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Synthesis of a small library of diketopiperazines as potential inhibitors of calpain

Yibin Zeng,[a,b] Qingshan Li,[a] Robert P. Hanzlik[a] and Jeffrey Aubé[a,b,*]

[a]*Department of Medicinal Chemistry, 1251 Wescoe Hall Drive, University of Kansas, Lawrence, KS 66045-7582, USA*
[b]*KU Chemical Methodology and Library Development Center of Excellence, 1501 Wakarusa Drive, University of Kansas, Lawrence, KS 66047, USA*

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Abstract—A small library of 2,5-diketopiperazines based on previously reported calpain inhibitors was synthesized. In addition, a concise total synthesis of the structurally related natural product phevalin (**2**) was accomplished. Despite literature reports that some of the compounds prepared were calpain inhibitors, none of the library members were found to have significant activity against recombinant human calpain I.

Calpains are a class of intracellular cytoplasmic non-lysosomal cysteine proteases expressed ubiquitously in mammalian cells. Among the 16 kinds of calpain identified thus far, μ-calpain (or calpain I) and m-calpain (or calpain II) are the two most thoroughly studied calpain isoforms. The two enzymes differ in their sensitivity to activation by calcium ions. Thus, calpain I is sensitive to activation by micromolar concentrations of calcium, whereas calpain II responds only to millimolar calcium concentrations.[1–3] Both isoforms are heterodimers made up of identical 30 kDa subunits but different 80 kDa subunits. Overactivation of calpain has been implicated in many pathological conditions such as stroke,[4] myocardial infarction,[4] Alzheimer's disease,[5] Parkinson's disease[5] and cancer.[6] Accordingly, selective inhibitors of calpain are of interest as pharmacological probes and as potential therapeutics.[7–9]

As part of our ongoing study of cysteine proteases and their associated medicinal chemistry, we were interested in the identification of selective non-peptide inhibitors of calpain. Several groups have reported small molecules having modest (micromolar) activity as calpain inhibitors (Fig. 1). These include the α-mercaptoacrylic acid PD 150606 (which binds remotely to the catalytic site of calpain)[10] and the natural products phevalin (**2**)[11] and penicillide.[12] The diketopiperazine dimer of *N*-methyltyrosine, **1**, has also been reported to inhibit calpain,[13] but this report has recently been questioned.[14]

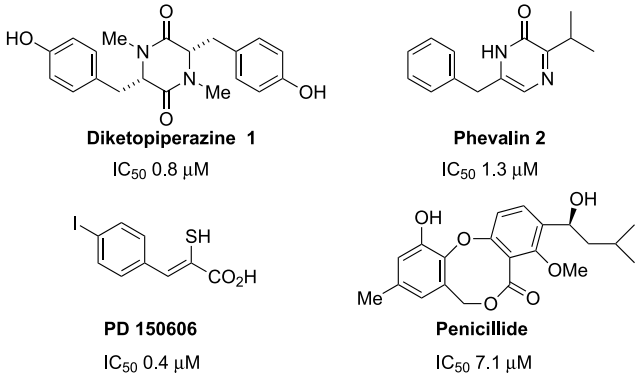

Figure 1. Some reported non-peptide inhibitors of calpain.

Despite some structural and/or binding site differences between these non-peptide calpain inhibitors, there are also some similarities. Specifically, the presence of an aromatic group two or three atoms away from either another aromatic group or a hydrogen bonding substituent suggests a possible motif for inhibitor design. In addition, the prominence of aromatic moieties in these molecules is consistent with the observed preference of calpain for hydrophobic residues in small peptide substrates and inhibitors.[7]

Keywords: Calpain; Peptidomimetic; Library diketopiperazine.
* Corresponding author. Tel.: +1 785 864 4496; fax: +1 785 864 5326; e-mail: jaube@ku.edu

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Taking into consideration that both diketopiperazine and phevalin are derived from 2,5-disubstituted piperazines (Fig. 2), we thought it possible to employ this common element as a scaffold for inhibitor synthesis. Thus, a small library of diketopiperazines (Fig. 3) based on compound **1** was synthesized. The library was specifically devised to examine the effects of N–Me versus N–H substitution (**1**, **4**, **6**, **8**, **10** vs **3**, **5**, **7**, **9**, **11**), stereochemistry (**14**, **15** vs **24**, **25**) and the hydrophobicity/hydrophilicity of the side chain, on calpain inhibition (e.g., **22**, **23**, **29**, **30** vs **17**–**21**).

Since no synthesis of phevalin (**2**) has been reported, we also devised a concise total synthesis to obtain this natural product. Because of conflicting reports[13,14] on its activity, the previously reported cyclic dimer of *N*-methyltyrosine (**1**) was also prepared. The peptide aldehyde Boc-Val-Phe-H (**33**) was prepared for use as a positive control in inhibition assays. The related aldehyde *Z*-Val-Phe-H (MDL 28170) is reported to be a slow tight-binding inhibitor of rat erythrocyte calpain (K_i = 10 nM).[15]

There are a number of reported routes for the parallel synthesis of diketopiperazines.[16] In the course of methodology verification, we found that a one-pot cyclization process of the dipeptide precursors could be deployed in a straightforward parallel synthesis protocol.[17] Thus the crude dipeptides were directly subjected to treatment with TFA to release the Boc group and allow subsequent ring closure (Scheme 1). The diketopiperazines were obtained in 35–60% yield over three steps. Twenty-four diketopiperazines were prepared in this manner. When needed, methylation was carried out using NaH/MeI in DMF,[18] and debenzylation by catalytic hydrogenation.[19]

A short total synthesis of phevalin[20] proceeded in a similar fashion described for the diketopiperazines except that an additional reduction of the Boc-Val-Phe-OMe to the corresponding aldehydes was carried out before Boc-deprotection and cyclization (Scheme 2). The in situ oxidation during the cyclization was a surprise to us.

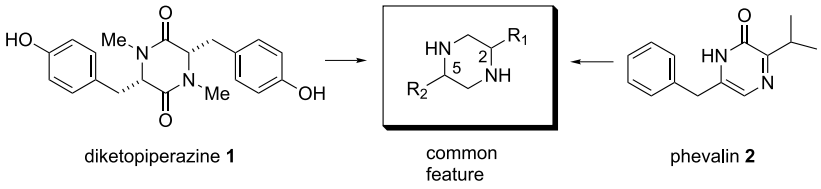


Figure 2. Structural similarity between diketopiperazine and phevalin.

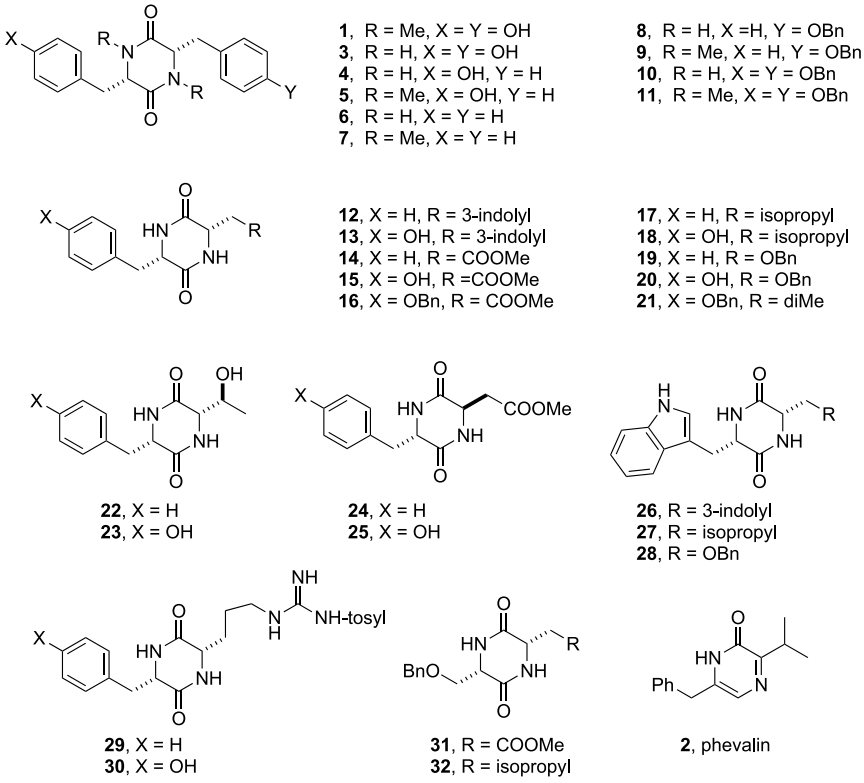
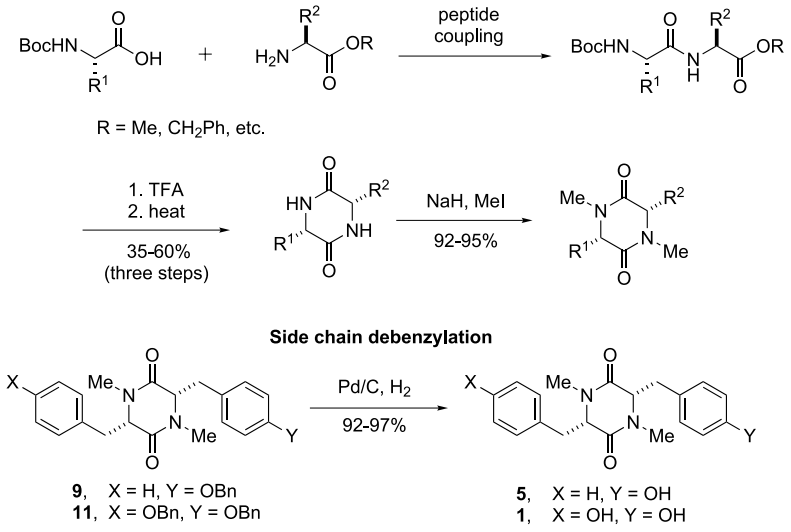
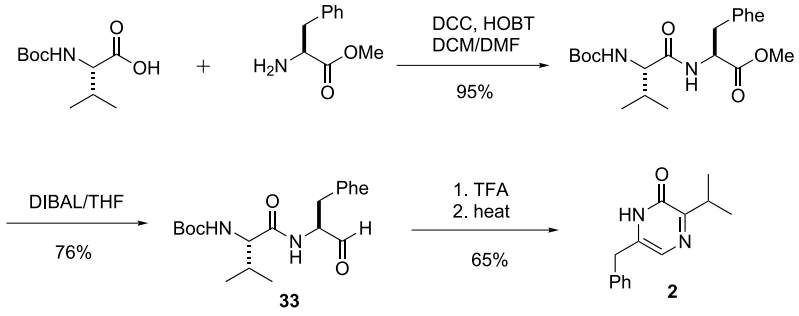


Figure 3. Structural list of compounds in the initial library.



Scheme 1. General synthesis of diketopiperazine analogues.



Scheme 2. Synthesis of phevalin.

Table 1. Inhibitory activity of analogues **1**–**32** against recombinant human calpain I

Compds	Highest conc tested (μM)	Percent inhibition observed[a]	Compds	Highest conc tested (μM)	Percent inhibition observed[a]
1	680	0	**17**	130	0
2	550	0	**18**	190	0
3	500	0	**19**	300	0
4	180	0	**20**	120	0
5	360	0	**21**	200	0
6	280	0	**22**	190	30
7	300	0	**23**	580	0
8	650	50	**24**	610	25
9	540	0	**25**	210	0
10	97	50	**26**	180	0
11	350	50	**27**	920	0
12	100	0	**28**	200	0
13	150	0	**29**	350	0
14	160	13	**30**	170	0
15	90	25	**31**	150	0
16	120	0	**32**	110	0

[a] Inhibition values are within ±10%.

Compounds **1**–**32** were evaluated for inhibition of calpain I in a continuous fluorescence assay essentially as described by Croce et al.[21] using recombinant calpain I produced by the baculovirus expression system[22] and Suc-Leu-Tyr-AMC[23] as the fluorogenic substrate. The increase in fluorescence was linear for at least 2 min, and inhibition was assessed from the decrease in initial rate compared to control incubations containing pure DMSO instead of inhibitor dissolved in DMSO. Peptide aldehyde **33**, used as a positive control compound, was found to decrease initial rates of substrate hydrolysis by 50% at a concentration of 1.2 μM in our assay system (see Table 1).

It was disappointing to find that phevalin (**2**), which had previously been reported to inhibit calpain, showed no detectable inhibition of recombinant human calpain I in our assay system. Likewise, and in agreement with Donkor and Sanders,[14] we found that diketopiperazine **1** also had negligible calpain inhibitory activity. The other diketopiperazine compounds synthesized and tested at best showed slight inhibitory activity, the best having IC_{50} values in the 0.1–1.0 mM range.

In summary, a small library of 2,5-diketopiperazines was synthesized as potential calpain inhibitors. However, neither these constrained dipeptide analogues nor phevalin showed any significant inhibition against recombinant human calpain I. On the other hand, the dipeptidyl aldehyde analogue Boc-Val-Phe-H was, as expected, a potent inhibitor. We have therefore refocused our efforts in the calpain inhibition area to structure-based methods and to a high-throughput screening approach.

Acknowledgments

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17. General procedure for the parallel synthesis of 3,6-disubstituted-2,5-piperazinediones. To a scintillation vial (20 mL) was added a Boc-protected amino acid (1 mmol), DCC (1 mmol), HOBT (1 mmol), and an amino acid ester (1 mmol), followed by addition of CH_2Cl_2 (5 mL), DMF (2 mL) and Et_3N (1 mmol). After flushing the vial with Ar, the reaction vessel was capped and shaken for 15 h. The mixture was then filtered through a 5 mL syringe tube cotton plug and washed with a small amount of EtOAc. The volume of the reaction mixture was reduced by flushing with a stream of argon; water (10 mL) was added and the mixture extracted with EtOAc. The organic layer was washed with 10% citric acid, H_2O, 5% $NaHCO_3$ and brine, dried (Na_2SO_4) and evaporated. The residue was dissolved in CH_2Cl_2 (3 mL) and transferred to another scintillation vial (20 mL), followed by the addition of TFA (0.4 mL). After flushing the vial with argon, the reaction mixture was capped and shaken for 2 h at room temperature. After evaporation of solvent by flushing with argon, 2-butanol (2 mL), toluene (0.5 mL) and Et_3N (1 mmol) were added to the residue. After flushing with argon, the vial was sealed with Teflon cap and shaken at 99 °C for 5 h. Most of 2,5-piperazinediones crystallized from the reaction mixture and were collected by filtration and washed with MeOH. Analytical samples were obtained by drying the crystals in vacuo over P_2O_5. The non-crystalline 2,5-piperazinediones were flashed through a silica gel column and evaporated to dryness to afford the desired compounds. (3*S*,6*S*)-3-Benzyl-6-(4′-benzyloxy-benzyl)-2,5-piperazinedione (**7**): From *N*-α-Boc-tyrosine-*O*-benzyl ether and phenylalanine methyl ester (200 mg, 50%) as a white crystal, mp 267–269 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.18 (dd, 1H, J = 6.0 Hz, 13.6 Hz), 2.28 (dd, 1H, J = 6.0 Hz, 13.6 Hz), 2.51–2.59 (m, 2H), 3.92 (br s, 1H), 3.98 (br s, 1H), 5.06 (s, 2H), 6.90–6.93 (m, 4H), 7.04–7.39 (m, 10H), 7.91 (d, 2H, J = 8.8 Hz, D_2O exchangeable); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 39.3, 56.3, 56.4, 69.9, 115.5, 127.3, 128.3, 129.0, 129.2, 129.4, 130.7, 131.7, 137.5, 138.0, 158.0, 167.0, 167.1; IR (KBr) 1676, 1659 cm^{-1}; MS (FAB) *m/e* 401 (M^++1); HRMS calcd for $C_{25}H_{25}N_2O_3$ (M^++H): 401.1865. Found: 401.1858. $[\alpha]_D$ −113 (*c* 0.65, DMSO).
18. The 1,4-dimethyl-2,5-piperazinediones were obtained by the following representative procedure. 1,4-Dihydro-2,5-piperazinedione (0.5–1 mmol) was suspended in DMF (10 mL) and cooled in an ice bath under Ar, NaH (60%, 3 equiv) was added and the mixture was stirred for 0.5 h followed by addition of MeI (5 equiv). The reaction mixture was allowed to warm up to room temperature over 3 h. Water (30 mL) was added and the reaction mixture extracted with EtOAc, which was sequentially washed with H_2O and brine. The organic layer was dried (Na_2SO_4) and concentrated; chromatography afforded the desired 1,4-dimethyl-2,5-piperazinediones. (3*S*,6*S*)-1,4-Dimethyl-3-benzyl-6-(4′-benzyloxy-benzyl)-2,5-piperazinedione

(**8**): From **7** (420 mg, 98%), as light yellow syrup: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.20–2.26 (m, 2H), 2.76–2.89 (m, 8H), 4.01–4.04 (m, 1H), 4.06–4.09 (m, 1H), 5.03 (s, 2H), 6.95–7.12 (m, 6H), 7.28–7.36 (m, 8H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 34.0, 38.5, 39.5, 64.7, 64.8, 70.4, 115.6, 127.7, 127.7, 128.4, 129.0, 129.3, 129.5, 130.0, 131.1, 137.2, 137.4, 158.5, 165.9, 165.9; IR (neat) 1658, 1512 cm^{-1}; MS (FAB) *m/e* 429 (M^++1); HRMS calcd for $C_{27}H_{29}N_2O_3$ (M^++H): 429.2178. Found: 429.2188. $[\alpha]_D$ −98 (*c* 0.8, MeOH).

19. Debenzylation was carried out in $CHCl_3$/MeOH at 45–50 psi H_2 pressure for 6 h with 10% Pd–C as catalyst. After filtration through Celite and concentration, the residue was recrystallized from MeOH/EtOAc to afford the desired debenzylated compounds. (3*S*,6*S*)-1,4-Dimethyl-3-benzyl-6-(4′-hydroxybenzyl)-2,5-piperazinedione (**4**): From **8** (290 mg, 97%), as a white crystal, mp 238–239 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.11–2.21 (m, 2H), 2.50–2.63 (m, 2H), 2.65 (s, 3H), 2.71 (s, 3H), 4.06–4.11 (m, 2H), 5.03 (s, 2H), 6.72 (d, 2H, J = 8.0 Hz), 6.84 (d, 2H, J = 8.0 Hz), 7.05–7.33 (m, 5H), 9.31 (s, 1H, D_2O exchangeable); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 33.3, 33.4, 37.8, 39.0, 63.7, 63.9, 116.2, 127.5, 127.8, 129.3, 130.3, 131.4, 138.4, 157.2, 165.6, 165.7; IR (KBr) 1667, 1644 cm^{-1}; MS (FAB) *m/e* 339 (M^++1); HRMS calcd for $C_{20}H_{23}N_2O_3$ (M^++H): 339.1709. Found: 339.1700. $[\alpha]_D$ −91 (*c* 1.0, DMSO). Compound **1** was obtained (180 mg, 99%) in a similar fashion as a light yellow crystals, mp 207–208 °C (lit.[14] 208 °C); $[\alpha]_D$ −118 (*c* 1.0, MeOH) (lit.[14] −114, *c* 1, MeOH). All other physical data matched those reported by Donkor and Sanders.[14]
20. Physical data for phevalin: R_f = 0.75 (95:5 $CHCl_3$/MeOH); mp 117–118 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26 (d, 6H, J = 6.7 Hz), 3.44 (m, 1H), 3.85 (s, 2H), 7.26–7.38 (m, 6H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 20.4, 30.5, 37.0, 122.8, 127.7, 129.3, 129.6, 136.4, 137.2, 157.9, 162.4; IR (neat) 1643, 1610 cm^{-1}; MS (FAB) *m/e* 229 (M^++1); HRMS calcd for $C_{14}H_{17}N_2O$ (M^++H): 229.1341. Found: 229.1316.
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23. A typical assay condition: To a cuvette was added 6 mL substrate (8 mM) + 460 mL buffer solution (Tris pH 7.3, 50 mM; EDTA, 0.5 mM; β ME, 10 mM) + 4 mL inhibitor or DMSO + 10 mL calpain (1 mg/mL) + 20 mL $CaCl_2$ (40 mM). λ_{ex} = 380 nm, λ_{em} = 460 nm.