Supplementary Information

Development of a highly sensitive, highly selective and high-throughput method for determination of total ascorbic acid

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3. Reference

1. Experimental

1-1. *Determination of AA with OPD*

The determination of ascorbic acid (AA) with OPD was performed according to the previously reported method of Vislisel *et al*.[1] Briefly, AA solutions prepared at various concentrations in acetate buffer (2.0 M, pH 5.5) were dispensed in aliquots of 40 μL into a 96-well black plate. Then 20 μL of 5.0 mM TEMPO/2.0 M acetate buffer (pH 5.5) was added to each well and incubated at room temperature for 10 min. After 10 min, 40 μL of 2.5 mM OPD/2.0 M acetate buffer (pH 5.5) was added to each well to initiate the reaction. Ten min after the start of the reaction, fluorescence intensity (Ex.: 345 nm, Em.: 425 nm) was measured by a microplate reader (Infinite 200 Pro M Nano plus, Tecan Japan Corporation, Tokyo, Japan).

1-2. *Study of conditions for reaction solvent and reaction temperature in the determination of AA with MDB*

The reaction solvents were 2.0 M acetate buffer (pH 5.5) and 100 mM (pH 5.5) citrate buffer. AA and α-keto acids prepared at 0.1-300 μM in acetate buffer or citrate buffer were aliquoted in 40 μL volumes in a 96-well black plate. After the addition of AA and α-keto acids, 20 μL of 5.0 mM TEMPO/2.0 M acetate buffer (pH 5.5) or 100 mM citrate buffer (pH 5.5) was added to each well and incubated for 10 min at room temperature. After 10 min, 40 μL of 2.5 mM MDB/2.0 M acetate buffer (pH 5.5) or 100 mM citrate buffer (pH 5.5) was added to each well and reacted with AA or α-keto acids for 2.5 h at room temperature or 60 °C. The fluorescence intensity was measured using an Infinite 200 Pro M Nano plus microplate reader at Ex./Em. of 367/446 nm.

1-3. ***Study of the condition for pH of the reaction solvent in the determination of AA with MDB***

The reaction was performed in 100 mM citrate buffer. AA and α-keto acids prepared at 0.1-300 μM in citrate buffer (pH 4.0, 4.5, 5.0, 5.5 or 6.0) were aliquoted in 40 μL volumes in a 96-well black plate. After the addition of AA and α-keto acids, 20 μL of 5.0 mM TEMPO/100 mM citrate buffer (pH 4.0, 4.5, 5.0, 5.5 or 6.0) was added to each well and incubated for 10 min at room temperature. After 10 min, 40 μL of 2.5 mM MDB /100 mM citrate buffer (pH 4.0, 4.5, 5.0, 5.5 or 6.0) was added to each well and reacted with AA or α-keto acids for 2.5 h at room temperature. Fluorescence intensity was measured using an Infinite 200 Pro M Nano plus microplate reader at Ex./Em. of 367/446 nm.

1-4. ***Study of the condition for reaction time in the determination of AA with MDB***

The reaction was performed in 100 mM citrate buffer (pH 5.0). AA and α-keto acids prepared at 10 μM in citrate buffer were aliquoted in 40 μL volumes in a 96-well black plate. After the addition of AA and α-keto acids, 20 μL of 5.0 mM TEMPO/100 mM citrate buffer (pH 5.0) was added to each well and incubated for 10 min at room temperature. After 10 min, 40 μL of 2.5 mM MDB/100 mM citrate buffer (pH 5.0) was added to each well and reacted with AA or α-keto acids at room temperature. Fluorescence intensity (Ex.: 367 nm, Em.: 446 nm) of the reaction solution was measured at 5 min, 10 min, 15 min, 30 min, 1 h, 1.5 h, 2 h and 2.5 h after the start of the reaction.

2. Results

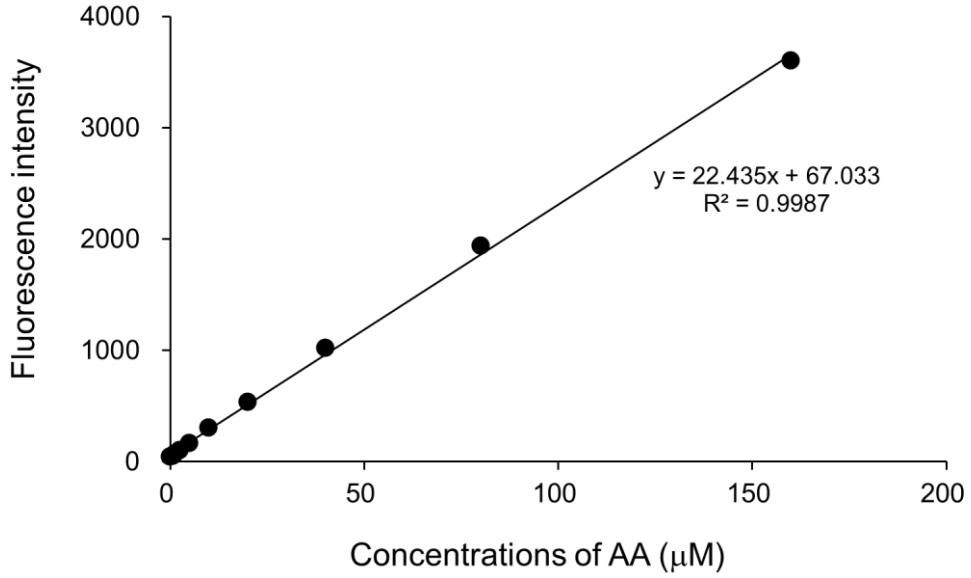

Fig. S1 Calibration curve of AA by OPD fluorescence derivatization.

The reaction was carried out in 2.0 M acetate buffer (pH 5.5). AA at 0.625-160 μM was dispensed into 96-well black plates, and the oxidation reaction was carried out by TEMPO. Then OPD was added and reacted with AA. Fluorescence intensity was measured by a microplate reader (Ex.: 367 nm, Em.: 446 nm). The calibration curve was prepared by plotting the concentrations of AA vs. the fluorescence intensities. Values are means ± SD for three independent experiments. The absence of an SD bar means that the SD bar is within the symbol.

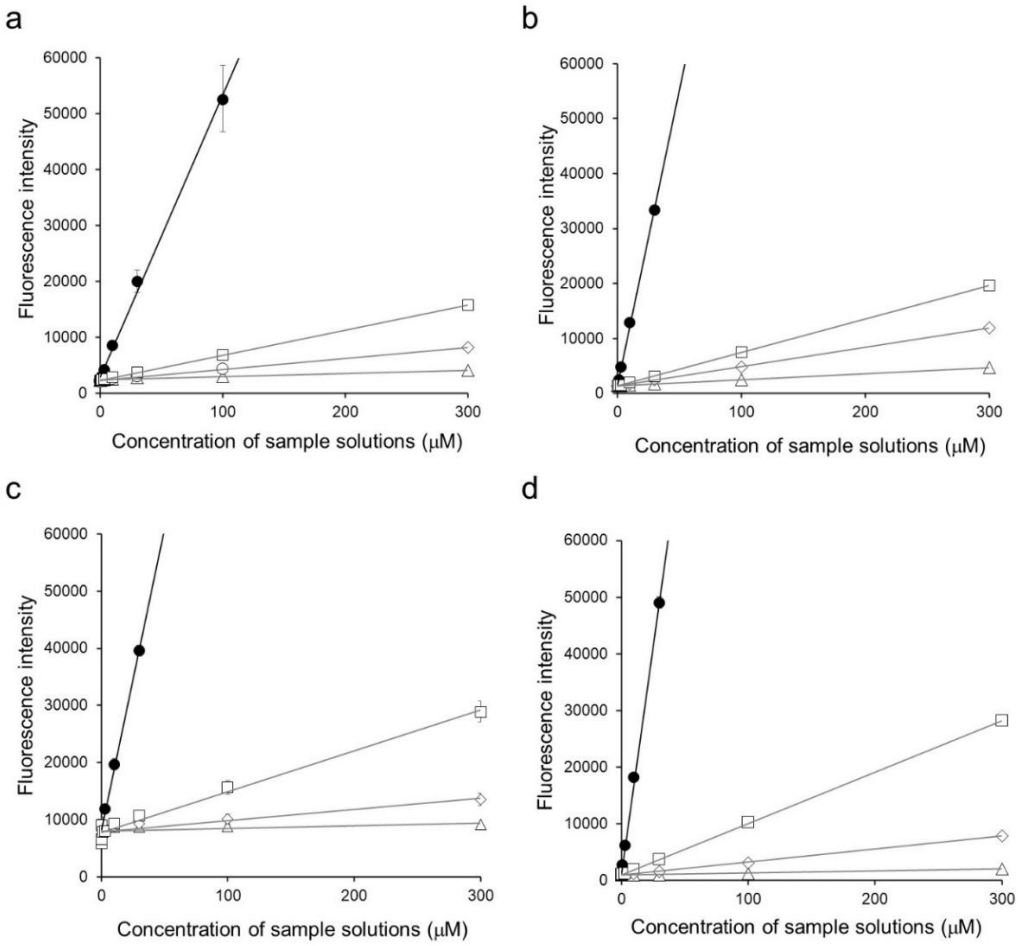

Fig. S2 Consideration of the reaction buffer and reaction temperature in the determination of AA and α-keto acids by MDB fluorescence derivatization.

The reaction was carried out in 2.0 M acetate buffer (pH 5.5) (a and b) or 100 mM citrate buffer (pH 5.5) (c and d). AA (●), α-ketoglutaric acid (△), oxaloacetic acid (◇), and pyruvic acid (□) solutions at 0.1, 0.3, 1, 3, 10, 30, 100 and 300 μM were dispensed into 96-well black plates. To each sample solution, TEMPO solution was added and incubated for oxidization. Then MDB solution was added to the oxidized sample to react at 60 °C (a and c) or room temperature (b and d) for 2.5 h. The fluorescence intensities of the reaction mixtures were analyzed in a microplate reader (Ex.: 367 nm, Em.: 446 nm). Values are means ± SD for three independent experiments. The absence of an SD bar means that the SD bar is within the symbol.

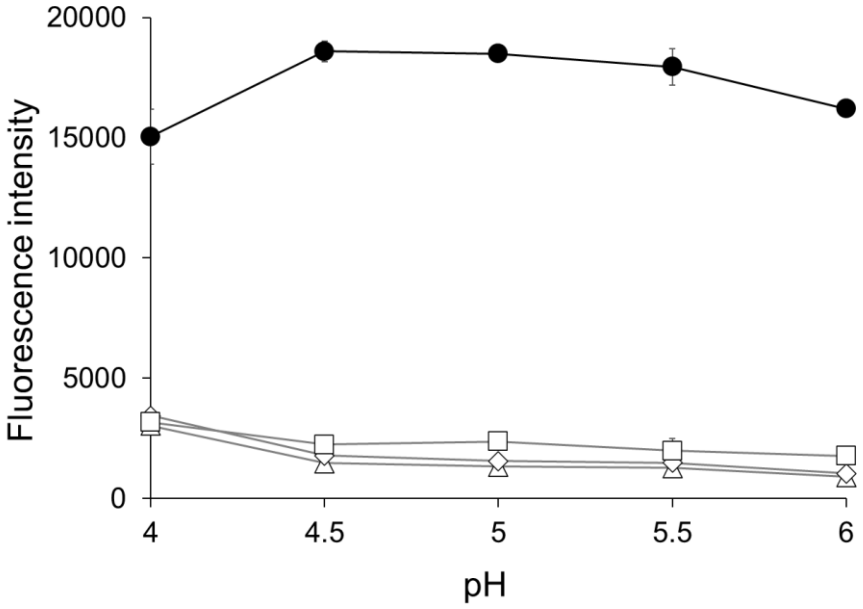

Fig. S3 Effect of citrate buffer pH on fluorescence intensities of MDB fluorescent derivatives with AA and α-keto acids.

MDB derivatization reactions of AA or α-keto acids were performed in citrate buffer (pH 4.0, 4.5, 5.0, 5.5 and 6.0). AA (●), α-ketoglutaric acid (△), oxaloacetic acid (◇), and pyruvic acid (□) solutions at 10 μM were dispensed into 96-well black plates. To each sample solution, TEMPO solution was added and incubated for oxidization. Then MDB solution was added to the oxidized sample to react at room temperature for 2.5 h. The fluorescence intensities of the reaction mixtures were analyzed in a microplate reader (Ex.: 367 nm, Em.: 446 nm). Values are means ± SD for three independent experiments. The absence of an SD bar means that the SD bar is within the symbol.

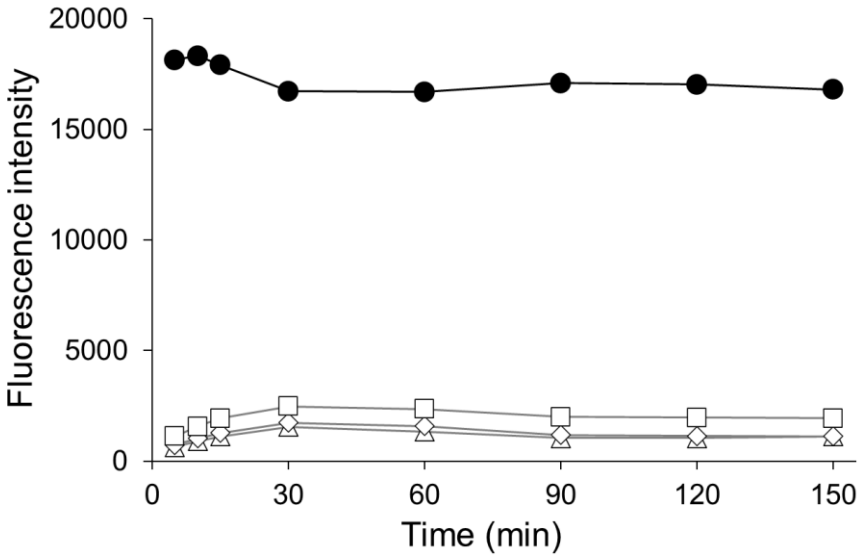

Fig. S4 Effect of reaction time on fluorescence intensities of MDB fluorescent derivatives with AA and α-keto acids.

MDB derivatization reactions of AA or α-keto acids were performed in citrate buffer (pH 5.0). AA (●), α-ketoglutaric acid (△), oxaloacetic acid (◇), and pyruvic acid (□) solutions at 10 μM were dispensed into 96-well black plates. To each sample solution, TEMPO solution was added and incubated for oxidization. Then MDB solution was added to the oxidized sample to react at room temperature for 5, 10, 15, 30, 60, 90, 120 and 150 min. Fluorescence intensities of the reaction mixtures were analyzed in a microplate reader (Ex.: 367 nm, Em.: 446 nm). Values are means ± SD for three independent experiments. The absence of an SD bar means that the SD bar is within the symbol.

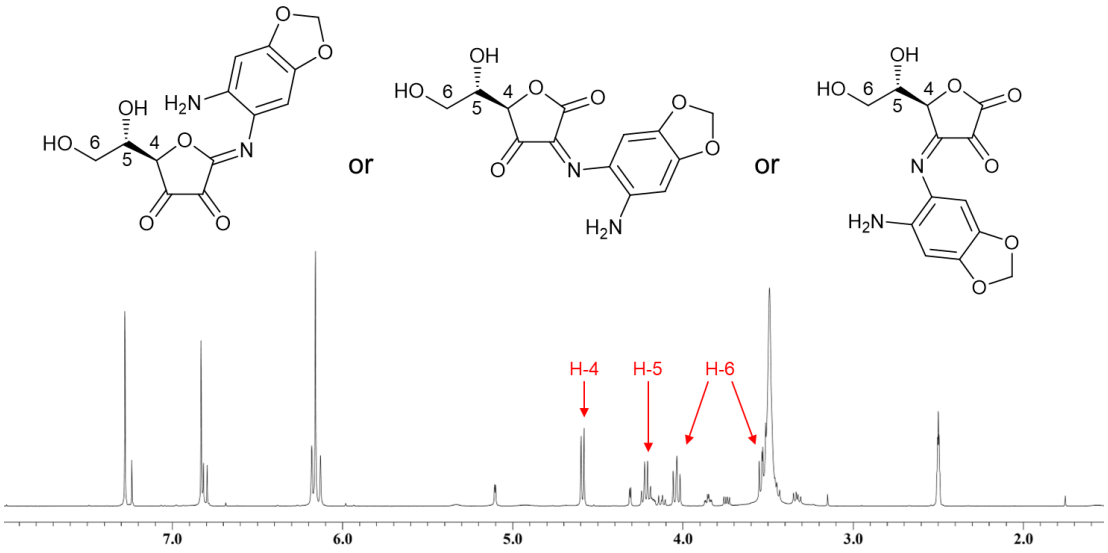

Fig. S5 ^{1}H-NMR spectrum of compound **1**.

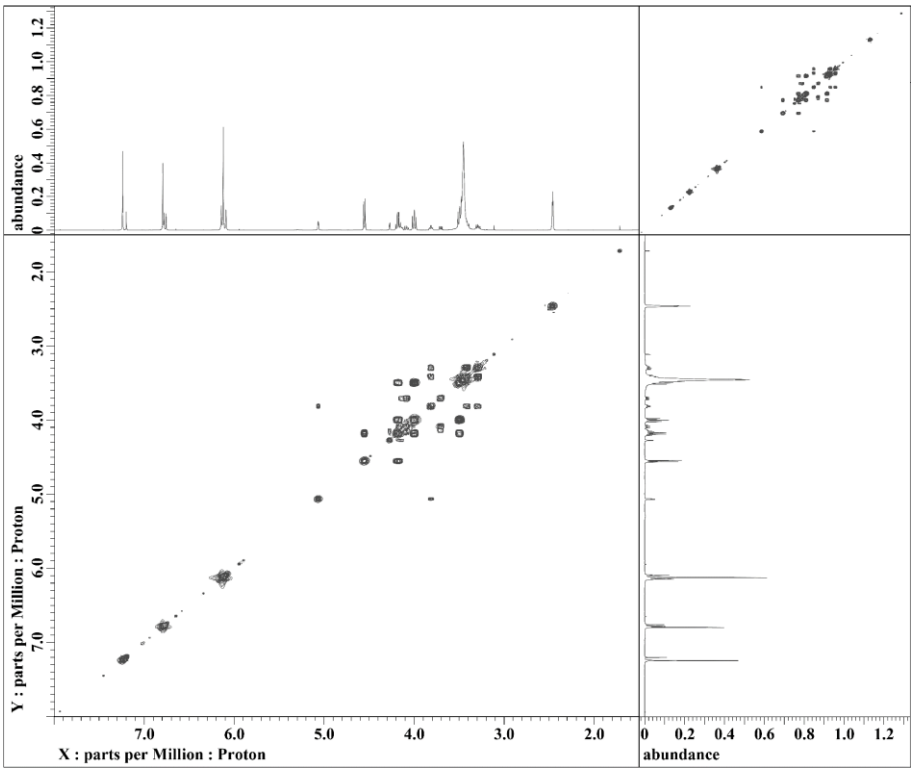

Fig. S6 ^{1}H-^{1}H COSY spectrum of compound **1**.

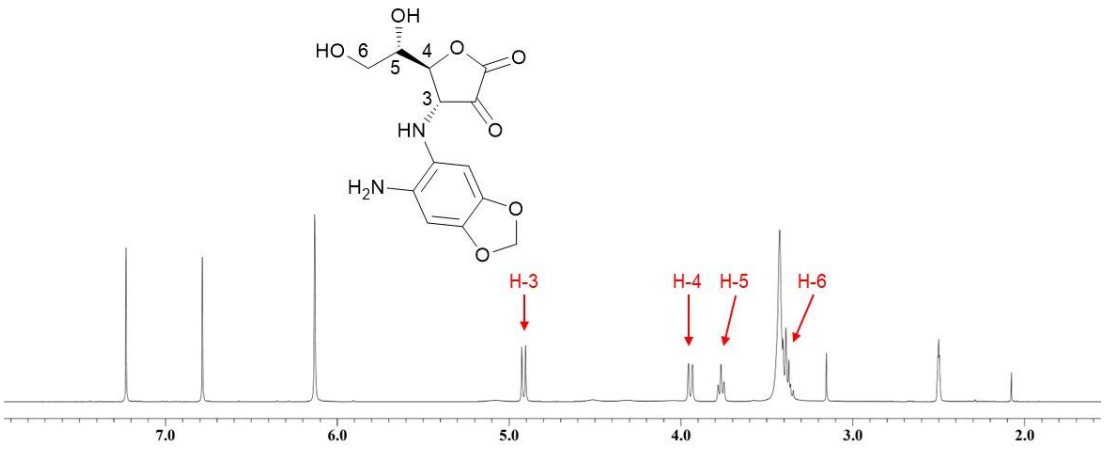

Fig. S7 ^{1}H-NMR spectrum of compound **2**.

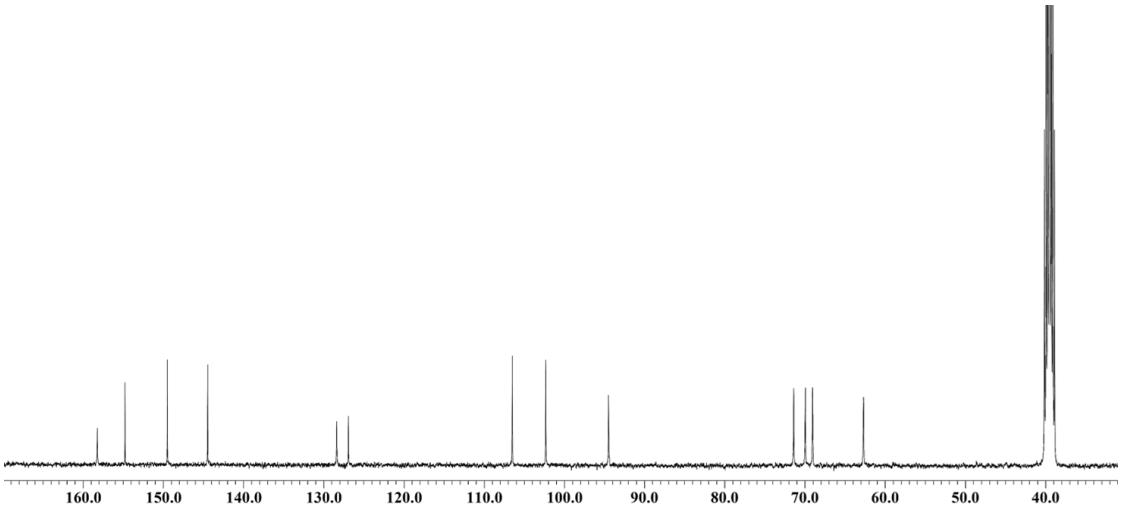

Fig. S8 ^{13}C-NMR spectrum of compound **2**.

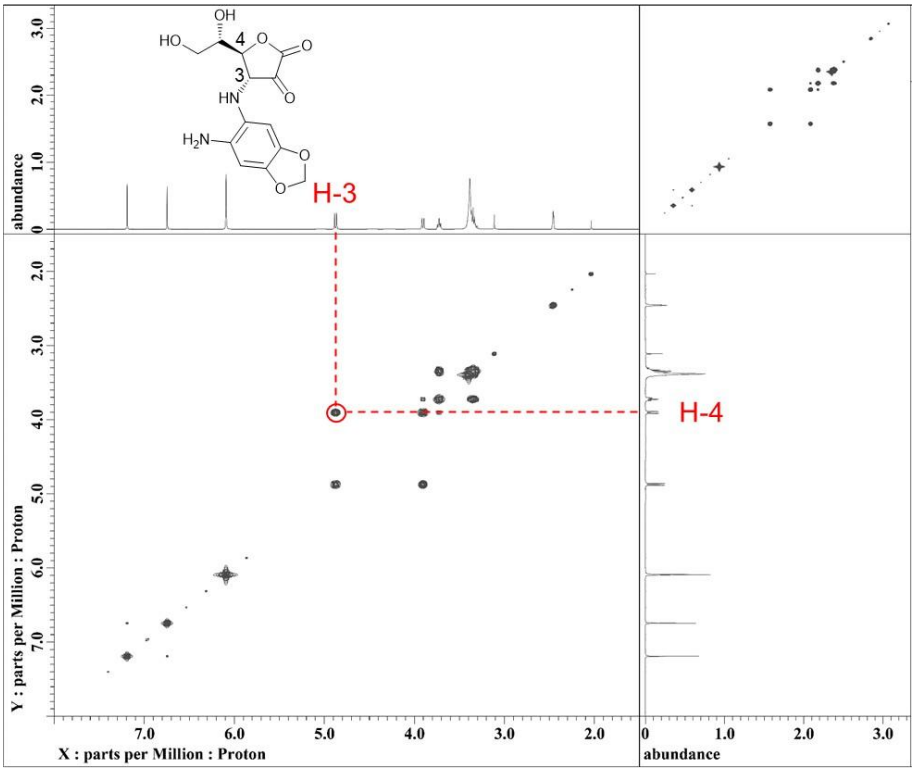

Fig. S9 ^{1}H-^{1}H COSY spectrum of compound **2**.

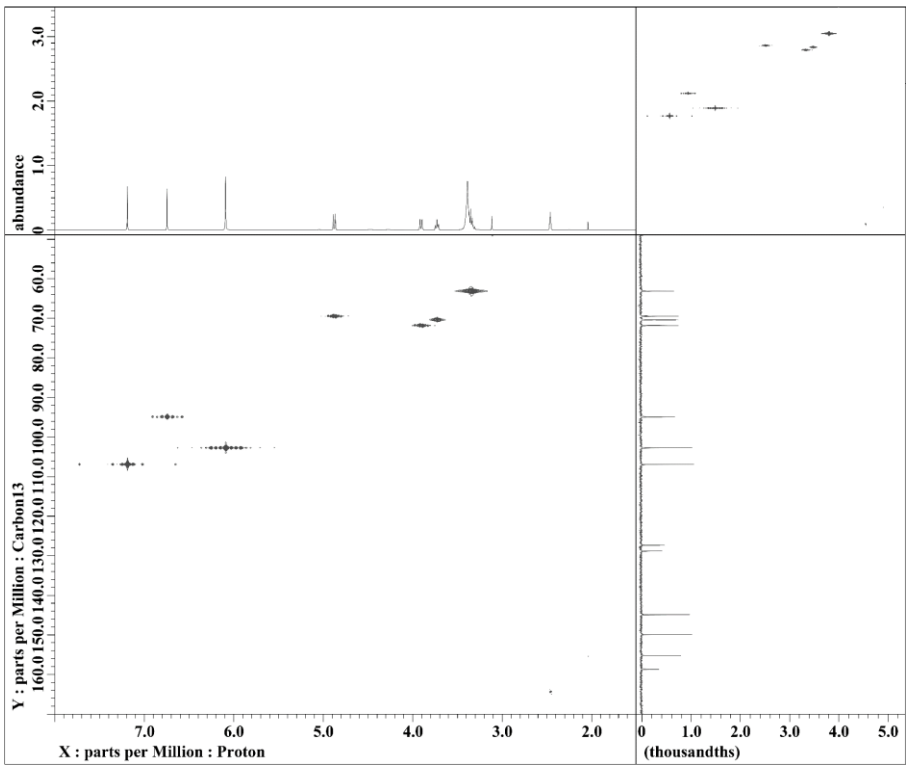

Fig. S10 HMQC spectrum of compound **2**.

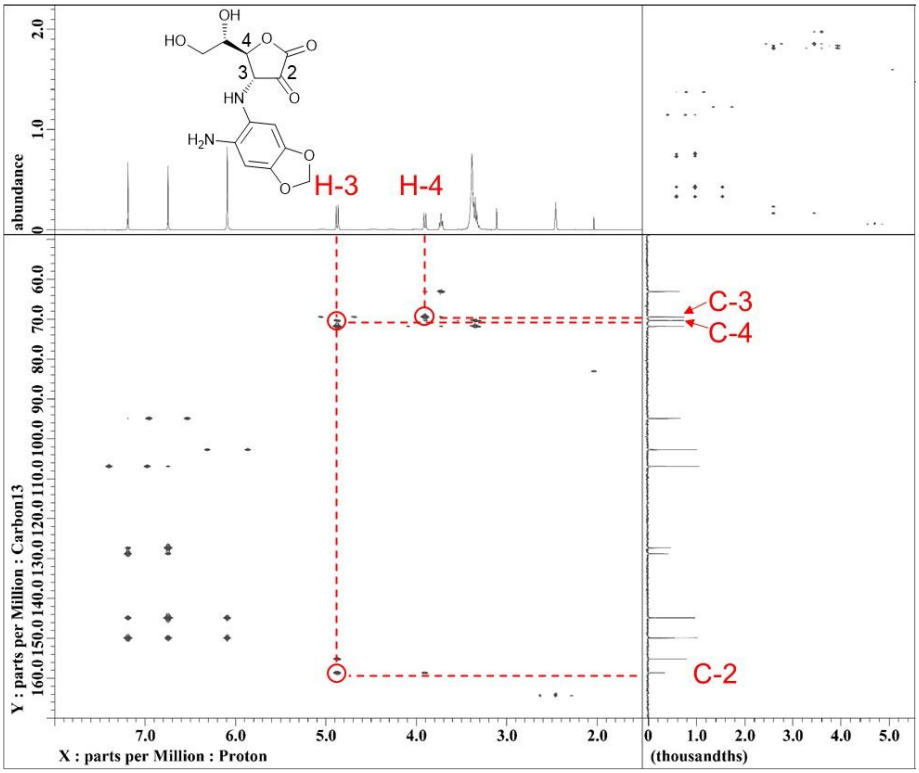

Fig. S11 HMBC spectrum of compound **2**.

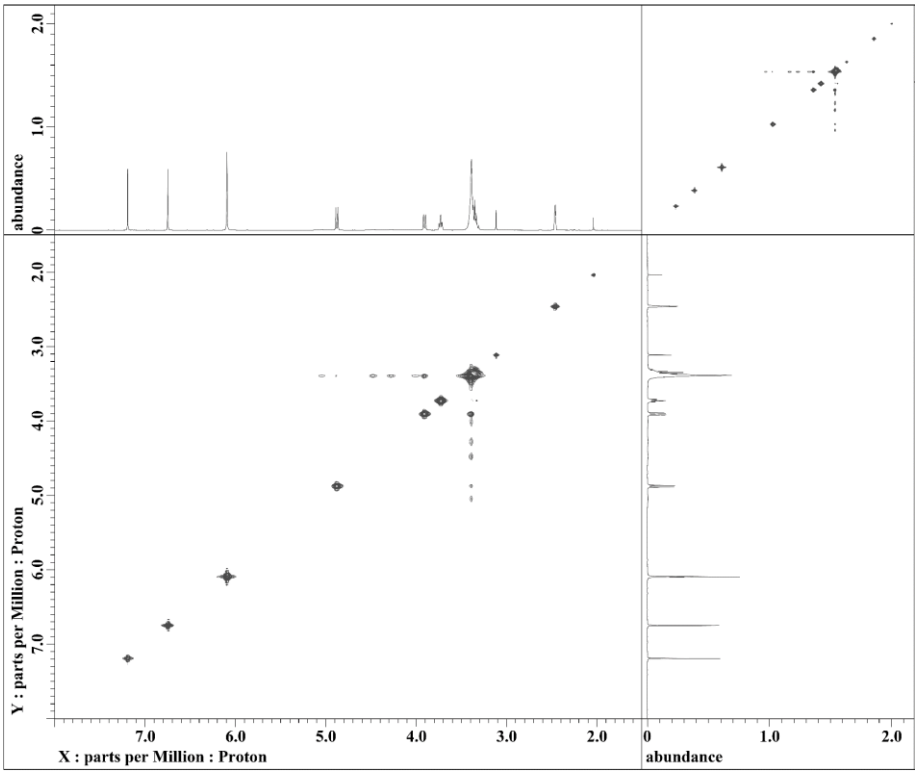

Fig. S12 NOESY spectrum of compound **2**.

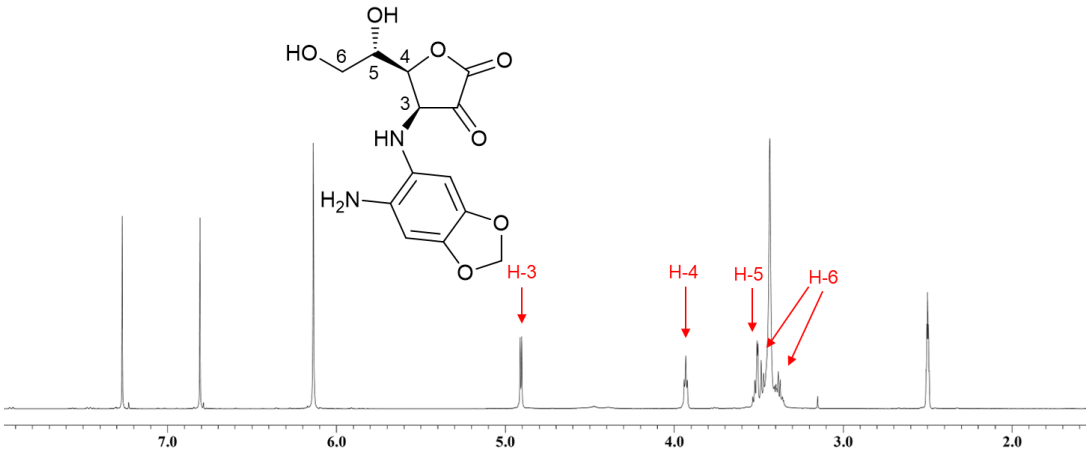

Fig. S13 ^{1}H-NMR spectrum of compound **3**.

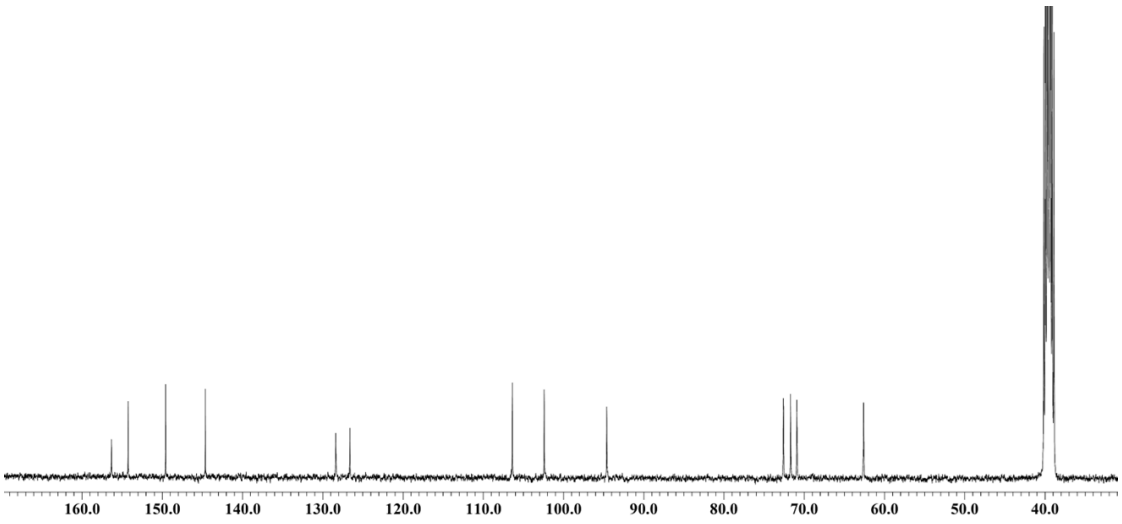

Fig. S14 ^{13}C-NMR spectrum of compound **3**.

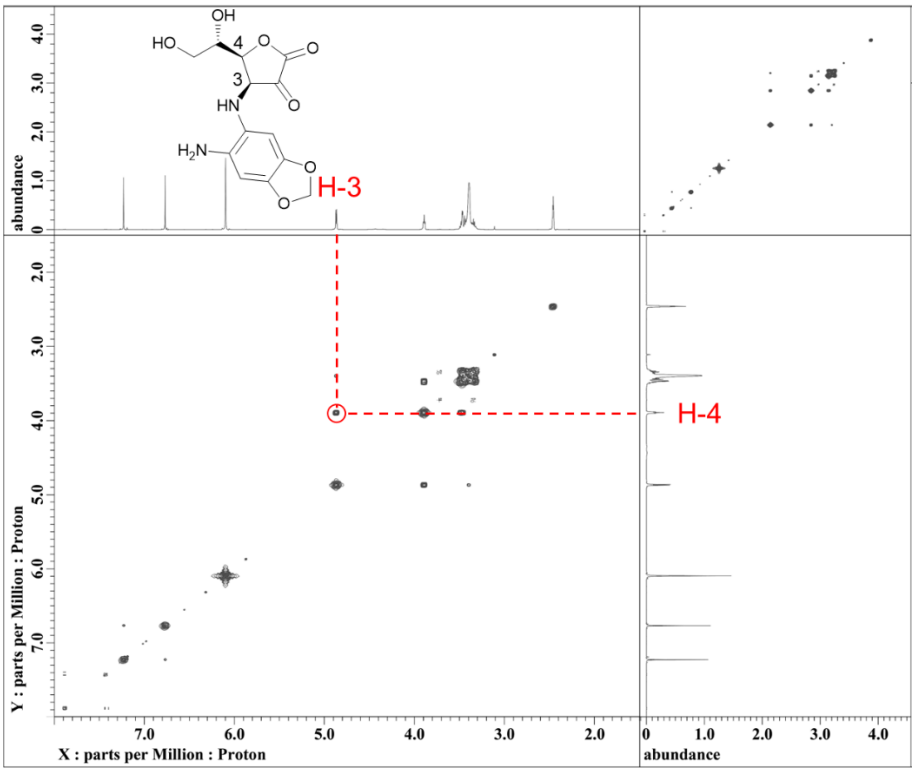

Fig. S15 ^{1}H-^{1}H COSY spectrum of compound **3**.

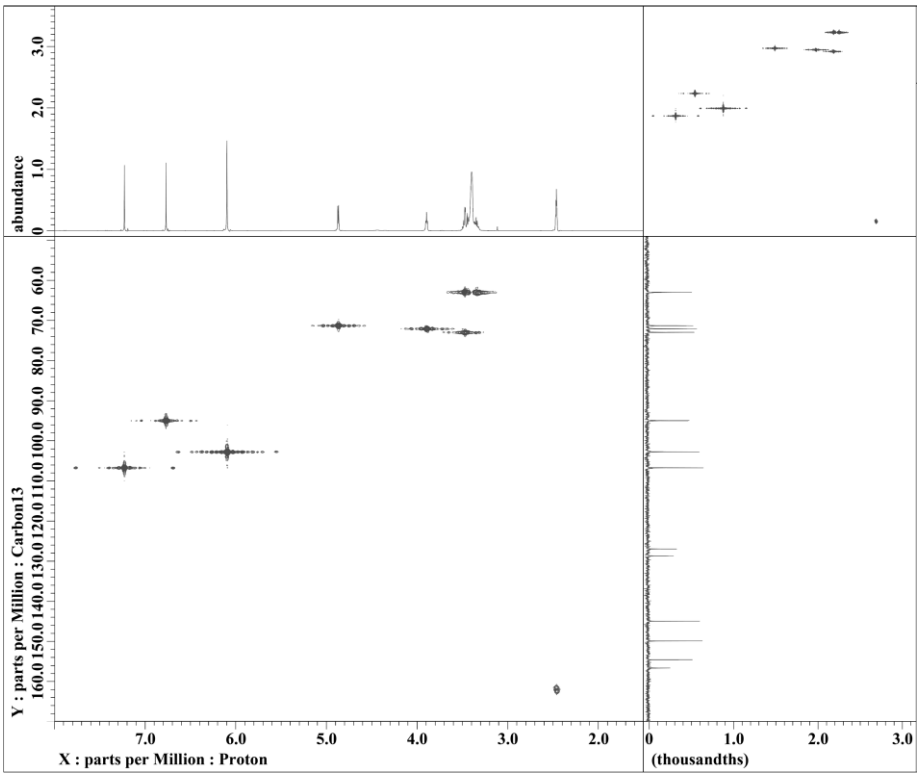

Fig. S16 HMQC spectrum of compound **3**.

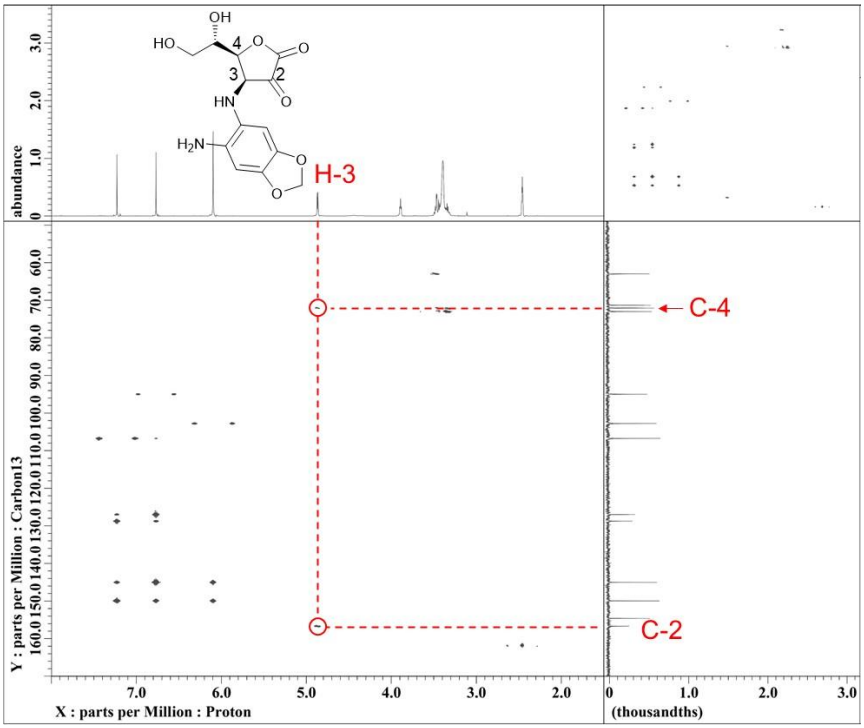

Fig. S17 HMBC spectrum of compound **3**.

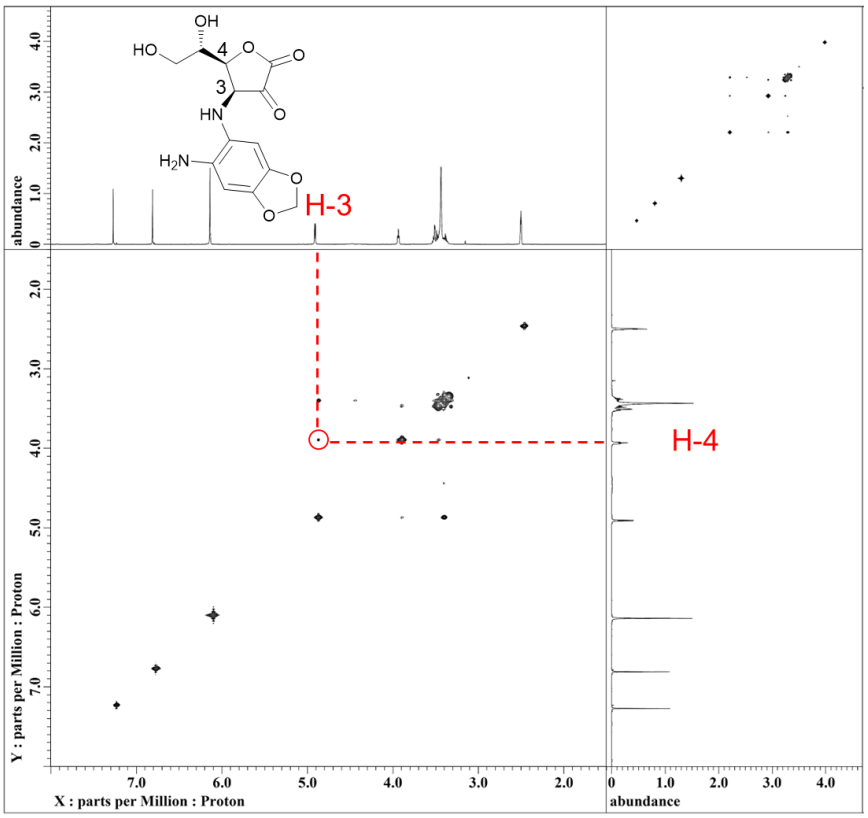

Fig. S18 NOESY spectrum of compound **3**.

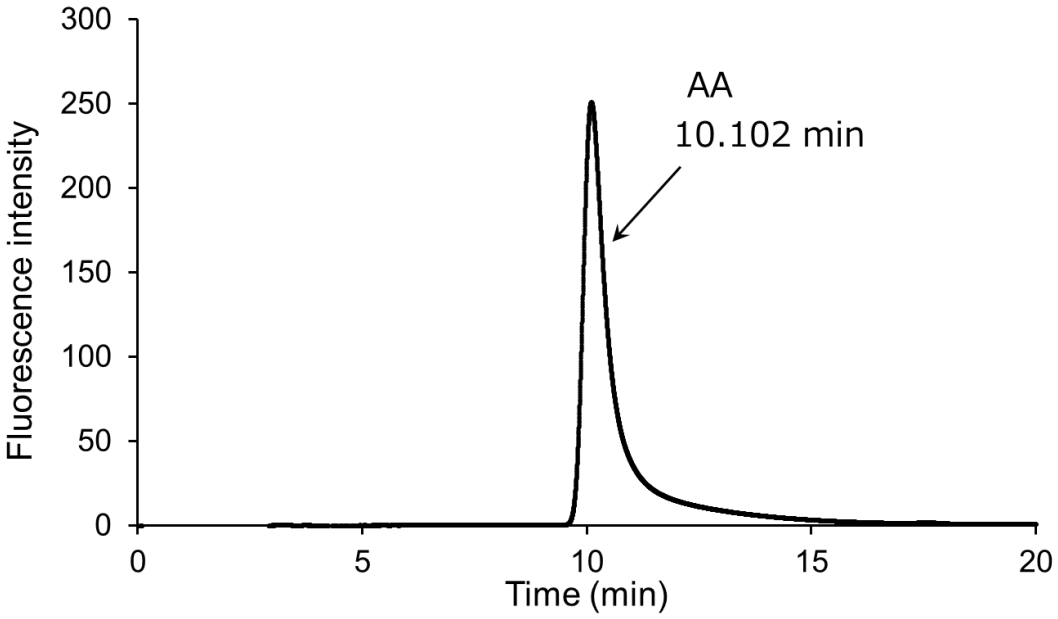

Fig. S19 HPLC analysis of reaction between AA and MDB.

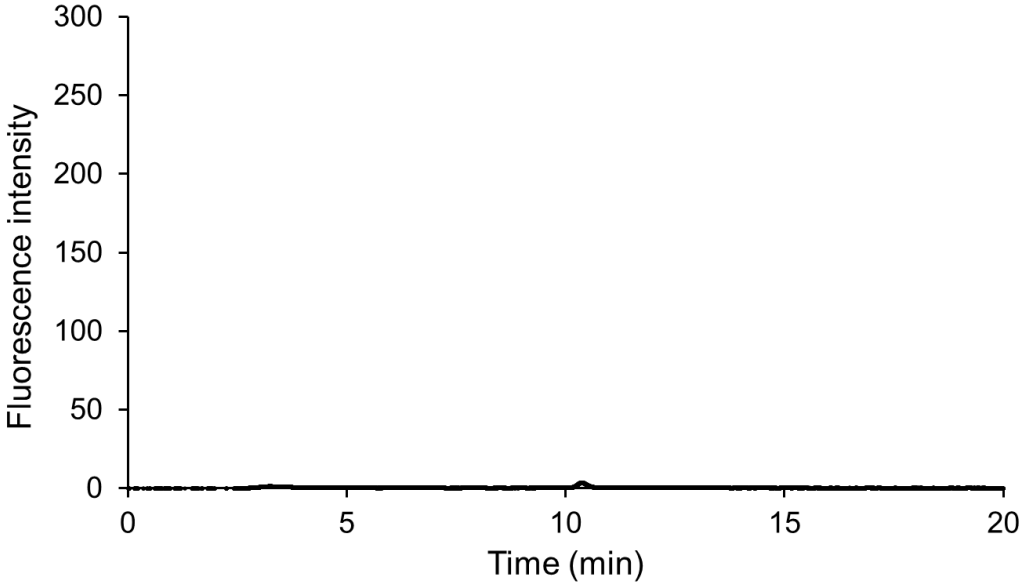

Fig. S20 HPLC analysis of reaction between α-ketoglutaric acid and MDB.

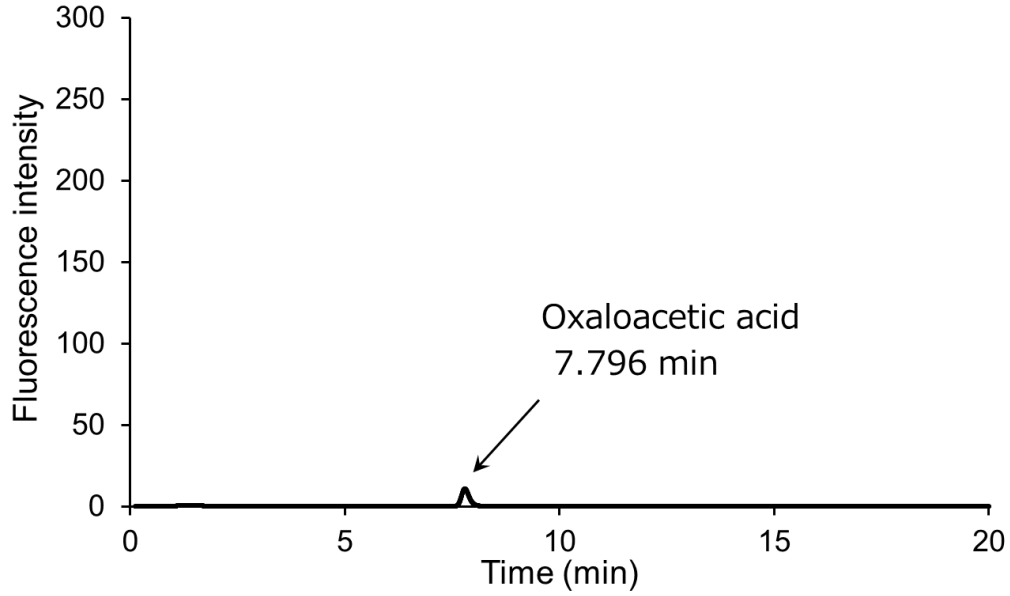

Fig. S21 HPLC analysis of reaction between oxaloacetic acid and MDB.

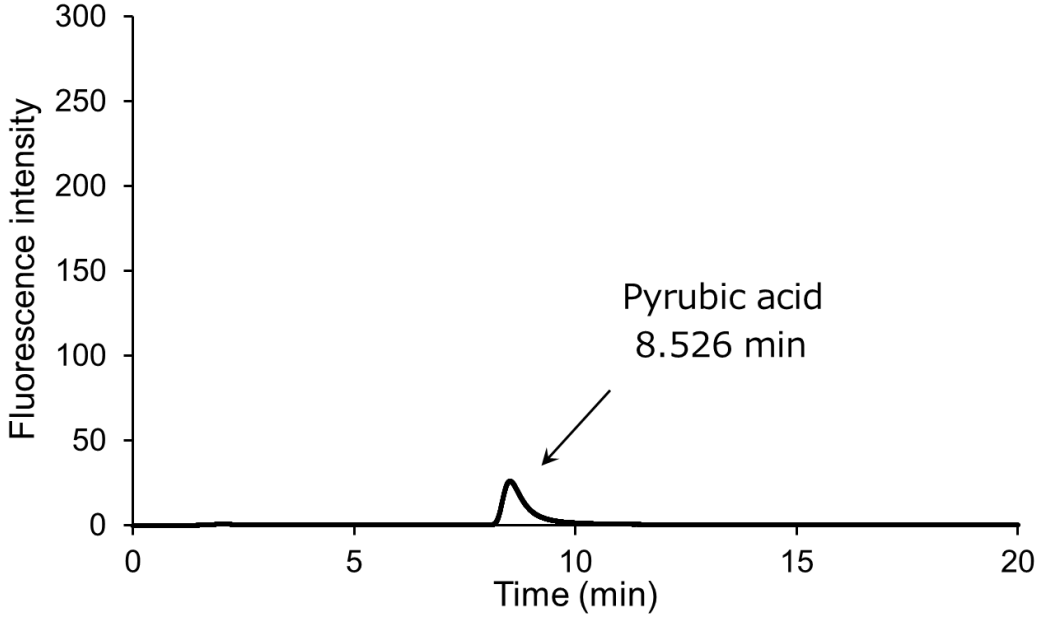

Fig. S22 HPLC analysis of reaction between pyruvic acid and MDB.

3. Reference

1. J. M. Vislisel, F. Q. Schafer and G. R. Buettner, *Anal. Biochem.*, 2007, **365**, 31-39.