favoring the flexibility of the side chain, was unfavorable which is shown by the K_i of 0.45 μM. A bulky naphthalene residue as the distal aromate was well tolerated (see **18e** and **18h**) indicating the considerable size of this lipophilic cave at the enzyme-binding site.

In order to optimize the conformation of the central and distal aromate, we examined the stilbene **18i** and the acetylene derivative **18j**, both representing more rigid structures. Both the E-stilbene **18i** and the acetylene **18j** were as potent as the alkyl derivative **18d**, which may raise doubts as to the significance of such rigidity. On the contrary, substitution of the distal aromate opened up additional opportunities for optimizations. The stilbene **18k**, which carries two methoxy groups at the distal phenyl ring, disclosed a K_i of 40 nM. Furthermore, the naphthalene **18l** exhibited a K_i of 15 nM and is roughly one and two orders of magnitude more potent than the stilbene **18i** and the alkyl derivative **18d**, respectively.

Since all of the above inhibitors were derived from aldehydes, which were used as a warhead to the active center of the calpain enzyme, we looked for more stable derivatives and selected ketoamides as the aldehyde surrogate.[18] Ketoamides corresponding to three potent aldehydes, **18b**, **18k** and **18l** were prepared. Interestingly, all three ketoamides **19a**, **19b** and **19c** retained the potency of the corresponding aldehydes within a two-fold range in calpain inhibition. In particular, the

Table 2. Results of selected compounds in enzyme inhibition using commercially available cathepsin B and L and Suc-Leu-Tyr-AMC as the substrate. Inhibition of the tyrosine kinase pp60src was determined in human thrombocytes[19]

	Calpain (K_i/nM)	Cathepsin B (K_i/nM)	Cathepsin L (K_i/nM)	pp60src (IC_{50}/μM)
19c	6	99	6100	1.4
MDL 28170 **1**	15	nd[a]	nd[a]	0.7

[a]Not determined.

naphthalene **19c** displayed a K_i of 6 nM and represents one of the most potent calpain inhibitors, which are not derived from aldehydes, reported so far.

Ketoamides are supposed to represent reversible enzyme inhibitors.[18] Indeed, according to its enzyme kinetics, the ketoamide derived inhibitor **19c** represents a reversible calpain inhibitor (data not shown). Many of the reported calpain inhibitors were found to be lacking owing to poor or modest selectivity versus related cysteine proteases such as cathepsines B and L.[11b] Accordingly, **19c** inhibited cathepsin B and cathepsin L with K_i's of 99 and 6100 nM, respectively, demonstrating moderate selectivity versus cathepsin B (15-fold), whereas selectivity versus cathepsin L is excellent (1000-fold). Compound **19c** did not block non-cysteine proteases even up to higher μM concentrations (data not shown). In comparison, the commonly used tool compound for calpain inhibition, MDL 28170 **1**, exhibited no selectivity versus these cysteine proteases and blocks calpain as well as cathepsines B and L at low nanomolaric concentrations.[19]

To determine the penetration of cellular membranes we evaluated the inhibition of the calpain-mediated degradation of the tyrosine kinase pp60src in human thromobocytes.[20] Both compounds, MDL 28170 **1** and **19c**, blocked pp60src degradation at low μM-concentrations with an IC_{50} of 0.7 and 1.4 μM, respectively.

In summary, we used the phenyl alanine carrying a benzoyl residue **17**, which showed only poor calpain inhibition, as the starting point for our investigations. Optimization of substitution in the *ortho*-position in the benzoyl moiety resulted in a novel class of potent calpain inhibitors. The most active inhibitor in this series, the naphthyl derivative **19c**, represents a reversible, cell penetrating inhibitor which also displays selectivity versus cathepsin B and L.

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