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Studies on aromatic compounds: inhibition of calpain I by biphenyl derivatives and peptide-biphenyl hybrids ☆

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Dedicated to the memory of our colleague Dr. J. C. del Amo and to all the other victims of the terrorist attack. (Madrid, 11th March 2004)

Abstract—With the objective to understand structural features responsible for the biological activity, novel nonelectrophilic biphenyl derivatives and peptide-biphenyl hybrids have been synthesized and evaluated as calpain I inhibitors. The preliminary results indicate that the presence of additional aromatic rings (besides the biphenyl system) makes these compounds potent calpain inhibitors with IC_{50} values in the nanomolar range.

Calpain I (μ-calpain) is an ubiquitous enzyme of the calpain family of cysteine proteases that is activated by micromolar amounts of Ca^{2+} ion.[1,2] Although its natural substrate is not yet known, this enzyme has been shown to hydrolyze a variety of proteins including cytoskeletal proteins, enzymes involved in signal transduction, membrane receptors and transcription factors.[3] The overactivation of calpain is implicated in a variety of pathophysiological conditions, including Alzheimer's disease, muscular dystrophy, multiple sclerosis, arthritis, cataract and other degenerative and ageing-related diseases.[4]

Although calpain I is a potential target for therapy, the field is underdeveloped; the reported calpain inhibitors are still few as compared to other proteases.[5] Most of the reported examples are based on similar strategies as in the design of other protease inhibitors,[6] that is to exploit electrophilic functionality able to react with the essential thiol group at the active centre of calpain. Accordingly, aldehydes,[7] 1,2-dicarbonyl compounds,[8] α-hetero-substituted ketones[9] and α,β-unsaturated carbonyl compounds,[10,11] frequently incorporated in short peptide chains, have been reported as calpain inhibitors. However this strategy has resulted in poor selectivity: many of the reported calpain inactivators are also inhibitors of other proteases. On the other hand, the distinctive mechanistic feature of calpain (i.e., activation by Ca^{2+}) has not been extensively used as a basis for the design.[12] On analyzing the structure of calpain inhibitors, it is observed the presence of aromatic rings (both in the main peptide chain and as substituents) in many synthetic inhibitors. Since aromatic compounds are known to interact with a variety of chemical species,[13] including Ca^{2+} ion,[14] we can use this structural feature for the design of nonelectrophilic calpain inhibitors.[15]

Our group has investigated aromatic compounds,[16] peptide derivatives[11,17–19] and calpain inhibitors[11,19] for several years. During the course of these three interests, we have recently synthesized compounds of type **A** (Fig. 1), termed *peptide-biphenyl hybrids*, where peptide chains are linked to the positions 2- and 2′- of the biphenyl system.[18] Our initial screening has shown that some peptide-biphenyl hybrids **A** are able to inhibit calpain I. The preliminary results indicate that the hybrids derived from aromatic amino acids, such as **1**–**3**, are potent and selective calpain I inhibitors, having IC_{50} values in the nanomolar scale.[19] This activity may arise from the

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☆ (a) Taken in part from the Ph. D. thesis of AC (Complutense University, Madrid, 2003) and the projected Ph. D. thesis of AM and MA. (b) A Spanish patent application (# 200301125, 14th May 2003) is pending.
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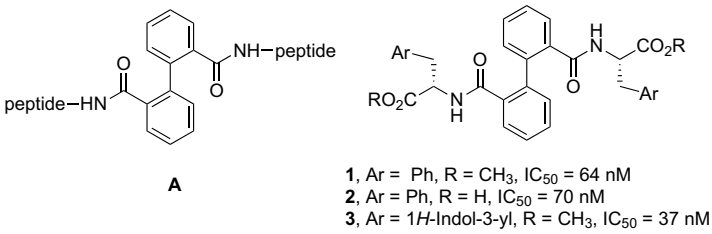


Figure 1. Generic structure (**A**) of peptide-biphenyl hybrids and representative compounds (**1**–**3**) along with their IC_{50} values as calpain inhibitors.

capacity of compounds **1**–**3** to interact with Ca^{2+} ion,[19c] hampering the activation of calpain. On continuing this research, we report herein further examples on the inhibition of calpain I by the peptide-biphenyl hybrids (**4**–**18**, Fig. 2) that shed light on the influence of the amino acid/peptide structures on the biological activity.

The synthesis of compounds **4**–**18** (Fig. 2) depends on if the two chains at the positions 2- and 2′- are identical or not. If the molecule possesses C_2-symmetry, the peptide-biphenyl hybrid was prepared by the reaction of biphenyl-2,2′-dicarboxylic acid (**19**) with excess of the corresponding *N*-unprotected amino acid or peptide

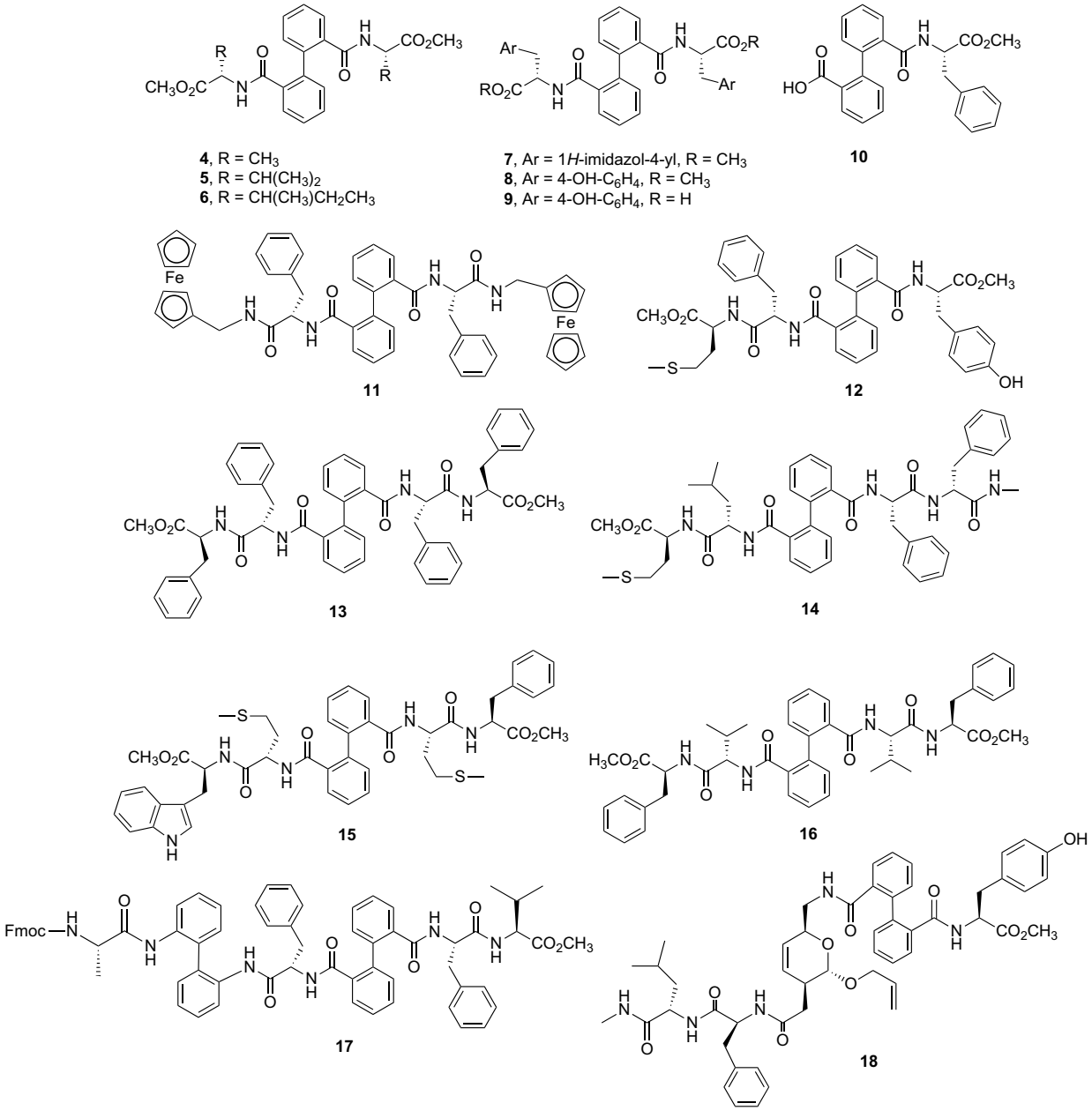
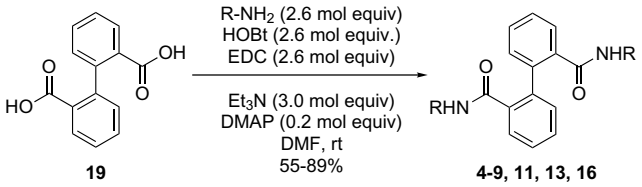


Figure 2. Structure of peptide-biphenyl hybrids essayed as calpain I inhibitors (see Table 1).



Scheme 1.

derivative according to standard peptide coupling methodology (EDC/HOBt/DMAP; Scheme 1). Following this method, the peptide-biphenyl hybrids **4**–**8**, **11**, **13** and **17** were synthesized in 79–94% yields.[20,21] The symmetric diacid **9** was prepared in quantitative yield by hydrolysis ($LiOH/H_2O$/THF) of the diester **8**.

If the substituents at positions 2- and 2′- of the peptide-biphenyl hybrid are different, the synthesis was carried out sequentially from the anhydride **20** (Scheme 2). First reaction with a slight excess of an *N*-unprotected amino acid/peptide to give mono-amides of type **B** (such as **10**, Fig. 2) and a second reaction with another *N*-unprotected amino acid/peptide in the presence of EDC/HOBt/DMAP. Following this method, the hybrids **12**, **14**, **15**, **17** and **18** were prepared in higher than 70% overall yield.[21]

Compounds **4**–**18** were tested as calpain inhibitors using a standard spectrofluorimetric method[22] with labelled casein as substrate and calpain I from porcine erythrocytes as enzyme. The results are indicated in Table 1. The peptide aldehyde *Z*-Leu-Phe–H (**21**), that is one of the most potent calpain inhibitors reported up to date (K_i in the order of 10 nM for human calpain, using fluorescent *N*-succinyl-dipeptides as substrates),[7,23] was also tested under the same experimental conditions. This result allows the direct comparison of the inhibitory capacity of the peptide-biphenyl hybrids with reported calpain inhibitors.

The results indicated in Table 1 permit to draw some conclusions:

(a) In our experiments, the known peptide aldehyde **21** has an IC_{50} value of 240 nM. The discrepancy with the reported result ($K_i \approx 10$ nM) is not surprising since two different parameters and two different substrates are used to gauge the biological activity; additionally, the sources of the enzyme are different in the reported examples (human) and in the current paper (porcine). Given that **21** is recognized as a very potent calpain inhibitor, we can conclude that the compounds having IC_{50} values lower than 240 nM are very active inhibitors.

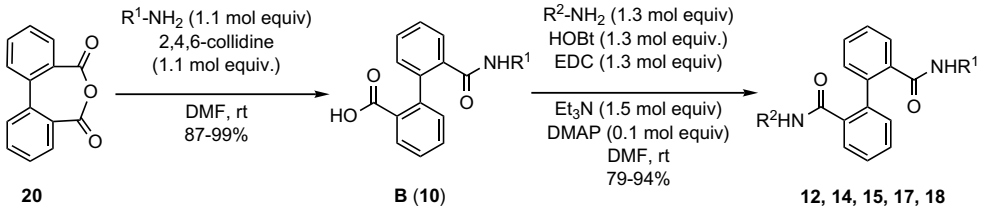
Table 1. Results of the inhibition of calpain I by peptide-biphenyl hybrids **4**–**18** and the peptide aldehyde **21**

Compound	IC_{50}
4	Noninhibitor
5	7 μM
6	Noninhibitor
7	Noninhibitor
8	Noninhibitor
9	200 μM
10	Noninhibitor
11	24 μM
12	Weak inhibitor
13	98 nM
14	Weak inhibitor
15	77 μM
16	24 nM
17	41 μM
18	116 μM
21	240 nM

(b) Most of the peptide-biphenyl hybrids with aliphatic amino acids do not inhibit calpain, as shown by the alanine (**4**) and isoleucine (**6**) derivatives. Other peptide-biphenyl hybrids with longer aliphatic peptide sequences at 2- and 2′- of the biphenyl system (i.e., Ala-Leu–OMe, D-Ala-D-Leu–OMe and Val-Ala-$Asp(OMe)_2$ derivatives, structures not shown) were not active as calpain inhibitors.[24] However, the valine derivative methyl ester **5** is a moderate inhibitor of calpain I.

(c) The comparison of the biological activities of compounds **1**, **3**, **7**,[25] and **8** show the striking dependence of the biological activity on the nature of the aromatic amino acid: the derivatives of phenylalanine **1** and of tryptophan **3** are very potent calpain I inhibitors (Fig. 1), while the analogues derived from histidine (**7**) and tyrosine (**8**) are inactive. The diacid **9** derived from tyrosine is a weak inhibitor.

(d) Compounds **10**–**18** are biphenyl derivatives with at least one phenylalanine residue, which can be considered as structural variants from the parent compound **1**. The acid **10** (inactive) is an analogue of **1** with a truncated chain and the diamide **11** (moderate activity), derived from ferrocene,[26] is a *C*-end modified derivative of **1** and **2**. The peptide-biphenyl hybrids **12**–**17** are analogues of **1** with extended peptide chains,[27] and they show the influence of the position of the phenylalanine residue, the nature of the other amino acids and the length of the peptide chain on the activity. It is found that the hybrids **12** and **14** are weak inhibitors,[28] and compounds **15**



Scheme 2.

and **17** are moderate inhibitors. On the other hand, the hybrids **13** and **16**, which have phenylalanine residues at each second position of the peptide chains, are excellent inhibitors with IC_{50} value in the nanomolar range. The compound **18**, which is a structurally more elaborated peptide-biphenyl hybrid having the aromatic amino acids separated by the biphenyl residue and a densely functionalized 3,6-dihydro-2*H*-pyran,[29] is a moderate inhibitor.

To conclude, the present paper reports further results on qualitative structure–activity relationship of peptide-biphenyl hybrids as calpain inhibitors. Although we have not performed a systematic structural study,[30] we have found that the most potent inhibitors are those having aromatic rings at the side chain, especially the derivatives of phenylalanine. Less efficient calpain inhibitors are the derivatives of tryptophan (i.e., **15**), whereas the derivatives of tyrosine and of histidine are either weak or noninhibitors. In contrast, the peptide-biphenyl hybrids derived from aliphatic amino acids are not inhibitors,[24] although the valine derivative **5** is an exception.[31] The combination of valine and phenylalanine residues in the peptide chains (i.e., the hybrid **16**) provides one of the most potent calpain inhibitors reported up to date (10 times more potent that the peptide aldehyde **21**). Whereas we can hypothesize that the inhibition of calpain by compounds bearing several aromatic rings can be due to its ability to coordinate Ca^{2+},[19c] the present results show that this is not the only cause, and a process of recognition of the inhibitor by the enzyme is also operating (as observed from the dependence of the biological activity on the sequence). Finally, it is worthwhile to remark that these compounds are some of the few calpain inhibitors without a highly-reactive electrophilic functionality, what might provide selectivity versus other proteases. The present results also illustrate the use of the biphenyl fragment as a privileged structure for the discovery of pharmaceuticals.[32] Work is in progress to further develop peptide-biphenyl hybrids as calpain inhibitors as well as to fully understand the relationship between structure and activity.[30]

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20. (a) Since that the purpose of our research was to investigate the properties of the target molecules we have not made any attempt to optimize the synthetic procedures. However, with the aim to gain some insight on the synthetic methodology for these compounds, we have prepared some peptide-biphenyl hybrids by more than one method. Although the more frequent reactions are those indicated in Schemes 1 and 2, other methods include: (a) The reaction of 1,1′-biphenyl-2,2′-dicarbonyl dichloride with 2.4 molar equiv of *N*-unprotected amino acid/peptide in the presence of Et_3N (60–90% yield)[18]; (b) The phenylalanine derivatives **11** and **13** were also synthesized from the acid **2** and the corresponding amine (HOBt/EDC/DMAP) in over 90% yield; (c) Nájera's methods (HOTT/DMF; see: Nájera, C.; Bailén, M. A.; Chinchilla, R.; Dodsworth, D. *J. Org. Chem.* **1999**, *64*, 8936) was quite suitable for the synthesis of peptide-biphenyl hybrids having valine at the *N*-end of the peptide chain (70–90% yield); we thank Professor Nájera for a generous gift of S-(1-oxido-2-pyridinyl)-1,1,3,3-teramethyluronium hexafluorophosphate (HOTT).
21. All the new compounds gave satisfactory analytical (C, H, N, ±0.3%) and spectroscopic (^{1}H NMR, ^{13}C NMR, IR, MS) data. (b) The synthesis of compound **12** illustrates the two main procedures used for the synthesis of the peptide-biphenyl hybrids. The experimental details are as follows. A solution of Tyr–OMe (1.13 g, 4.90 mmol) and 2,4,6-collidine (0.65 mL, 4.90 mmol) in dry DMF (12.5 mL) were dropwise added to a solution of **20** (1.0 g, 4.46 mmol) in dry DMF (12.5 mL) under argon atmosphere. The mixture was stirred at room temperature for 15 h. Then, the solvent was removed under reduced pressure, the residue was taken up with EtOAc and washed with 5% aqueous HCl (twice). The organic phase was dried ($MgSO_4$). After filtration and solvent removal, the residue was purified by crystallization from CH_2Cl_2 to give pure acid (1.8 g, 97 % yield.) of type **B** (with R^1 corresponding to Tyr–OMe). A mixture of the intermediate acid (800 mg, 1.91 mmol) and the trifluoroacetate salt of Phe-Cys–OMe (1.05 g, 1.91 mmol) in dry DMF (17 mL) was sequentially treated with HOBt (335 mg, 2.48 mmol), Et_3N (0.40 mL, 2.85 mmol), EDC (495 mg, 2.48 mmol) and DMAP (24 mg, 0.16 mmol) at room temperature under argon atmosphere. The mixture was stirred at room temperature for 32 h. Then, the solvent was removed under reduced pressure; the residue was taken up with EtOAc and washed with 5% aqueous HCl, water, saturated aqueous $NaHCO_3$ and brine. The organic phase was dried ($MgSO_4$). After filtration and solvent removal, the residue was purified by chromatography (1:1 to 1:4 gradient of hexane/EtOAc) to give **12** as a white solid (1.28 g, 94% yield).
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