Supporting Information

Peptidyl α-Ketoamides with Nucleobases, Methylpiperazine, and Dimethylaminoalkyl Substituents as Calpain Inhibitors

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Contents

General Procedure for Synthesis of Dipeptide Methyl Esters. HOBt (1.5 eq) was added to a stirred solution of Cbz-Leu-OH (1 eq) in DMF at -15 °C. The hydrochloride salt of the amino acid methyl ester was pretreated with NMM (1.5 eq) at -15 °C DMF prior to addition. The reagent DCC (1.5 eq) was added to the solution and allowed to react for 16 h at RT. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, and saturated NaCl prior to being dried over $MgSO_4$, and concentrated. Purification on a silica gel column with the proper eluent gave the product with yields of 68-76%.

Cbz-Leu-Abu-OMe was purified by column chromatography on silica gel using 9:1 EtOAc:hexane as the eluent; white solid, yield 68%. ^{1}H NMR ($CDCl_3$): 0.85-0.94 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.48-1.91 (m, 5H, 2 x CH_2 and CH), 3.74 (s, 3H, OCH_3), 4.23 (m, 1H, α-H), 4.53 (m, 1H, α-H), 5.10 (s, 2H, CH_2), 5.26 (d, 1H, NH), 6.55 (d, 1H, NH), 7.31-7.37 (m, 5H, Ph). MS (ESI) *m/z* 365.2 ($[M+H]^+$).

Cbz-Leu-Phe-OMe was purified by column chromatography on silica gel using 1:1 EtOAc:hexane as the eluent; white solid, yield 76%. ^{1}H NMR ($CDCl_3$): 0.90 (d, 6H, 2 x Leu-CH_3), 1.46 (m, 1H, CH), 1.62 (m, 2H, CH_2), 3.03-3.15 (m, 2H, CH_2), 3.70 (s, 3H, OCH_3), 4.19 (m, 1H, α-H), 4.84 (m, 1H, α-H), 5.05-5.12 (m, 2H, CH_2), 5.20 (d, 1H, NH), 7.08 (d, 1H, NH), 7.22-7.34 (m, 5H, Ph). MS (ESI) *m/z* 427.2 ($[M+H]^+$).

General Procedure for the Synthesis of Dipeptide Acids. The dipeptide methyl esters were hydrolyzed in MeOH using 1 M aqueous NaOH (1.1 eq) under standard deblocking conditions.

Cbz-Leu-Abu-OH, white solid, yield 89%. ^{1}H NMR (DMSO-d_6): 0.79-0.84 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.41-1.73 (m, 5H, 2 x CH_2 and CH), 4.07-4.10 (m, 2H, 2 x α-H), 5.00 (s, 2H, CH_2), 7.32-7.40 (m, 6H, Ph and NH), 7.98-8.07 (d, 1H, NH). MS (ESI) *m/z* 351.1 ($[M+H]^+$).

Cbz-Leu-Phe-OH, white solid, yield 85%. ^{1}H NMR (DMSO-d_6): 0.87 (d, 6H, 2 x Leu-CH_3), 1.45-1.61 (m, 3H, CH_2 and CH), 2.96-3.20 (m, 2H, CH_2), 4.26 (m, 1H, α-H), 4.81 (m, 1H, α-H), 5.06-5.13 (m, 2H, CH_2), 5.54 (d, 1H, NH), 6.88 (d, 1H, NH), 7.10-7.34 (m, 5H, Ph). MS (ESI) *m/z* 413.1 ($[M+H]^+$).

Synthesis of 9-(3-aminopropyl)adenine.[1] A mixture of adenine (1 eq), 1-bromo-3-chloropropane (4.3 eq), and potassium carbonate (2.35 eq) in DMF (200 mL) was stirred at RT under argon for 4 days, filtrated, and evaporated to dryness. The crude product was washed with water and dried. Recrystallization from ethanol gave 9-(3-chloropropyl)adenine in 59% yield. MS (ESI) *m/z* 212.0 ($[M+H]^+$).

A mixture of 9-(3-chloropropyl)adenine (1 eq) and sodium azide (3 eq) in DMF was stirred at 80 °C for 24 hours, cooled to RT, and filtered. The solid was washed with CH_2Cl_2. The solvent was removed from the combined filtrates and the residue was redissolved in water and sonicated. The aqueous layer was extracted with CH_2Cl_2 (3 x 60 mL). After removing solvent, the crude product was recrystallized from ethanol to give 9-(3-azidopropyl)adenine as a white crystalline solid in 81% yield. ^{1}H NMR (DMSO-d_6): 2.04 (m, 2H, CH_2), 3.36 (m, 2H, CH_2), 4.19 (m, 2H, CH_2), 7.22 (s, 2H, NH_2), 8.12 (s, 2H, CH of adenine).

A mixture of 9-(3-azidopropyl)adenine and 5 % palladium on carbon in methanol was reacted with hydrogen gas at RT for 22 hours. The catalyst was removed by

filtration, the solvent removed to give 9-(3-aminopropyl)adenine as a white solid in 76% yield. ^{1}H NMR (DMSO-d_6): 1.80 (m, 2H, CH_2), 2.45 (m, 2H, CH_2), 3.35 (s, 2H, NH_2), 4.20 (m, 2H, CH_2), 7.20 (s, 2H, NH_2), 8.10 (s, 2H, CH). MS (ESI) *m/z* 193.0 ($[M+H]^+$).

Synthesis of 1-(3-Aminopropyl)cytosine.[2] A mixture of N-acetylcytosine (1eq), 1-bromo-3-chloropropane (1.5 eq), and potassium carbonate (1.5 eq) in DMF was stirred at RT under argon for 4 days, filtered and evaporated to dryness. The crude product was purified by column chromatography and gave 1-(3-chloropropyl)-N-acetylcytosine in 69% yield. MS (ESI) *m/z* 230.0 ($[M+H]^+$).

A mixture of 1-(3-chloropropyl)-N-acetylcytosine and sodium azide in acetonitrile was refluxed for 24 hours, cooled to RT, and filtered. The crude product was purified by silica gel column chromatography and gave 1-(3-azidopropyl)-N-acetylcytosine as a white solid in 64% yield . ^{1}H NMR (DMSO-d_6): 1.85-1.91 (m, 2H, CH_2), 2.06 (s, 3H, CH_3), 3.35-3.39 (t, 2H, CH_2), 3.81-3.84 (t, 2H, CH_2), 7.12 (d, 1H, CH), 8.04 (s, 1H, CH), 10.79 (s, 1H, NH). MS (ESI) *m/z* 237.0 ($[M+H]^+$).

A mixture of 1-(3-azidopropyl)-N-acetylcytosine was reacted with 7N ammonia solution in MeOH at RT for 2 days to give 1-(3-azidopropyl)cytosine as a white solid in 56% yield. ^{1}H NMR (DMSO-d_6): 1.73-1.83 (m, 2H, CH_2), 3.31-3.34 (t, 2H, CH_2), 3.64-3.67 (t, 2H, CH_2), 5.62 (d, 1H, CH), 7.01 (d, 2H, NH_2), 7.53 (s, 1H, CH). MS (ESI) *m/z* 195.0 ($[M+H]^+$).

A mixture of 1-(3-azidopropyl)cytosine and 5% palladium on carbon in MeOH was reacted with hydrogen gas at RT for 8 h. The catalyst was removed by filtration, the solvent was removed to give 1-(3-aminopropyl)cytosine as a white solid in 82% yield. ^{1}H NMR (DMSO-d_6): 1.66 (m, 2H, CH_2), 2.96 (t, 2H, CH_2), 2.96 (s, 2H, CH_2), 3.57-3.67

(m, 4H, CH_2 and NH_2), 5.59 (d, 1H, CH), 6.97 (d, 2H, NH_2), 7.54 (d, 1H, CH). MS (ESI) *m/z* 169.9 ($[M+H]^+$).

Synthesis of 1-(3-Aminopropyl)-4-methylpiperazine.[3] A solution of N-(3-bromopropyl)phthalimide (8.04 g, 30 mM) in xylene (60 mL) was added dropwise to a solution of 1-methylpiperazine (6.61 g, 66 mM) in xylene (90 mL) at 70 °C. The mixture was heated under reflux for 20 hours, precipitate was removed by filtration and the filtrate was concentrated. The crude product was purified by silica gel chromatography with 9:1 CH_2Cl_2:MeOH to give the product N-(3-(4-methylpiperazin-1-yl)propyl)phthalimide as an oil in 72% yield.

A solution of N-(3-(4-methylpiperazin-1-yl)propyl)phthalimide (6.2 g, 21.6 mM) and hydrazine monohydrate (1.13 g, 26 mM) in EtOH (60 mL) and MeOH (60 mL) was refluxed for 4 hours. After cooling to RT, concentrated HCl (2.4 mL) was added and the mixture heated under reflux for another hour. After removing the solvent, water (100 mL) was added, the mixture stirred and insoluble material removed by filtration. Solid K_2CO_3 (1.2 eq) and CH_2Cl_2 (100 mL) was added to the aqueous layer; the mixture was stirred and filtered. The organic layer was washed with water (3 x 20 mL). The combined aqueous layers were washed with Et_2O. Water was removed from the organic layers, which were then dried and evaporated to give 1-(3-aminopropyl)-4-methylpiperazine as oil in 39% yield. ^{1}H NMR ($CDCl_3$): 1.55 (m, 2H, CH_2), 2.20 (s, 3H, CH_3), 2.34 (t, 2H, CH_2), 2.67 (t, 2H, CH_2). MS (ESI+): 157.9 ($[M+H]^+$).

3-(Benzyloxycarbonyl-L-leucylamino)-N-(3-(6-amino-9H-purin-9-yl)propyl)-2-oxopentanamide (4a, Cbz-Leu-Abu-CONH-$(CH_2)_3$-adenin-9-yl). The ketoamide product Cbz-Leu-Abu-CONH-$(CH_2)_3$-adenin-9-yl was obtained from 9-(3-

aminopropyl)adenine and the ketoacid Cbz-Leu-Abu-COOH using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2:MeOH as the eluent, then recrystallized from CH_3COOEt/hexane to give a white solid (27% yield). ^1H NMR ($CDCl_3$): 0.91 (m, 9H, CH_3 of Leu and Abu), 1.60-1.80 (m, 5H, CH_2 and CH of Leu and Abu), 2.00 (m, 2H, CH_2), 3.20 (2H, CH_2), 4.24 (m, 3H, CH_2 and α-H), 5.11 (s, 2H, Cbz), 5.20 (m, 1H, α-H), 6.20 (s, 1H, NH), 6.80 (b, 1H, NH), 7.20-7.40 (m, 6H, Ph and NH), 7.85 (d, 1H, CH of adenine), 8.36 (d, 1H, CH of adenine). Purity >90% by LC-MS. HRMS (FAB) Calcd. for $C_{27}H_{37}N_8O_5$: 553.2856. Observed *m/z* 553.2881 ($[M+H]^+$).

3-(Benzyloxycarbonyl-L-leucylamino)-N-(3-(4-methylpiperazin-1-yl)propyl)-2-oxopentanamide (4d, Cbz-Leu-Abu-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl). The ketoamide product Cbz-Leu-Abu-CONH-$(CH_2)_3$-(4-methylpiperazin-1-yl) was synthesized from 1-(3-aminopropyl)-4-methylpiperazine and Cbz-Leu-Abu-COOH using the EDC/HOBt coupling method, purified twice by column chromatography on silica gel using 80:20 CH_2Cl_2:MeOH and 85:15 CH_2Cl_2:MeOH as the eluent to give a yellow semi-solid in 16% yield. ^1H NMR ($CDCl_3$): 0.91 (m, 9H, CH_3 of Leu and Abu), 1.60-1.80 (m, 5H, CH_2 and CH), 2.00 (m, 2H, CH_2), 2.44 (s, 3H, CH_3 of piperazine), 2.50-2.65 (m, 8H, CH_2 of piperazine), 3.30 (m, 2H, CH_2), 4.20 (m, 3H, CH_2 and α-H), 5.10 (s, 2H, Cbz), 5.15 (m, 1H, α-H), 6.70 (b, 1H, NH), 7.20-7.30 (m, 6H, Ph and NH), 8.60 (b, 1H, NH). Purity >95% by LC-MS. HRMS (FAB) for $C_{27}H_{44}N_5O_5$: *m/z* 518.3301 ($[M+H]^+$).

1. Zhang, Q. Z.; Hua, G. X.; Bhattacharyya, P.; Slawin, A. M. Z.; Woollins, J. D. Syntheses and Coordination Chemistry of Aminomethylphosphine Derivatives of Adenine. Eur. J. Inorg. Chem. 2003, 2003, 2426-2437.

2. Summers, W. A.; Lee, J. Y.; Burr, J. G. Synthesis of Fluorescent Labeled Derivatives of Aminopropylpyrimidines. J. Org. Chem. 1975, 40, 1559-1561.

3. Hou, D. R.; Cheng, H. Y.; Wang, E. C. Efficient Syntheses of Oncinotine and Neooncinotine. J. Org. Chem. 2004, 69, 6094-6099.

Statistical analysis of the results in Table 1 were analyzed using the VassarStats website for statistical computation. The URL is http://faculty.vassar.edu/lowry/VassarStats.html. Specifically a one-way ANOVA with a post-hoc Tukey HSD (honestly significant differences) test was performed. This website was developed by Professor Richard Lowry. VassarStats only allows 5 date sets to be entered simultaneously and the data in Table A, B, and C was determined by entering multiple different data sets into the program. In general, inhibition constants for calpain I, calpain II, and or cathepsin B that differed by less than a factor of approximately two were considered to be non-significant in this analysis. In the case of Table D, three data sets (calpain I, calpain II, and cathepsin B; one row in Table 1) were used in the one-way ANOVA with a post-hoc Tukey HSD test to generate each line in Table D.

Table A. Cal I vs. Cal I. One Way Analysis of Variance (ANOVA) on Calpain I Inhibition Date (Column 2 of Table 1) Using the Tukey Post-Hoc HSD Test. The values are significance (P) values. Nonsig indicates a non-significant difference based on this test. The 99% confidence level is indicated by <.01 and the 95% confidence level is indicated by <.05. The mean (inhibitor potency) is sorted for lowest value to highest value left to right and top to bottom. In general, values that differed by approximately a factor of less than two were non-significant in this test. All the others values were significant.

Cal I vs. Cal I	**4b**	**5b**	**4a**	**5a**	**1**	**4c**	**5e**	**5c**	**4d**	**5f**	**5d**
Mean	0.023	0.041	0.053	0.055	0.15	0.165	0.226	0.48	0.64	0.711	1.37
	4b	**5b**	**4a**	**5a**	**1**	**4c**	**5e**	**5c**	**4d**	**5f**	**5d**
4b	*	nonsig	<.05	<.01	<.01	<.01	<.01	<.01	<.01	<.01	<.01
5b		*	nonsig	nonsig	<.01	<.05	<.01	<.01	<.01	<.01	<.01
4a			*	nonsig	<.01	<.01	<.01	<.01	<.01	<.01	<.01
5a				*	<.01	<.01	<.01	<.01	<.01	<.01	<.01
1					*	nonsig	<0.5	<.01	<.01	<.01	<.01
4c						*	<0.5	<.01	<.01	<.01	<.01
5e							*	<.01	<.01	<.01	<.01
5c								*	nonsig	<.01	<.01
4d									*	nonsig	<.01
5f										*	<.01
5d											*

Table B. Cal II vs. Cal II. One Way Analysis of Variance (ANOVA) on Calpain II Inhibition Date (Column 3 of Table 1) Using the Tukey Post-Hoc HSD Test. The values are significance (P) values. Nonsig indicates a non-significant difference based on this test. The 99% confidence level is indicated by <.01 and the 95% confidence level is indicated by <.05. The mean (inhibitor potency) is sorted for lowest value to highest value left to right and top to bottom. In general, values that differed by approximately a factor of less than two were non-significant in this test. All others values were significant.

Cal II vs. Cal II	**1**	**5a**	**4a**	**4b**	**5b**	**4d**	**5c**	**5e**	**4c**	**5f**	**5d**
Mean	0.041	0.068	0.07	0.077	0.209	0.286	0.438	0.844	1.14	3.52	6.36
	1	**5a**	**4a**	**4b**	**5b**	**4d**	**5c**	**5e**	**4c**	**5f**	**5d**
1	*	nonsig	nonsig	<.01	<.01	<.01	<.01	<.01	<.01	<.01	<.01
5a		*	nonsig	nonsig	<.01	<.01	<.01	<.05	<.01	<.01	<.01
4a			*	nonsig	<.01	<.05	<.01	<.01	<.01	<.01	<.01
4b				*	<.01	<.01	<.01	<0.05	<.01	<.01	<.01
5b					*	nonsig	nonsig	<.01	<.01	<.01	<.01
4d						*	nonsig	<.01	<.01	<.01	<.01
5c							*	<.01	<.01	<.01	<.01
5e								*	<.01	<.01	<.01
4c									*	<.01	<.01
5f										*	<.01
5d											*

Table C. Cat B vs. Cat B. One Way Analysis of Variance (ANOVA) on Calpain I Inhibition Date (Column 4 of Table 1) Using the Tukey Post-Hoc HSD Test. The values are significance (P) values. Nonsig indicates a non-significant difference based on this test. The 99% confidence level is indicated by <.01 and the 95% confidence level is indicated by <.05. The mean (inhibitor potency) is sorted for lowest value to highest value left to right and top to bottom. In general, values that differed by approximately a factor of two were non-significant in this test. All other values are significant.

Cat B vs. Cat B	**5c**	**4c**	**4a**	**4b**	**4d**	**5a**	**5b**	**1**	**5e**	**5d**	**5f**
Mean	0.44	0.75	0.8	0.88	1.42	1.75	2.34	6.9	75.5	111	475
	5c	**4c**	**4a**	**4b**	**4d**	**5a**	**5b**	**1**	**5e**	**5d**	**5f**
5c	*	nonsig	<.05	<.01	<.01	<.01	<.01	<.01	<.01	<.01	<.01
4c		*	nonsig	nonsig	<.01	<.01	<.01	<.01	<.01	<.01	<.01
4a			*	nonsig	<.01	<0.05	<.01	<.01	<.01	<.01	<.01
4b				*	<.01	nonsig	<.01	<.01	<.01	<.01	<.01
4d					*	nonsig	nonsig	<.01	<.01	<.01	<.01
5a						*	nonsig	<.01	<.01	<.01	<.01
5b							*	<.01	<.01	<.01	<.01
1								*	<.01	<.05	<.01
5e									*	nonsig	<.01
5d										*	<.01
5f											*

Table D. Cal I vs. Cal II vs. Cat B. One Way Analysis of Variance (ANOVA) on Calpain I, Calpain II, and Cathepsin B Inhibition Data (Column 2, 3, and 4 of Table 1) Using the Tukey Post-Hoc HSD Test. The values are significance (P) values. Each row was analyzed separately. Nonsig indicate non-significant difference based on this test. The 99% confidence level is indicated by <.01 and the 95% confidence level is indicated by <0.5. The table is sorted for lowest value to highest value top to bottom using the calpain I mean (inhibitor potency). In general, the difference between values for calpain I and calpain II with the same inhibitor were non-significant by this analysis with three exceptions. With one exception, the differences between calpain I and cathepsin B, or calpain II and cathepsin B were significant in this analysis.

Rows	**Cal I**	**Cal II**	**Cat B**	**Cal I vs. Cal II**	**Cal I vs. Cat B**	**Cal II vs. Cat B**
4b	0.023	0.077	0.88	<.01	<.01	<.01
5b	0.041	0.209	2.34	<.05	<.01	<.01
4a	0.053	0.07	0.8	nonsig	<.01	<.01
5a	0.055	0.068	1.75	nonsig	<.01	<.01
1	0.15	0.041	6.9	nonsig	<.01	<.01
4c	0.165	1.14	0.75	<.01	<.01	<.01
5e	0.226	0.844	75.5	nonsig	<.01	<.01
5c	0.48	0.438	0.44	nonsig	nonsig	nonsig
4d	0.64	0.286	1.42	nonsig	<.05	<.01
5f	0.711	3.52	475	nonsig	<.01	<.01
5d	1.37	6.36	111	nonsig	<.01	<.01