Supporting Information for

Ageladine A: an Anti-angiogenic matrixmetalloproteinase inhibitor from the Marine Sponge

Agelas nakamurai

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Experimental Section

General Experimental Procedures. FAB mass spectra were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using glycerol as a matrix. Positive ion mode HR-FABMS were obtained at a resolution of 5000 using PEG 400 as a marker. Optical rotations were determined on a Jasco DIP-1000 digital polarimeter in MeOH. UV spectra were recorded on a SHIMADZU BioSpec-1600 UV photometer in MeOH. NMR spectra were recorded on a JEOL [600 NMR spectrometer at 600 MHz for ^1^H and 150 MHz for ^1^C. Chemical shifts of ^1^H and ^1^C NMR spectra were referenced to the solvent peaks: [H] 3.30 and [C] 49.0 for CD$_3$OD. Fluorescent spectrum and fluorescent measurements of enzyme inhibition assay were performed on a Molecular Devices SPECTRA MAX GEMINI fluorescence photometer.

Animal Material. The sponge was collected by hand using SCUBA at a depth of 10-15 m off Kuchinoerabu-jima Island, southern Japan (30° 28’ N; 130° 13’ E) in July 1993. The specimens were immediately frozen and preserved at -20 °C until extraction. The sponge was identified as Agelas nakamura Hoshino (Agelasida, Agelasidae); a voucher specimen was deposited at Zoological Museum, University of Amsterdam (ZMA POR. 16996).

Extraction and Isolation. The frozen specimen (400 g) was homogenized and extracted with MeOH, CHCl$_3$/MeOH (1:1), and acetone (1.2 L x 2, each). The combined extracts were concentrated and partitioned between CHCl$_3$ and H$_2$O, and the aqueous layer was further extracted with n-BuOH. The CHCl$_3$ and n-BuOH extracts were combined and partitioned between n-hexane and 90% aqueous MeOH. The active aqueous MeOH layer was fractionated by ODS flash chromatography using stepwise elution of aqueous MeOH, in which fractions eluted with 40 % to 60 % aqueous MeOH were combined and chromatographed on a Sephadex LH-20 column with MeOH to obtain 9 fractions. The active last two fractions were combined and successively fractionated by ODS HPLC on Cosmosil AR-II with 30 % aqueous MeCN containing 0.05 % TFA. Obtained active fraction was finally purified by reversed phase chromatography on Asahipak GS-320 with a solvent system of 25 % aqueous MeCN containing 0.05 % TFA to yield ageladine A (1: 4.9 mg, 1.2 x 10$^{-3}$ % yield based on wet weight).

Ageladine A (1): yellow powder; UV (MeOH) $\lambda_{max}$ 381 nm (1.22 x 10$^4$), 250 nm (9.96 x 10$^3$), 227
Methylation of Ageladine A. Ageladine A (20 mg) was dissolved in 10 mL of ice cooled THF, and the solution was poured into round bottom flask containing 150 mg of NaH. The solution was stirred at 0 °C for 30 min and at rt for 1 h. To the reaction mixture was added 300 μL of MeI, and the mixture was stirred at room temperature overnight. The reaction was quenched by the addition of H₂O (5 mL), and the mixture was briefly evaporated. To the residue was added 10 mL of H₂O and extracted three times with 15 mL of CHCl₃. The combined organic extracts were fractionated by two steps of reversed phase HPLC on Asahipak GS-320 column with 30 % MeCN containing 0.05 % TFA to afford three methylated derivatives 2-4.

1, 14, 15-Trimethylageladine A (2): yellowish powder; UV (MeOH) λ_max 357 nm (ε 3.46 x 10³), 237 nm (ε 9.13 x 10³); ¹H NMR data (CD₃OD) δ 8.22 (d, J = 6.3 Hz, 1H), 7.67 (d, J = 6.3 Hz, 1H), 6.81 (s, 1H), 3.76 (s, 3H), 3.71 (s, 3H), 3.12 (s, 3H); ¹³C NMR data (CD₃OD) δ 162.0, 148.2, 140.7, 133.5, 130.3, 126.2, 116.8, 111.9, 104.8, 37.0, 30.0, 29.8; HR-FABMS (glycerol) m/z 399.9606 (calcd for C_{13}H_{14}N₅S²⁹Br³¹Br 399.9595).

1, 7, 14, 15-Tetramethylageladine A (3): yellowish powder; UV (MeOH) λ_max 331 nm (ε 5.80 x 10³), 239 nm (ε 1.73 x 10³); ¹H NMR data (CD₃OD) δ 8.46 (d, J = 6.7 Hz, 1H), 7.70 (d, J = 6.7 Hz, 1H), 6.72 (s, 1H), 4.09 (s, 3H), 3.70 (s, 3H), 3.53 (s, 3H), 3.07 (s, 3H); ¹³C NMR data (CD₃OD) δ 161.8, 147.2, 143.7, 139.0, 131.5, 123.1, 117.0, 110.2, 105.3, 46.0, 35.9, 30.0, 29.8; HR-FABMS (glycerol) m/z 413.9758 (calcd for C_{12}H_{16}N₅S²⁹Br³¹Br 413.9752).

1, 4, 7, 14, 15-Pentamethylageladine A (4): yellowish powder; UV (MeOH) λ_max 331 nm (ε 2.35 x 10³), 239 nm (ε 1.13 x 10³); ¹H NMR data (CD₃OD) δ 8.50 (d, J = 6.5 Hz, 1H), 7.73 (d, J = 6.5 Hz, 1H), 3.99 (s, 3H), 3.71 (s, 3H), 3.42 (s, 3H), 3.06 (s, 3H), 1.91 (s, 3H); ¹³C NMR data (CD₃OD) δ 162.1, 147.5, 144.5, 139.2, 131.3, 123.7, 120.2, 109.3, 105.3, 45.3, 35.3, 31.0, 30.8, 11.7; HR-FABMS
(glycerol) m/z 427.9921 (calcd for C_{14}H_{16}N_{5}^{79}Br^{81}Br 427.9909).

**MMP-2 Inhibition Assay.** Purified recombinant MMP-2 which was expressed by insect cells and fluorescent substrate MOCAc-Pro-Leu-Gly-Leu-A_{3}pr(Dnp)-Ala-Arg-NH_{2} purchased from Peptide Institute Inc., Osaka, were used. Inhibition assay of MMP-2 was carried out by the modified procedure of Knight *et al.*[Knight, C. G.; Willenbrock, F.; Murphy, G. *FEBS Lett.* **1992**, *296*, 263-266.] Test samples (2 μL) were added to wells of 96-well microtiter plates, each of which contained 100 μL of TNC buffer (50 mM Tris-HCl pH 7.5 + 150 mM NaCl + 10 mM CaCl_{2} + 0.02% NaN_{3} + 0.05% Brij-35). Aliquots of 50 μL of enzyme solution (5 ng/mL) were added to this solution, and pre-incubated at 37 °C for 10 min. After pre-incubation, 50 μL of substrate solution (10 μM) was added to the mixture to begin the reaction. The fluorescence values were measured at an excitation of 328 nm and an emission of 393 nm after incubation at 37 °C for 1 h.

**Kinetic Analysis.** Kinetic analysis was performed based on the MMP-2 inhibition assay, using six substrate concentrations from 0.5 to 7.5 μM and seven ageladine concentrations from 0 to 10 μg/mL. The lines were crossed on X-axis in Lineweaver-Burk Plot, thus indicating the non-competitive inhibition mechanism of ageladine A.

**Zn^{2+} Titration Experiment.** Six aliquots of ageladine A (5 μmol each) were dissolved in 170 μL of CD_{3}OD and 0.0, 0.05, 0.2, 0.5, 1.0, and 2.0 equivalent of ZnCl_{2} was added to each solution. Their \(^{1}\text{H}\) NMR spectra were measured. As control experiments, the \(^{1}\text{H}\) NMR spectra of 1,10-phenanthroline (Zn^{2+} chelator) and 4,7-phenanthroline (not Zn^{2+} chelator) were similarly observed.

**In vitro vascular formation assay.**

In vitro vascular formation assay was performed following the procedure by Yamashita et al. [Yamashita, J. *et al.* *Nature*, **2000**, *408*, 92-96]. Maintenance, differentiation of CCE ES cells (gift from M. J. Evans) and cell sorting were as described [Nishikawa *et al.* *Development*, **1998**, *125*, 747-1757]. Sorted Flk1\(^{+}\) cells (4 x 10\(^{5}\) cells/ml) were incubated in differentiation medium (alpha-MEM supplemented with 10% FCS and 5 x 10\(^{5}\) M 2-mercaptoethanol) containing 50 ng/ml VEGF on uncoated petri dishes for 12 h to induce aggregate formation. Aggregates were resuspended in 2 x
differentiation medium and then mixed with the same volume of 3 mg/mL collagen I-A gel (final concentration 1.5 mg/mL) (Nitta Gelatin, Osaka, Japan). Two hundred fifty μl of this mixture was applied onto a lucent insert disc, Cell Disk (Sumitomo Bakelite, Tokyo, Japan), in 24-well dishes. After 30 minutes incubation at 37 °C to allow polymerization of collagen gel, 740 μl differentiation medium with VEGF (final 50 ng/ml) and 10 μl of sample solution were added to each well. Culture medium and sample solution were replaced every two days.
Figure S1. $^1$H NMR spectrum of ageladine A (1) in CD$_3$OD.

Figure S2. $^{13}$C NMR spectrum of ageladine A (1) in CD$_3$OD.
**Figure S3.** COSY spectrum of ageladine A (1) in CD$_3$OD.

**Figure S4.** HMQC spectrum of ageladine A (1) in CD$_3$OD.
Figure S5. HMBC spectrum of ageladine A (1) in CD$_3$OD.

Figure S6. FABMS spectrum of ageladine A (1) (positive mode).
Figure S7. $^1$H NMR spectrum of 1, 14, 15-trimethyl ageladine A (2) in CD$_3$OD.

Figure S8. HMQC spectrum of 1, 14, 15-trimethyl ageladine A (2) in CD$_3$OD.
Figure S9. HMBC spectrum of 1, 14, 15-trimethyl ageladine A (2) in CD$_3$OD.

Figure S10. $^1$H NMR spectrum of 1, 7, 14, 15-tetramethyl ageladine A (3) in CD$_3$OD.
Figure S11. HMQC spectrum of 1, 7, 14, 15-tetramethyl ageladine A (3) in CD$_3$OD.

Figure S12. HMBC spectrum of 1, 7, 14, 15-tetramethyl ageladine A (3) in CD$_3$OD.
**Figure S13.** $^1$H NMR spectrum of 1, 4, 7, 14, 15-pentamethyl ageladine A (4) in CD$_3$OD.

**Figure S14.** HMQC spectrum of 1, 4, 7, 14, 15-pentamethyl ageladine A (4) in CD$_3$OD.
Figure S15. HMBC spectrum of 1, 4, 7, 14, 15-pentamethyl ageladine A (4) in CD$_3$OD.

Figure S16. $^1$H NMR spectra of ageladine A (1) and ZnCl$_2$ mixture.
Figure S17. $^1$H NMR spectra of 1,10-phenanthroline and ZnCl$_2$ mixture.

Figure S18. $^1$H NMR spectra of 4,7-phenanthroline and ZnCl$_2$ mixture.
Figure S19. Lineweaver-Burk plot for ageladine A (1) against MMP-2.