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Nitro-based selective inhibitors against matrix metalloproteinase-7 over matrix metalloproteinase-1

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Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases, which catalyze the cleavage of extracellular matrix and thus tightly associate with various physiological and pathological processes. MMP family contain at least 28 members with only MMP-1, -2 and -7 experimentally validated as targets for cancer therapy. As reported in previous, inhibition of MMP-1 could result in various side effects including musculoskeletal syndrome, which should be spared. However, the "broad spectrum" inhibitors, which typically utilize hydroxymatic acid as strong zinc-binding group (ZBG), have been unapproved due to the lack of selectivity. Further, because both MMP-1 and -7 have the similar S_1' hydrophobic pockets, it remains to be a challenge to explore the selective inhibition of MMP-7 over -1 up to now. In the presented paper, we firstly introduced nitro as a ZBG in the inhibition of MMP. A series of nitro-based dipeptidic compounds were thus synthesized and evaluated as MMP inhibitors. Combined with the kinetic assays results and computation analysis of the binding mode of the inhibitors with the active sites of enzymes, it was disclosed that a reasonable adjustment of the P_3' side chains of the nitro-based MMP inhibitors could improve the selectivity for inhibition of MMP-7 over MMP-1.

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that play the key roles in tissue remodeling associated with various physiological and pathological processes such as inflammation, multiple sclerosis, cancer, and so on.[1] Inhibitors against MMPs have been thus extensively pursued as the promising drug candidates for various pharmaceutical applications including cancer therapy.[2] Among the 28 members of MMP family, MMP-1, MMP-2 and MMP-7 have been validated as the anticancer drug targets as suggested by Overall et al.[3] It means that the MMP inhibitors directed to cancer therapy should selectively inhibit the three MMPs. However, most of MMP inhibitors of the first generation utilized the hydroxymatic acid (HA) as zinc binding group (ZBG), which generally resulted in the "broad spectrum" inhibition due to the strong chelation ability of HA.[4] Typically, a broad-based inhibitor (**Marimastat, Chart 1**) could inhibit various MMPs and result in various side effects including severe musculoskeletal pains.[5] MMP-1 inhibition was then suggested as the origin of the side effects and should be thus spared.[6] Failure of the reported MMP inhibitors in clinical trials might be ascribed to the deficiency of selectivity. Application of the non-HA ZBGs were then suggested to improve the selectivity of MMP inhibitors.[2d] In a word, it is necessary to develop the selective MMP-2 or MMP-7 inhibitors spared MMP-1 utilizing a non-HA ZBG such as exemplified in the selective inhibition of MMP-2.[7]

In the presented paper, we choose MMP-7 as target enzyme because MMP-7 has been identified as the specific enzyme secreted by the tumor cells, and could induce the evolution of tumor cells to cancer cells.[8] In the other hand, nitro is utilized as the ZBG, which has been successfully introduced in the inhibition of the other zinc-proteases.[9] Here we hope to present our efforts to develop the nitro-based inhibitors against MMP-7 over MMP-1.

For the design rationale to MMP inhibitors, many reported MMP inhibitors possess a ZBG attached to a peptide framework that binds to the "primed" binding regions,[10] where the depth of the S_1' pocket is regarded as the key determinant for discrimination of various MMPs.[11] Thus, a general approach to selective MMP inhibition concentrated on the size of the P_1' side chain of MMP inhibitors to satisfy the "so-called" S_1' specificity for different MMPs. However, because both MMP-1 and MMP-7 have the similar S_1' pockets,[2a,12] it seems infeasible that optimization of the structures of the P_1' side chains of the inhibitors to obtain the selective inhibition of MMP-7 over MMP-1, which maybe the reason that selective inhibition of MMP-7 has been rarely reported as now.

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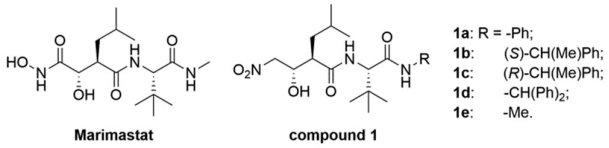


Marimastat compound 1

Chart 1

According to the suggested ranking of the MMP sub-pockets based on the possibility of achieving selective interactions: $S_1'>S_3'>S_2'$,[13] we then designed a series of nitro-based dipeptides compounds **1a-d** (**Chart 1**), which possessed different P_3' side chains, to investigate the possible selective inhibition of MMP-7 over MMP-1.

Experimental

Materials and Methods

Unless otherwise noted, all chemicals were commercially available and used as received without further purification.

Flash chromatography was performed with 100-200 mesh silica gel (Qingdao, China) and thin-layer chromatography (TLC) was carried out on silica coated glass sheets (Qingdao silica gel 60 F-254). Melting points were taken on a Thomas-Hoover capillary melting point apparatus and uncorrected. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded with a Bruker AV 300 (300 MHz) instrument using tetramethylsilane as the internal standard. IR spectra were recorded on a Perkin-Elmer 1300 FT-IR spectrometer. High-resolution mass spectra were taken on a Shimadzu GC-MS-QP 2010. Elemental analyses were performed at Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, China.

Synthesis

(2*R*,3*S*)-2-*iso*-Butyl-3,4-epoxybutanoic acid methyl ester (2).

Prepared according to the literature starting from L-maleic acid.[14] Oil. $[\alpha]_D^{21}$ = – 7.5° (*c* 1.60, $CHCl_3$). IR (film): 864, 949, 1130, 1171, 1196, 1236, 1276, 1331, 1440, 1469, 1738 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.89 (d, *J* = 6.4 Hz, 3H), 0.91 (d, *J* = 6.4 Hz, 3H), 1.36-1.45 (m, 1H), 1.56-1.73 (m, 2H), 2.26 (dd, *J* = 4.8, 10.4 Hz, 1H), 2.58 (dd, *J* = 2.6, 10.4 Hz, 1H), 2.83-2.86 (m, 1H), 3.10-3.15 (m, 1H), 3.73 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 22.18, 22.72, 25.96, 37.93, 46.53, 46.94, 51.91, 52.97, 174.20. HRMS calcd for $C_9H_{16}O_3$: 172.1099. Found: 172.1107.

(2*R*, 3*S*)-2-iso-Butyl-3,4-epoxybutanoic acid *p*-nitrophenyl ester (4).

Compound **2** (110 mg, 0.64 mmol) was dissolved in the mixture of MeOH and THF (4 mL, V_{MeOH} :V_{THF} = 3:1) followed by addition of 1 mL aqueous LiOH (20 mg, 0.84 mmol). The reaction mixture was kept stirring at room temperature for 6 hours. The reaction was quenched by addition of 5 mL water and extracted with ether (5 mL × 2). The remained water layer was acidified to pH = 1 with 10 % citric acid solution at 0 °C. After extracted with ether (5 mL × 5), the combined organic layer was dried over anhydrous Na_2SO_4. Evaporation under reduced pressure provided an oil as compound **3** (88.1 mg), which was then used in the subsequent esterification without further purification and characterization.

To the solution of CH_2Cl_2 containing compound **3**, DCC (1.1 eq) was added at 0 °C. After stirring for 40 minutes, a solution of CH_2Cl_2 containing *p*-nitro phenol (1.0 eq) was added. The reaction mixture was then kept in stirring at room temperature overnight. The reaction mixture were poured into 5 % $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic layer was dried over anhydrous Na_2SO_4. Evaporation under reduced pressure gave an oil, which was then purified by flash column chromatography (silica gel, *n*-hexane/EtOAc = 3:1) to provide an oil as products. Yield: 72.4 %. $[\alpha]_D^{18}$ = – 24.6° (*c* 2.00, $CHCl_3$). IR (film): 866, 1012, 1113, 1161, 1192, 1346, 1489, 1524, 1591, 1614, 1765 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.01 (d, *J* = 6.4 Hz, 3H), 1.03 (d, *J* = 6.4 Hz, 3H), 1.59-1.63 (m, 1H), 1.73-1.92 (m, 2H), 2.48-2.57 (m, 1H), 2.68 (dd, *J* = 2.6, 10.4 Hz, 1H), 2.94 (dd, *J* = 4.0, 10.4 Hz, 1H), 3.24-3.29 (m, 1H), 7.33 (d, *J* = 9.0 Hz, 2H), 8.31 (d, *J* = 9.0 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 22.01, 22.48, 25.91, 37.80, 46.49, 46.88, 52.50, 122.46, 125.00, 145.41, 154.80, 171.04. HRMS calcd for $C_{14}H_{17}O_5$: 279.1107. Found: 279.1112.

(2*R*,3*S*)-4-Bromo-2-iso-butyl-4-hydroxybutanoic acid *p*-nitrophenyl ester (5).

To 2 mL solution of MeCN containing compound **4** (70 mg, 0.25 mmol), 1.0 eq. $Me_2S{\cdot}Br_2$ was slowly added at 0 °C. The reaction mixture were kept for stirring at room temperature until completion detected by TLC. Addition of 30 mL EtOAc followed by washing with brine, the organic layer was then dried over anhydrous Na_2SO_4. After removing the solvents under reduced pressure, the residue was then purified by flash column chromatography (silica gel, *n*-hexane/EtOAc = 5:1) to provide an oil as products. Yield: 78.1 %. $[\alpha]_D^{18}$ = – 35.7° (*c* 1.52, $CHCl_3$). IR (film): 1113, 1207, 1348, 1526, 1593, 1616, 1757 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.02 (d, *J* = 6.4 Hz, 3H), 1.05 (d, *J* = 6.4 Hz, 3H), 1.35-1.44 (m, 1H), 1.68-1.88 (m, 2H), 2.69 (d, *J* = 6.4 Hz, 1H), 3.01-3.03 (m, 1H), 3.62 (dd, *J* = 5.8, 10.8 Hz, 1H), 3.72 (dd, *J* = 5.6, 10.8 Hz, 1H), 4.04-4.07 (m, 1H), 7.32 (d, *J* = 9.0 Hz, 2H), 8.31 (d, *J* = 9.0 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 21.65, 23.32, 26.43, 37.49, 38.14, 48.47, 72.12, 122.28, 125.28, 145.53, 155.21, 172.08. HRMS calcd for $C_{14}H_{18}BrNO_5$: 359.0368. Found: 359.0377.

General procedure for the synthesis of the bromo-substituted dipeptides (6a-d).

To 2 mL solution of THF containing compound **5** (180 mg, 0.5 mmol), (*S*)-*tert*-Leu-NHR (0.75 mmol, 1.5 eq.) and NaOAc (61.5 mg, 0.75 mmol, 1.5 eq.) was added. After standing at 30 °C for 12 hours, the reaction mixture were neutralized with 10 % Na_2CO_3. After extracted by ether, the combined organic layer was washed by brine and then dried over anhydrous Na_2SO_4. After removing the solvents under reduced pressure, the residue was then purified by flash column chromatography (silica gel, *n*-hexane/EtOAc = 1:1) to give the compound **6**.

[(2'*R*,3'*S*)-4'-Bromo-2'-*iso*-butyl-3'-hydroxy]butyryl-(*S*)-*tert*-leucyl-phenylamide (**6a**). Oil. $[\alpha]_D^{19}$ = – 22.2° (*c* 1.52, $CHCl_3$). IR (film): 755, 877, 1023, 1248, 1442, 1469, 1488, 1546, 1647, 1688, 2980, 3298 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.88 (d, *J* =

6.4 Hz, 3H), 0.93 (d, *J* = 6.4 Hz, 3H), 1.12(s, 9H), 1.59-1.68 (m, 3H), 2.78-2.83 (m, 1H), 3.33 (dd, *J* = 8.0, 10.8 Hz, 1H), 3.58 (dd, *J* = 6.4, 10.8 Hz, 1H), 4.02-4.06 (m, 1H), 4.46 (d, *J* = 9.0 Hz, 1H), 6.83 (d, *J* = 6.8 Hz, 1H), 7.09-7.34 (m, 5H), 7.50 (d, *J* = 9.0 Hz, 1H), 7.97 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ*: 18.67, 22.38, 25.07, 26.72, 34.87, 35.59, 39.04, 46.76, 64.77, 120.21, 124.72, 129.00, 137.23, 168.81, 174.80. HRMS calcd for $C_{20}H_{31}BrN_2O_3$: 426.1518. Found: 426.1516. Anal. $C_{20}H_{31}BrN_2O_3$ requires C, 56.21; H, 7.31; N, 6.55. Found: C, 56.57; H, 7.26; N, 7.26.

[(2'*R*,3'*S*)-4'-Bromo-2'-*iso*-butyl-3'-hydroxy]butyryl-(*S*)-*tert*-leucyl-[(1''*S*)-phenyl]-ethylamide (**6b**). M.p.: 156-157.5 °C. $[\alpha]_D^{19}$ = – 28.8° (*c* 1.34, $CHCl_3$). IR (film): 688, 700, 768, 1114, 1215, 1530, 1643, 1696, 2897, 2963, 3302 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.93 (d, *J* = 6.4 Hz, 3H), 0.95 (d, *J* = 6.4 Hz, 3H), 0.97 (s, 9H), 1.50 (d, *J* = 6.4 Hz, 3H), 1.55-1.65 (m, 2H), 1.70-1.76 (m, 1H), 2.75-2.81 (m, 1H), 3.27 (dd, *J* = 7.6, 9.8 Hz, 1H), 3.54 (dd, *J* = 4.6, 9.8 Hz, 1H), 3.85-3.89 (m, 1H), 4.12 (d, *J* = 6.2 Hz, 1H), 4.14-4.20 (m, 1H), 5.12 (d, *J* = 7.4 Hz, 1H), 5.86 (d, *J* = 6.2 Hz, 1H), 6.54 (d, *J* = 9.6 Hz, 1H), 7.32-7.37 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ*: 21.36, 22.48, 22.71, 25.79, 26.67, 34.57, 35.32, 39.11, 46.48, 49.08, 60.81, 72.26, 126.28, 127.55, 128.71, 142.57, 169.18, 174.55. HRMS calcd for $C_{22}H_{35}BrN_2O_3$: 454.1831. Found: 454.1836. Anal. $C_{22}H_{35}BrN_2O_3$ requires C, 58.02; H, 7.75; N, 6.15. Found: C, 58.19; H, 7.69; N, 5.97.

[(2'*R*,3'*S*)-4'-Bromo-2'-*iso*-butyl-3'-hydroxy]butyryl-(*S*)-*tert*-leucyl-[(1''*R*)-phenyl]-ethylamide (**6c**). M.p.: 162-163 °C. $[\alpha]_D^{23}$ = +31.9° (*c* 1.20, $CHCl_3$). IR (film): 1110, 1215, 1496, 1517, 1641, 1647, 2960, 3018, 3305 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.84 (d, *J* = 6.4 Hz, 3H), 0.88 (d, *J* = 6.4 Hz, 3H), 1.07(s, 9H), 1.43-1.49 (m, 2H), 1.50 (d, *J* = 6.4 Hz, 3H), 1.67-1.71 (m, 1H), 2.70-2.75 (m, 1H), 3.22 (dd, *J* = 7.4, 10.0 Hz, 1H), 3.46 (dd, *J* = 4.4, 10.0 Hz, 1H), 3.82-3.85 (m, 1H), 4.14 (d, *J* = 6.8 Hz, 1H), 4.16-4.20 (m, 1H), 5.12 (d, *J* = 7.8 Hz, 1H), 5.97 (d, *J* = 7.8 Hz, 1H), 6.53 (d, *J* = 9.2 Hz, 1H), 7.29-7.34 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ*: 21.62, 22.30, 22.73, 25.68, 25.71, 34.53, 35.29, 39.02, 46.43, 48.98, 72.23, 126.02, 127.42, 128.69, 142.53, 169.17, 174.46. HRMS calcd for $C_{22}H_{35}BrN_2O_3$: 454.1831. Found: 454.1842. Anal. $C_{22}H_{35}BrN_2O_3$ requires C, 58.02; H, 7.75; N, 6.15. Found: C, 58.16; H, 7.73; N, 6.58.

[(2'*R*,3'*S*)-4'-Bromo-2'-*iso*-butyl-3'-hydroxy]butyryl-(*S*)-*tert*-leucyl-[(1'',1'')-diphenyl]-methylamide (**6d**). M.p.: 167-168 °C. $[\alpha]_D^{18}$ = – 18.3° (*c* 1.48, $CHCl_3$). IR (film): 689, 700, 796, 1215, 1495, 1518, 1643, 2961, 3294 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.90 (d, *J* = 6.4 Hz, 3H), 0.92 (d, *J* = 6.4 Hz, 3H), 1.04 (s, 9H), 1.43-1.55 (m, 2H), 1.67-1.75 (m, 1H), 2.70-2.74 (m, 1H), 3.23 (dd, *J* = 8.0, 10.2 Hz, 1H), 3.47 (dd, *J* = 5.8, 10.2 Hz, 1H), 3.82-3.87 (m, 1H), 4.02 (d, *J* = 8.0 Hz, 1H), 4.27 (d, *J* = 9.4 Hz, 1H), 6.23 (d, *J* = 8.0 Hz, 1H), 6.33 (d, *J* = 8.0 Hz, 1H), 6.52 (d, *J* = 9.4 Hz, 1H), 7.19-7.25 (m, 5H), 7.30-7.36 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ*: 22.31, 22.82, 25.71, 26.67, 34.61, 35.42, 39.03, 46.57, 57.19, 60.85, 72.21, 127.13, 127.49, 127.63, 127.72, 128.63, 128.79, 140.79, 141.03, 168.45, 174.49. HRMS calcd for $C_{27}H_{37}BrN_2O_3$: 516.1988. Found: 516.1992. Anal. $C_{27}H_{37}BrN_2O_3$ requires C, 62.67; H, 7.21; N, 5.41. Found: C, 62.71; H, 7.27; N, 5.66.

[(2'*R*,3'*S*)-4'-Bromo-2'-*iso*-butyl-3'-hydroxy]butyryl-(*S*)-*tert*-leucyl-methylamide (**6e**). Oil. $[\alpha]_D^{21}$ = – 10.6° (*c* 0.58, $CHCl_3$). IR (film): 753, 806, 1310, 1497, 1587, 1614, 2954, 3491 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.84 (d, *J* = 6.4 Hz, 3H), 0.88 (d, *J* = 6.4 Hz, 3H), 0.95 (s, 9H), 1.45-1.62 (m, 3H), 2.31-2.52 (m, 1H), 2.71 (d, *J* = 9.2 Hz, 3H), 3.24 (dd, *J* = 7.6, 10.0 Hz, 1H), 3.43 (dd, *J* = 6.8, 10.0 Hz, 1H), 3.91-4.05 (m, 1H), 4.14 (d, *J* = 7.0 Hz, 1H), 5.27 (br, 1H), 6.33 (d, *J* = 7.0 Hz, 1H), 6.58 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ*: 22.42, 23.60, 25.79, 27.02, 34.66, 35.38, 38.98, 46.72, 58.92, 65.72, 169.50, 173.13. HRMS calcd for $C_{15}H_{29}BrN_2O_3$: 364.1362. Found: 364.1368. Anal. $C_{15}H_{29}BrN_2O_3$ requires C, 49.32; H, 8.00; N, 7.67. Found: C, 49.36; H, 8.05; N, 8.13.

General procedure for the synthesis of the nitro-substituted dipeptides (1a-d).

To 1 mL solution of DMF containing compound **6** (0.25 mmol), $NaNO_2$ (10 eq.) and phloroglucinol (1.1 eq.) was added. After standing at 50 °C for 16 hours, the reaction mixture were cooled to room temperature and then added 20 mL EtOAc. After washed by brine, the organic layer was then dried over anhydrous Na_2SO_4. After removing the solvents under reduced pressure, the residue was then purified by flash column chromatography (silica gel, *n*-hexane/EtOAc = 1:1) to give the compound **1**.

[(2'*R*,3'*S*)-2'-*iso*-Butyl-3'-hydroxy-4'-nitro]butyryl-(*S*)-*tert*-leucyl-phenylamide (**1a**). M.p.: 155-156 °C. $[\alpha]_D^{23}$ = – 26.4° (*c*0.88, $CHCl_3$). IR (film): 669, 798, 1080, 1147, 1211, 1310, 1378, 1454, 1539, 1578, 1643, 1661, 2872, 2962, 3315 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.87(d, *J* = 6.4 Hz, 3H), 0.89 (d, *J* = 6.4 Hz, 3H), 1.12(s, 9H), 1.53-1.63 (m, 3H), 2.49-2.54 (m, 1H), 3.80-3.84 (m, 1H), 4.32-4.67 (m, 4H), 6.99 (d, *J* = 8.8 Hz, 1H), 7.11-7.15 (m, 1H), 7.31-7.35 (m, 2H), 7.45-7.49 (m, 2H), 7.96 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ*: 22.16, 22.64, 25.63, 26.75, 34.71, 38.69, 47.25, 61.68, 69.53, 79.15, 120.27, 124.88, 129.03, 137.05, 169.03, 174.22. HRMS calcd for $C_{20}H_{31}N_3O_5$: 393.2264. Found: 393.2270. Anal. $C_{20}H_{31}N_3O_5$ requires C, 61.05; H, 7.94; N, 10.68. Found: C, 61.09; H, 8.01; N, 11.15.

[(2'*R*,3'*S*)-2'-*iso*-Butyl-3'-hydroxy-4'-nitro]butyryl-(*S*)-*tert*-leucyl-[(1''*S*)-phenyl]-ethylamide (**1b**). M.p.: 163-164 °C. $[\alpha]_D^{19}$ = – 26.5° (*c*0.56, $CHCl_3$). IR (film): 669, 798, 1080, 1147, 1211, 1310, 1378, 1454, 1539, 1578, 1643, 1661, 2872, 2962, 3315 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.91 (d, *J* = 6.4 Hz, 3H), 0.95 (d, *J* = 6.4 Hz, 3H), 0.96 (s, 9H), 1.41-1.47 (m, 1H), 1.51 (d, *J* = 6.8 Hz, 3H), 1.65-1.72 (m, 1H), 1.85-1.94 (m, 1H), 2.37-2.40 (m, 1H), 4.14 (d, J = 9.0 Hz, 1H), 4.31-4.42 (m, 3H), 4.48-4.53 (m, 1H), 5.01 (dd, *J* = 7.0, 12.8 Hz, 1H), 5.07-5.14 (m, 1H), 5.96 (d, *J* = 9.0Hz, 1H), 6.62 (d, *J* = 9.4 Hz, 1H), 7.31-7.37 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ*: 21.29, 22.37, 22.68, 25.72, 26.69, 34.50, 38.74, 46.93, 61.16, 69.63, 79.18, 125.28, 127.65, 128.76, 142.42, 169.18, 173.93. HRMS calcd for $C_{22}H_{35}N_3O_5$: 421.2577. Found: 421.2572. Anal. $C_{22}H_{35}N_3O_5$ requires C, 62.69; H, 8.37; N, 9.97. Found: C, 62.77; H, 8.34; N, 10.05.

[(2'*R*,3'*S*)-2'-*iso*-Butyl-3'-hydroxy-4'-nitro]butyryl-(*S*)-*tert*-leucyl-[(1''*R*)-phenyl]-ethylamide (**1c**). M.p.: 145-146 °C. $[\alpha]_D^{23}$ = +42.6° (*c*0.62, $CHCl_3$). IR (film): 700, 759, 1022, 1093, 1211, 1238, 1259, 1371, 1384, 1537, 1633, 1643, 2872, 2928, 3309 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.85 (d, *J* = 6.4 Hz, 3H), 0.87 (d, *J* = 6.4 Hz, 3H), 1.05(s, 9H), 1.41-1.47 (m, 1H), 1.52 (d, *J* = 7.0 Hz, 3H), 1.60-1.71 (m, 1H), 1.85-1.91 (m, 1H), 2.37-2.40 (m,

1H), 4.19 (d, *J* = 9.0 Hz, 1H), 4.31-4.42 (m, 3H), 4.48-4.53 (m, 1H), 5.10 (dd, *J* = 7.0, 12.8 Hz, 1H), 6.31 (d, *J* = 9.0 Hz, 1H), 6.75 (d, *J* = 9.0 Hz, 1H), 7.22-7.34 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ*: 21.62, 22.19, 22.74, 25.72, 34.42, 38.64, 47.23, 49.11, 61.03, 79.16, 126.05, 127.49, 128.68, 142.51, 169.34, 173.80. HRMS calcd for $C_{22}H_{35}N_3O_5$: 421.2577. Found: 421.2581. Anal. $C_{22}H_{35}N_3O_5$ requires C, 62.69; H, 8.37; N, 9.97. Found: C, 62.62; H, 8.34; N, 9.38.

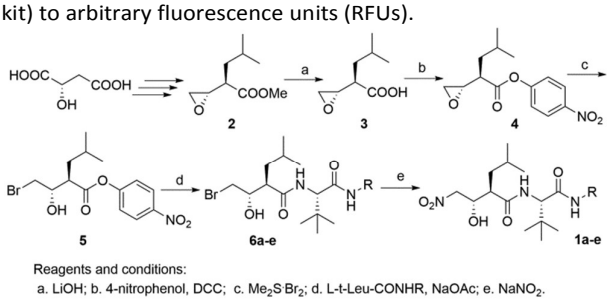
[(2'*R*,3'*S*)-2'-*iso*-Butyl-3'-hydroxy-4'-nitro]butyryl-(*S*)-*tert*-leucyl-[(1'',1'')-diphenyl]-methylamide (**1d**). M.p.: 159-161 °C. $[\alpha]_D^{21}$ = – 23.6° (*c* 0.68, $CHCl_3$). IR (film): 785, 1217, 1389, 1500, 1631, 2962, 3314 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.89 (d, *J* = 6.4 Hz, 3H), 0.91 (d, *J* = 6.4 Hz, 3H), 1.03 (s, 9H), 1.41-1.52 (m, 1H), 1.55-1.69 (m, 2H), 2.36-2.42 (m, 1H), 4.13-4.25 (m 2H), 4.34-4.45 (m, 3H), 6.23 (d, *J* = 9.0 Hz, 1H), 6.52 (d, *J* = 7.8 Hz, 1H), 6.64 (d, *J* = 9.0 Hz, 1H), 7.20-7.25 (m, 5H), 7.30-7.36 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ*: 22.22, 22.78, 25.67, 26.70, 34.48, 38.70, 47.12, 57.35, 61.21, 69.61, 79.08, 127.14, 127.57, 127.63, 127.78, 128.65, 128.82, 140.72, 140.96, 169.55, 173.84. HRMS calcd for $C_{27}H_{37}N_3O_5$: 483.2733. Found: 483.2736. Anal. $C_{27}H_{37}BrN_2O_3$ requires C, 67.06; H, 7.71; N, 8.69. Found: C, 67.31; H, 7.78; N, 9.12.

[(2'*R*,3'*S*)-2'-*iso*-Butyl-3'-hydroxy-4'-nitro]butyryl-(*S*)-*tert*-leucyl-methylamide (**1e**). M.p.: 138-139 °C. $[\alpha]_D^{21}$ = – 11.9° (*c* 0.21, $CHCl_3$). IR (film): 989, 1211, 1312, 1490, 1521, 1578, 1630, 2950, 3422 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.84 (d, *J* = 6.4 Hz, 3H), 0.88 (d, *J* = 6.4 Hz, 3H), 1.09 (s, 9H), 1.47-1.68 (m, 3H), 2.21-2.34 (m, 1H), 2.76 (d, *J* = 9.2 Hz, 3H), 3.91-4.09 (m, 2H), 4.16 (d, *J* = 7.0 Hz, 1H), 4.37 (dd, *J* = 8.2, 13.0 Hz, 1H), 5.10 (br, 1H), 6.15 (d, *J* = 7.0 Hz, 1H), 6.81 (d, *J* = 7.4 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) *δ*: 22.46, 23.14, 24.51, 25.88, 32.41, 35.66, 37.98, 47.44, 60.18, 82.31, 168.25, 174.33. HRMS calcd for $C_{15}H_{29}N_3O_5$: 331.2107. Found: 331.2111. Anal. $C_{15}H_{29}N_3O_5$ requires C, 54.36; H, 8.82; N, 12.68. Found: C, 54.39; H, 8.81; N, 13.08.

Kinetics

Human recombinant MMP-1, -2 and MMP-7 were purchased from Enzo Life Science and used without further purification for kinetic assays. Fluorescent substrate {Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 [Mca = (7-methoxycourmarin-4-yl)-acetyl; Dpa = *N*-3-(2,4-dinitrophenyl)-L-α,β-diaminopropionyl)]} and 50 mM HEPES buffer solution (pH = 7.5, containing 10 mM $CaCl_2$ and 0.05 % Brij-35) used for kinetic assays were obtained from MMP inhibitor Profiling Kit (BML-AK-016). BioTek FLx 800 fluorescence plate reader with 96-well plates was used for fluorescent profiling.

Enzyme stock solutions were diluted with the assay buffer solution to the desired concentrations as mentioned in Instruction Manual BML-AK016 of Enzo Life Science. All nitro-based inhibitors were dissolved in DMSO and further diluted into the assay buffer. Content of DMSO in the final measurement was kept as 1 % (v/v). Before kinetic assay, the following controls were firstly set up (positive control, inhibitor control, vehicle control, test compound control, and substrate control). In the other hand, we also built a standard curve relating Mca-Pro-Leu-OH (a calibration standard packing in the kit) to arbitrary fluorescence units (RFUs).



Scheme 1 Synthesis of the presented nitro-based MMP inhibitors

Before addition of the fluorescent substrate, the enzyme was incubated with varying concentrations of inhibitors (0.2-2.0 K_i) for 1 hour at 37 °C. The enzymatic hydrolysis were agitated by shaking for 1 second after each fluorescence measurement. The final concentrations of MMP-1, -2 and -7 were 0.765 U/μL, 0.047 U/μL, and 0.064 U/μL, respectively (1 U = 100 pmol/min@37°C, 100 μM thiopeptide, as described in the kit). Upon cleavage of the fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (final concentration = 4 μM) at the Gly-Leu bond, Mca fluorescence (λ_{ex} = 340 nm, λ_{ex} = 400 nm) was measured at 60-second intervals for 60 minutes. According to the plots of RFUs versus time for each sample, the range of initial time points was determined during which the reaction was linear. The slope of each line was fitted as the initial velocity (v) of the enzymatic hydrolysis using OriginPro 7.5. We measured the velocity for each concentration of the tested inhibitors in triplet. The averaged slope was used as v in the subsequent determination of K_i values. Here v and v_0 are the initial velocity in the presence and absence of inhibitor, respectively.

Molecular Docking

The X-ray structures of enzymes were obtained from the Protein Data Bank (PDB ID: 966C[15] and 1MMQ[16] for MMP-1 and -7, respectively). After extracting the bound inhibitor from the original structure, water molecules and the possibly missing residues were added using Swiss-pdb Viewer 3.7.[17] Before docking, the inhibitors 1 were optimized at the B3LYP/6-31G* level[18] using Gaussian 09.[19]

The ligands were docked onto the active site of enzyme using AutoDock 4.0.[20] The partial atomic charges for the ligands were obtained at the B3LYP/6-31(G)* level. The nonbonded zinc parameters used were as follows: radius = 0.87 Å, well depth = 0.35 kcal/mol, charge = 0.95 e.[21] The grid size was set to 80×80×80 Å points with a grid spacing of 0.375 Å centered on the zinc ion at the active site of enzyme. The docking simulations were performed using the Lamarckian genetic algorithm with 200 solutions for each ligands obtained. The best poses were based on two criteria, the distance of the nitro group from the catalytic zinc ion and the binding free energies.

Table 1 Inhibitory constants (K_i) of the tested compounds against MMPs. The errors were estimated according to the corresponding SD of the slope after the linear fitting

Compound	K_i /(nM)			Selectivity
	MMP-7	MMP-1	MMP-2	
1a	9.4 ± 0.2	249 ± 8.2	155 ± 5.7	26.5
1b	3.7 ± 0.2	141 ± 5.1	116 ± 4.2	38.1
1c	8.9 ± 0.1	198 ± 4.8	106 ± 3.3	22.2
1d	14.8 ± 0.3	358 ± 8.2	130 ± 4.8	24.2
1e	158 ± 7.4	192 ± 5.7	190 ± 5.1	1.22

Results and discussion

Chemistry

Synthesis of the designed nitro-modified dipeptides compounds was shown in **Scheme 1**. Our initial attempt to obtain 2-*iso*-butyl-3-hydroxy-4-nitrobutanoic acid failed (ESI). We then selected the corresponding activated carboxylate, i.e. 2-*iso*-butyl-3-hydroxy-4-nitrobutanoic acid *p*-nitrophenyl ester, as the substrate of the condensation with the L-*t*-Leu amides. Compound **5** was thus prepared for the subsequent nitro substitution. However, we found that a great deal of *p*-nitro phenol appeared in the reaction of sodium nitrate with compound **5**, which indicated that the *p*-nitrophenoxy anion should be the better leaving group than the bromo group at the 4-position. Therefore, we revised our synthetic route as shown. It should be noted that the condensation with the L-*t*-Leu amides could only proceed in the existence of NaOAc. We believed that a possible anhydride intermediate might be crucial to the smooth attacking of the amine group of the L-*t*-Leu amides to the carbonyl of compound **5**. Final nitro-substitution of compounds **6a-e** gave the needed compounds **1a-e**, respectively.

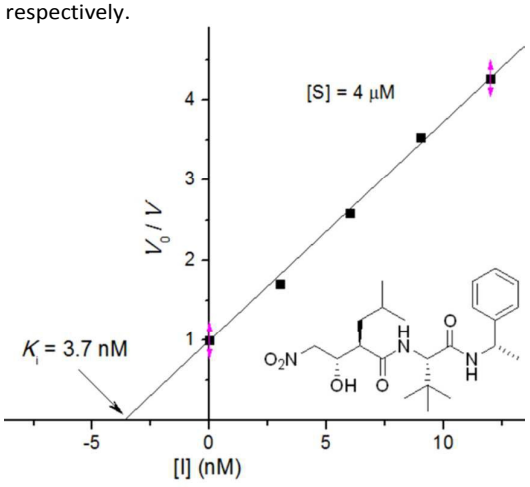


Fig 1. Kinetics of compounds **1b** as inhibitor against MMP-7. The final concentrations of MMP-7 was 0.064 U/μL. Final concentration of the fluorescent substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) was kept as 4 μM. All kinetic assay were performed in 50 mM HEPES buffer solution (pH = 7.5, containing 10 mM $CaCl_2$ and 0.05 % Brij-35) containing 1 % DMSO (v/v).

MMP Inhibition

Inhibitory activities of compounds **1a-e** were thus examined in vitro against recombinant human MMP-1, -2 and -7 using fluorogenic substrate assay. The final concentration of the substrate was controlled as 4 μM, which was far less than the K_m values for each tested MMP.[22] A competitive inhibition could be thus described by the rate equation (Eq. 1),

$$d[P]/dt = k_{cat}[E][S]/K_m(1+[I]/K_i) \quad \text{Eq. 1}$$

where a first-order rate constant was found as $k_{cat}[E]/K_m(1+[I]/K_i)$. Accordingly, the ratio of the first-order rate constants in the absence and presence of inhibitor was given as shown in Eq. 2.[23] The inhibitory constant (K_i) could be then extracted from a linear plot of v_0/v versus [I]. Fig. 1 shown a typical Dixon plot for inhibition of MMP-7 by compound **1b** as inhibitor.

$$v_0/v = k_0/k = 1+[I]/K_i \quad \text{Eq. 2}$$

All K_i values were collected in **Table 1**. Firstly, compared with **Marimastat** (IC_{50} values were 20 and 5 nM on MMP-7 and -1, respectively)[2f], the inhibitory activities of compound **1e** against both enzymes significantly decreased, which could be ascribed to the weaker coordination of nitro group to the catalytic zinc than HA. Secondly, compounds **1** exhibited the similar inhibitory potency with submicromolar level against MMP-1 and -2, whereas the K_i values of compounds **1a-d** on MMP-7 ranged from 3.7 to 14.8 nM. It means that compounds **1a-d** were more potent inhibitors against MMP-7 than MMP-1. If we defined the selectivity as the ratio of K_i(MMP-1)/K_i(MMP-7), it could be observed that the selectivity of compound **1b** was the highest as 38.1. Selective inhibition of MMP-7 over MMP-1 is thus proved to be feasible by adjustment of the sizes of P_3' side chains. Especially, compound **1b** exhibited the highest inhibitory activities against MMP-7, which was about twofold potent than its diastereromer **1c**. It is a very interesting finding that the stereochemistry of the P_3' chains has such subtle effects on the inhibition of MMP-7, which may be further utilized in the design of MMP inhibitors. Finally, it should be

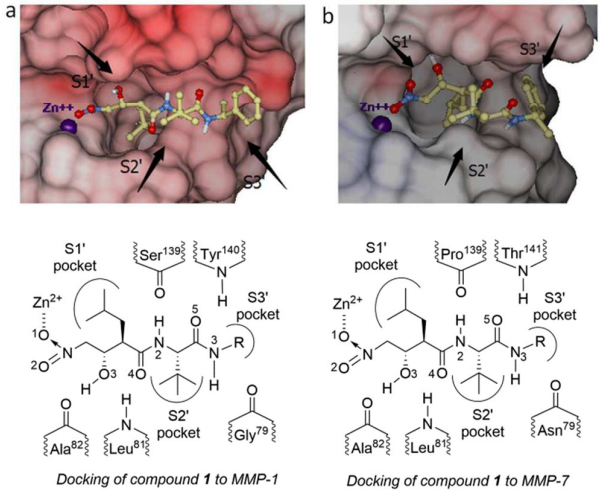


Fig. 2 Up: The binding mode of compounds **1b** with MMP-1 (left) and MMP-7 (right), respectively, predicated by Auto Dock; down: schematic representation of the important interactions of compound **1** with the active sites of MMP-1 and -7.

Table 2 Predicated interactions of compounds **1a-e** with the residues at the active sites of MMP-1 and MMP-7.

		MMP-1		MMP-7	
Compound	Atom	Residue	Distance (Å)	Residue	Distance (Å)
1a	O1	Zinc	3.06	Zinc	3.08
	O2	Zinc		Zinc	
	O3	Ala^{82}	3.08	Ala^{82}	1.84
	O4	Leu^{81}	2.81	Leu^{81}	3.08
	N2	Ser^{139}	4.02	Pro^{139}	2.18
	O5	Tyr^{140}		Thr^{141}	3.05
	N3	Gly^{79}		Asn^{79}	2.67
1b	O1	Zinc	3.05	Zinc	3.05
	O2	Zinc		Zinc	
	O3	Ala^{82}	2.97	Ala^{82}	1.67
	O4	Leu^{81}	3.86	Leu^{81}	3.01
	N2	Ser^{139}	2.62	Pro^{139}	2.21
	O5	Tyr^{140}		Thr^{141}	3.05
	N3	Gly^{79}	3.72	Asn^{79}	1.91
1c	O1	Zinc	3.08	Zinc	3.13
	O2	Zinc		Zinc	
	O3	Ala^{82}	2.15	Ala^{82}	2.03
	O4	Leu^{81}		Leu^{81}	3.05
	N2	Ser^{139}	3.11	Pro^{139}	3.36
	O5	Tyr^{140}	2.19	Thr^{141}	3.00
	N3	Gly^{79}		Asn^{79}	
1d	O1	Zinc	3.14	Zinc	3.06
	O2	Zinc		Zinc	
	O3	Ala^{82}	4.02	Ala^{82}	3.08
	O4	Leu^{81}	3.44	Leu^{81}	2.75
	N2	Ser^{139}		Pro^{139}	3.70
	O5	Tyr^{140}		Thr^{141}	3.38
	N3	Gly^{79}	3.46	Asn^{79}	2.17
1e	O1	Zinc	3.11	Zinc	3.15
	O2	Zinc		Zinc	
	O3	Ala^{82}	2.51	Ala^{82}	2.66
	O4	Leu^{81}	2.60	Leu^{81}	
	N2	Ser^{139}		Pro^{139}	4.20
	O5	Tyr^{140}	3.99	Thr^{141}	4.05
	N3	Gly^{79}	3.80	Asn^{79}	3.95

noted that the change of P_3' from methyl to aromatic group could significantly improve the inhibitory potency on MMP-7 as well as the selectivity.

Computational Analysis

Since Browner's group firstly reported the crystal structures of the complexes of MMP-7 and inhibitors in 1995,[24] the other related X-ray crystallography studies have been rarely reported, which may be ascribed to the difficulties to obtain the single crystal of MMP-7. Although we have incubated the complexes of the other zinc-proteases and the nitro-based inhibitors,[9] our group also failed to achieve the single crystal of MMP-7. To investigate the effects of the P_3' chains of compounds **1** on the inhibitory activities of MMPs, we then conducted computational docking of the presented inhibitors into the active sites of MMP-1 and -7, respectively. The predicated interactions between the typical inhibitor **1b** and MMPs were depicted in **Fig. 2**. Docking results could be divided to two classes of the binding modes of the inhibitors at the active site of MMP, where one was featured by the bidentate chelation of nitro to the catalytic zinc ion and the other featured by the monodentate mode. However, using the experimentally exhibited inhibitory potency orders as the standard, it was surprisingly to be found that the series with the monodentate coordination were in good agreement. Such coordinative mode of the nitro has been rarely reported in the previous structural analysis of the complexes of the other zinc-proteases with inhibitors.[9] We thus assumed that a longer peptide chain should provide more hydrogen bonding and/or more hydrophobic effects to remedy the loss of the chelated coordination to the catalytic zinc in binding energies. Such strategy has been successfully applied in the selective inhibitors against MMP-13 as reported.[25]

All possible interactions between the inhibitors and the residues (the catalytic zinc and the other amino acids residues) at the active site of the two MMPs were collected in Table 2. Although the exhibited docking results could not precisely show the details of the enzyme-inhibitor complexes compared with the X-ray diffraction, we then focused on the possible main interactions between the inhibitor and the residues at the active site of enzyme. According to the docked conformations of compounds **1** at the active sites of MMP-1 and -7, it could be found that both the number and the strength of hydrogen bonds contributed significantly to the inhibitory potencies. Compared with the active site of MMP-1, a relatively large S_3' hydrophobic pocket of MMP-7 subjected to accommodate an aromatic ring with less loss of the vital hydrogen bonds to the amino acids residues at the active sites of enzymes.

Conclusions

We firstly introduced nitro as ZBG in the inhibition of MMP. According to the ranking of the sub-pockets based on achieving selectivity in MMP inhibition, a series of nitro-based dipeptides compounds were designed as MMP inhibitors by adjustment of the P_3' chains. Synthesis of the designed compounds was finally achieved utilized the activated carboxylate as the key intermediate. Kinetic assay disclosed that the nitro-based compounds exhibited weaker inhibitory activities compared with the HA-based inhibitor of MMP. Further we found that aromatic side chains as the P_3' could enhanced both the potency for MMP-7 and the selectivity of MMP-7 over MMP-1. Further docking studies shown that both the number and the strength of hydrogen bonds should be curial to the selective inhibition of MMP-7 without regard for the slight variations in zinc binding mode. We noted that the presented nitro-based compounds **1a-d** inhibited MMP-7 with moderate selectivity. However, the results were clearly enough indicated that the delicate modification of the side chains of inhibitors could improve the selective inhibition of MMP-7 over MMP-1. Further advances are highly anticipated based on both experimental and theoretical exploration.

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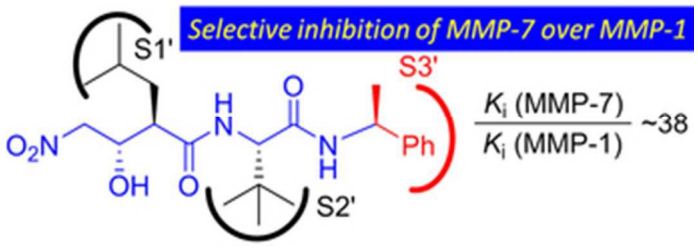

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