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Synthesis of a Reported Calpain Inhibitor Isolated from *Streptomyces griseus*

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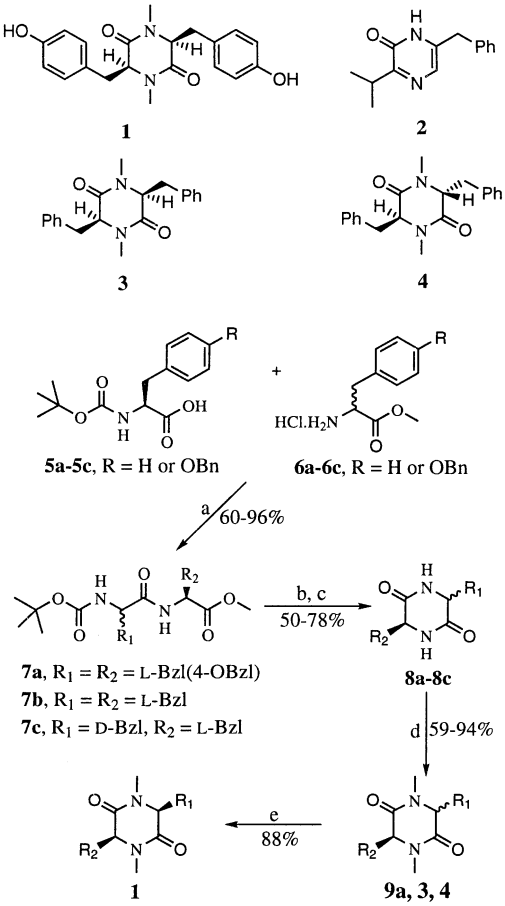
Abstract—The reported diketopiperazine calpain inhibitor, *cis*-L-L-3,6-bis-(4-hydroxybenzyl)-1,4-dimethylpiperazine-2,5-dione **1**, and its analogues **3** and **4** were synthesized from the corresponding amino acids. The previously assigned structure of **1** is confirmed but neither synthetic **1** nor its *N*-methylphenylalanine analogues **3** and **4** inhibit porcine erythrocyte calpain I.

Calpain (EC 3.4.22.17) is a unique cytosolic calcium-activated cysteine protease that is widely distributed in mammalian cells.[1–3] The enzyme has been implicated in various neurodegenerative disorders and several other pathological conditions.[4–7] Calpain has therefore attracted considerable interest as a therapeutic target. Several synthetic, semi-synthetic, as well as natural products have been investigated as inhibitors of the enzyme.[8,9] Diketopiperazine **1** of *N*-methyltyrosine was isolated from *Streptomyces griseus* (SC488) and reported to inhibit calpain ($IC_{50} = 0.8\,\mu M$).[10] Phevalin **2**, which bears some structural resemblance to diketopiperazine **1**, was isolated from *Streptomyces* species and also reported to inhibit calpain with an IC_{50} of $1.2\,\mu M$.[11] These natural products attracted our attention as interesting leads for the discovery of novel non-peptide inhibitors of calpain. In this communication we describe the total synthesis of diketopiperazine **1** and its *N*-methylphenylalanine analogues **3** and **4** and their calpain inhibitory activity. Marcuccio and Elix[12] previously reported compounds **3** and **4** but no calpain inhibitory data were presented.

The diketopiperazine **1** of *N*-methyltyrosine and its *N*-methylphenylalanine analogues **3** and **4** were synthesized from the appropriately protected amino acids **5a**–**5c** and **6a**–**6c** (see Scheme 1). Coupling of *t*-boc-protected amino acids **5a**–**5c** with amino acid methyl esters **6a**–**6c** followed by purification of the crude product by column chromatography (silica gel) with



Scheme 1. (a) EDC/CH_2Cl_2/Et_3N or CDI/anhyd THF/DIEA/N_2; (b) 96% formic acid; (c) *sec*-butanol/toluene (4:1), reflux; (d) NaH/MeI/DMF; (e) H_2, 10% Pd/C, MeOH. For **1**, $R_1 = R_2 =$ Bzl (4-OH).

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EtOAc/hexanes (1:1) as eluant afforded dipeptides **7a**–**7c**.[13] It was observed in the synthesis of **7a** that utilization of carbonyldiimidazole (CDI) instead of ethylcarbodiimide hydrochloride (EDC) as the coupling agent afforded higher yields of the corresponding dipeptide. Following a modification of the procedure of Nitecki et al.,[14] compounds **7a**–**7c** were selectively deprotected with 96% formic acid and the resulting dipeptide amino esters were transformed to diketopiperazines **8a**–**8c** by refluxing in a mixture of *sec*-butanol/toluene (4:1).[15] The diketopiperazines (**8a**–**8c**) were *N*-methylated using NaH/MeI in DMF as described by Marcuccio and Elix[12] to give the *N*-methylphenylalanine analogues **3** and **4** as well as **9a**.[16] Catalytic hydrogenolysis of **9a** over 10% Pd/C at 60 psi afforded the diketopiperazine **1** of *N*-methyltyrosine as a yellow tinted solid.[17]

We expected that, like the diketopiperazine isolated from *S. griseus*, **1** and **3** should have a *cis* or LL-configuration since they were derived from dipeptides composed of L-amino acids while **4** should have a *trans* or DL-configuration since it was derived from a dipeptide composed of D- and L-amino acids. Indeed, the ^{1}H NMR spectrum of **1** was identical to that of the diketopiperazine isolated from *S. griseus*.[10] The spectra of **3** and **4** were also in agreement with literature values.[12] Additionally, the splitting pattern and the resonance positions of the geminal methylene protons and the methine protons of **1** and **3** were similar and different from those of **4**, which has a *trans* or DL-configuration (see Fig. 1). Furthermore, the specific rotation of the compounds confirmed their assigned configuration. Compounds **1** and **3** had specific rotations of $-114.64°$ and $-98.24°$, respectively, due to their LL-configuration while **4** did not rotate plane polarized light due to its DL-configuration.

Despite the similarity in the ^{1}H NMR data of synthetic diketopiperazine **1** and the natural product, **1** was obtained as a solid while the natural product was reported as an oil.[10] Also, synthetic diketopiperazine **1** and its *N*-methylphenylalanine analogues **3** and **4** did not inhibit porcine erythrocyte calpain I up to 1 mM inhibitor concentration under our assay conditions.[18] This is at odds with the reported calpain inhibitory activity ($IC_{50} = 0.8\,\mu M$) of the diketopiperazine isolated from *Streptomyces griseus*. Casein and calpain I isolated from purified human RBC were used as the substrate and the enzyme, respectively, to determine the calpain inhibitory potency of the diketopiperazine isolated from the natural product.[10] Suc-Leu-Tyr-AMC and calpain I isolated from porcine erythrocytes were used as the substrate and the enzyme, respectively, in the assay system that we used to evaluate the calpain inhibitory potency of the synthetic diketopiperazines **1**, **3**, and **4**.[18] We have previously used our calpain assay system to duplicate the calpain inhibitory potency of a known α-ketoamide (Z-Leu-Phe-$CONHCH_2CH_2Ph$) calpain inhibitor.[19,20] Thus, despite the differences in the assay protocols the synthetic diketopiperazines should have inhibited calpain if they are true calpain inhibitors. We suggest that an unidentified contaminant of the diketopiperazine isolated from *Streptomyces griseus* is responsible for the observed calpain inhibitory activity reported by Alvarez et al.[10] Indeed, the fact that synthetic diketopiperazine **1** is a solid and the diketopiperazine isolated from the natural product is an oil is consistent with the possibility of an impurity in the natural

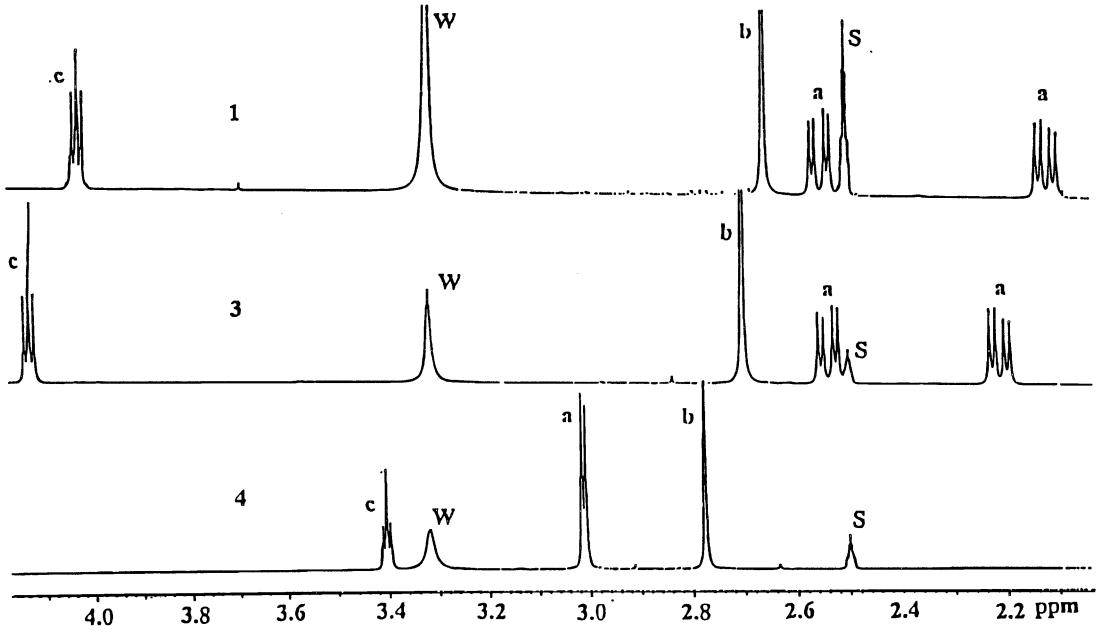

Figure 1. ^{1}H NMR (500 MHz) spectra showing the aliphatic regions of **1**, **3**, and **4**. S, solvent (DMSO-d_6); W, water; a, methylene protons; b, N–CH_3 protons; c, methine protons.

product which may be responsible for its calpain inhibitory action.

Acknowledgements

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13. The ^{1}H NMR spectra of all the compounds in this study were recorded on a Bruker 300 MHz instrument. (a) *t*-Boc-L-tyrosyl(*O*-benzyl)-L-tyrosine(*O*-benzyl)methyl ester **7a** was obtained in 96% yield as a white solid of melting point 163–165 °C. ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9H, *t*-Boc), 2.92–3.0 (m, 4H, PhCH_2CH), 3.66 (s, 3H, OCH_3), 4.28 (m, 1H, C*H*CONH), 4.73 (dd, 1H, C*H*COOCH$_3$), 5.01 (d, 4H, PhCH_2O), 6.81–7.12 (m, 8H, phenyl protons), 7.27–7.42 (m, 10H, phenyl protons). Anal. calcd for $C_{38}H_{42}N_2O_7$: C, 71.45; H, 6.63; N, 4.39. Found: C, 71.64; H, 6.57; N, 4.31. (b) *t*-Boc-L-phenylalanyl-L-phenylalanine methyl ester **7b** was obtained in 60% yield as a white solid of melting point 116–117 °C. ^{1}H NMR ($CDCl_3$) δ 1.39 (s, 9H, *t*-Boc), 2.98–3.12 (m, 4H, PhCH_2CH), 3.67 (s, 3H, OCH_3), 4.32 (m, 1H, C*H*CONH), 4.78 (dd, 1H, C*H*COOCH$_3$), 6.95–7.30 (m, 10H, phenyl protons). Anal. calcd for $C_{24}H_{30}N_2O_5$: C, 67.59; H, 7.09; N, 6.57. Found: C, 67.34; H, 7.03; N, 6.47. (c) *t*-Boc-D-phenylalanyl-L-phenylalanine methyl ester **7c** was obtained in 63% yield as a white solid of melting point 124–126 °C. ^{1}H NMR ($CDCl_3$) δ 1.38 (s, 9H, *t*-Boc), 2.90–3.11 (m, 4H, PhCH_2CH), 3.67 (s, 3H, OCH_3), 4.35 (m, 1H, C*H*CONH), 4.83 (dd, 1H, C*H*COOCH$_3$), 6.94–7.30 (m, 10H, phenyl protons). Anal. calcd for $C_{24}H_{30}N_2O_5$: C, 67.59; H, 7.09; N, 6.57. Found: C, 67.34; H, 7.04; N, 6.54
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15. (a) *cis*-L-L-3,6-Bis-(4-benzyloxybenzyl)piperazine-2,5-dione **8a** was obtained in 84% yield. Mp > 260 °C. ^{1}H NMR (TFA-*d*) δ 2.15 (m, 2H, PhCH_2CH), 2.87 (d, 2H, PhCH_2CH), 4.47 (s, 2H, PhCH$_2$C*H*), 5.19 (s, 4H, PhCH_2O), 6.96–7.35 (m, 18H, phenyl protons). Anal. calcd for $C_{32}H_{30}N_2O_4$: C, 75.87; H, 5.97; N, 5.53. Found: C, 75.81; H, 6.04; N, 5.56. (b) *cis*-L-L-3,6-Dibenzylpiperazine-2,5-dione **8b** was obtained in 87% yield. Mp > 300 °C. ^{1}H NMR (TFA-*d*) δ 2.25 (m, 2H, PhCH_2CH), 3.01 (d, 2H, PhCH_2CH), 4.52 (s, 2H, PhCH$_2$C*H*), 7.05–7.34 (m, 10H, phenyl protons). Anal. calcd for $C_{18}H_{18}N_2O_2$: C, 73.45; H, 6.16; N, 9.52. Found: C, 73.32; H, 6.25; N, 9.43. (c) *trans*-D-L-3,6-Dibenzylpiperazine-2,5-dione **8c** was obtained in 50% yield. Mp 294 °C. ^{1}H NMR (TFA-*d*) δ 3.03–3.20 (m, 4H, PhCH_2CH), 3.86 (d, 2H, PhCH$_2$C*H*), 7.00–7.28 (m, 10H, phenyl protons). Anal. calcd for $C_{18}H_{18}N_2O_2$: C, 73.45; H, 6.16; N, 9.52. Found: C, 73.58; H, 6.22; N, 9.56.
16. (a) *cis*-L-L-3,6-Bis-(4-benzyloxybenzyl)-1,4-dimethylpiperazine-2,5-dione **9a** was obtained in 59% yield as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 2.22 (dd, 2H, 4-BzlOPhCH_2CH, J = 6.6, 14.2), 2.75 (s, 6H, NCH_3), 2.80 (dd, 2H, 4-BzlOPhCH_2CH, J = 4.0, 14.2), 4.01 (dd, 2H, 4-BzlOPhCH$_2$C*H*, J = 4.0, 6.6), 5.02 (s, 4H, PhCH_2O), 6.91–7.02 (m, 8H, phenyl protons), 7.27–7.37 (m, 10H, phenyl protons). Anal. calcd for $C_{34}H_{34}N_2O_4$·0.2EtOAc: C, 75.69; H, 6.50; N, 5.07. Found: C, 75.31; H, 6.47; N, 5.02. (b) *cis*-L-L-3,6-Dibenzyl-1,4-dimethylpiperazine-2,5-dione **2** was obtained in 94% yield. Mp 144–146 °C. ^{1}H NMR ($CDCl_3$) δ 2.22 (dd, 2H, PhCH_2CH, J = 6.5, 14.2), 2.76 (s, 6H, NCH_3), 2.84 (dd, 2H, PhCH_2CH, J = 4.2, 14.2) 4.06 (dd, 2H, PhCH$_2$C*H*, J = 4.2, 6.5), 7.07–7.35 (m, 10H, phenyl protons); ^{13}C NMR ($CDCl_3$) δ 33.5 (NCH_3), 39.0 (CH_2), 64.3 (CH), 127.2, 128.8, 129.0, 129.6, 137.0 (Ar), 165.4 (CO). $[\alpha]_D^{23}$ −98.24° (*c* 1, CH_3OH). Anal. calcd for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.26; H, 6.92; N, 8.72. (c) *trans*-D-L-3,6-Dibenzyl-1,4-dimethylpiperazine-2,5-dione **3** was obtained in 89% yield. Mp 184–187 °C. ^{1}H NMR ($CDCl_3$) δ 2.84 (s, 6H, NCH_3), 2.96 (dd, 2H, PhCH_2CH, J = 4.2, 14.1 Hz), 3.21 (dd, 2H, PhCH_2CH, J = 3.1, 14.1 Hz), 3.39 (t, 2H, PhCH$_2$C*H*, J = 3.6), 6.95–7.27 (m, 10H, phenyl protons); ^{13}C NMR ($CDCl_3$) δ 32.2 (NCH_3), 36.9 (CH_2), 61.7 (CH), 127.3, 128.3, 129.6, 134.8 (Ar), 165.2 (CO). $[\alpha]_D^{26}$ +0.05° (*c* 1, CH_3OH). Anal. calcd for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.58; H, 6.93; N, 8.74.
17. *cis*-L-L-3,6-Bis-(4-hydroxybenzyl)-1,4-dimethylpiperazine-2,5-dione **1** was obtained in 88% yield as a yellow tinted solid. Mp 208 °C. ^{1}H NMR (CH_3OH-d_4) δ 2.14 (dd, 2H, 4-OHPhCH$_2$CH, J = 6.4, 14.2 Hz), 2.75 (s, 6H, NCH_3), 2.76 (dd, 2H, 4-OHPhCH$_2$CH, J = 4.2, 14.2 Hz), 4.09 (dd, 2H, 4-OHPhCH$_2$C*H*, J = 4.2, 6.4 Hz), 6.75 (d, 4H, phenyl protons), 6.91 (d, 4H, phenyl protons). ^{1}H NMR (DMSO-d_6) δ 2.03 (dd, 2H, 4-OHPhCH_2CH, J = 6.0, 14.2 Hz), 2.48 (dd, 2H, 4-OHPhCH_2CH, J = 4.5, 14.2 Hz), 2.58 (s, 6H, NCH_3), 3.96 (t, 2H, 4-OHPhCH$_2$C*H*, J = 5.3), 6.61–6.78 (dd, 8H, phenyl protons), 9.22 (s, 2H, PhO*H*); ^{13}C NMR (DMSO-d_6) δ 32.6 (NCH_3), 37.3 (CH_2), 63.1 (CH), 115.3, 127.2, 130.4, 156.3 (Ar), 164.9 (CO); MS: calcd for $C_{20}H_{22}N_2O_4$ + H = 355.4; Found: 355.3. $[\alpha]_D^{25}$ −114.64° (*c* 1, CH_3OH). Anal. calcd for $C_{20}H_{22}N_2O_4$·1H_2O: C, 64.50; H, 6.50; N, 7.52. Found: C, 64.27; H, 6.49; N, 7.51.
18. Calpain activity was monitored in a reaction mixture containing 50 mM Tris HCl (pH 7.4), 50 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.2 or 1.0 mM Suc-Leu-Tyr-AMC (Calbiochem), 2 μg porcine erythrocyte calpain I (Calbiochem), varying concentrations of inhibitor dissolved in DMSO (2% total concentration) and 5 mM $CaCl_2$ in a final volume of 250 μL in a polystyrene microtiter plate. Assays were initiated by addition of $CaCl_2$ and the increase in fluorescence (γ_{ex} = 370 nm, γ_{em} = 440 nm) was monitored at ambient temperature using a SPECTRAmax Gemini fluorescence plate reader (Molecular Devices).
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