Metabolism of Gambogic Acid in Rats: a Rare Intestinal Metabolic Pathway Responsible for its Final Disposition

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Supplemental experimental

***In vitro* rat intestinal contents incubation** The rats without dose were euthanized by cervical dislocation followed by removing the total intestinal tract immediately. The total intestinal contents of each rat were then collected and homogenized in 5 mL of water. The reactions were initiated by the addition of 100 μL of 1.0 mg/mL GA solution in the homogenate and carried out at 37 °C water bath. After approximately 24 h, 1.0 mL of sample from each tube was extracted with the same procedures as the plasma sample preparation, and then analyzed by LC-MS/MS. This experiment was performed in duplicate. The LC-MS/MS method was performed using an Agilent Technologies Series 6410B LC-MS/MS system (Agilent Technologies, Palo Alto, CA, USA), including an Agilent 1200 rapid LC system and an Agilent 6410B triple-quadrupole mass spectrometer equipped with an electrospray source. A Sepax HPC18 column, 3 μm, 150 × 2.1 mm i.d. (Sepax Technologies, Inc, Newark, USA) protected by a SecurityGuard C18 column, 5 μm, 4 × 2.0 mm i.d. (Phenomenex, Torrance, CA, USA) was used for all measurements. The Signal acquisition, peak integration and concentration determination were performed using the MassHunter supplied by Agilent Technologies. The mobile phase, delivered at a constant flow rate of 0.25 mL/min, consisted of a gradient of solvent A (acetonitrile) in solvent B (10 mM ammonium acetate in water), as follows: 0 to 15 min, 50 % A; 20 to 45 min, 90 % A; 45.1 to 60 min, 50 % A. The column temperature was maintained at 25 °C. The triple-quadrupole mass spectrometer equipped with an ESI source was set with the drying gas (N_2) flow of 10 L/min, nebulizer pressure of 40 psig, drying gas

temperature of 350 °C, capillary voltage of 3.5 kV and the negative ion mode. The fragmentor voltage was set at 140 V. The ESI-MS was performed in the selected-ion monitoring (SIM) mode using the target ions of m/z 709.3 for M21 and M22, full scan mode, and product ion scanning mode, respectively.

Study of the *in vitro* formation mechanism of M11 Incubations with NADPH consisted of GA (50 μM), and NADPH (0.1, 1, and 10 mM) in 0.2 mL of 0.1 M PBS buffer (pH 7.4). GA in 5.0 μL of acetonitrile was added to the incubation tubes containing NADPH solution. After mixing, the tubes were incubated for 1 h at 37 °C with air and without oxygen (Expelling the air with a gentle N_2 stream), respectively. All incubations were performed in triplicate. The incubation was stopped by adding 1mL ethyl acetate. Following centrifugation and separation, the organic phase was evaporated to dryness under a stream of nitrogen in a water bath of 40 °C. The residue was reconstituted in 150 μL of mobile phase, and a 20 μL aliquot was injected into the HPLC system. The HPLC analysis was performed using an Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA). A Venusil C18 column, 5 μm, 250 × 4.6 mm i.d. (Agela Technologies, Newark, DE, USA) protected by a SecurityGuard C18 column, 5 μm, 4 × 3.0 mm i.d. (Phenomenex, Torrance, CA, USA) was used for all measurements. The Signal acquisition, peak integration and concentration determination were performed using the ChemStation software (10.02 A) supplied by Agilent Technologies. The mobile phase was 10 mM ammonium acetate water solution - acetonitrile (10:90, v/v) at a flow rate of 1.5 mL/min. The column

temperature was maintained at 25°C. M11 was measured at 320 nm.

Legends of supplemental Figures:

Fig.S1 Full scan MS^2 chromatograms of the synthesized reference standards of M21(A) and M22 (B), and the fecal sample after 4 mg/kg i.v. dose.

Fig.S2 Typical SIM chromatogram of M21 and M22 in the rat intestinal contents incubation sample.

Fig.S3 Proposed fragmentation pathway of M1 (A) and the product ion scanning spectrum (B) in the positive ion ESI mode (Inset) the Q1 full scan spectrum of M1.

Fig.S4 Expanded region of the chemical shifts exhibiting the glutathionyl group in the ^{1}H-NMR spectrum (A) and ^{13}C-NMR of 10-GGA (M1 reference) in DMSO-d_6.

Fig.S5 Proposed fragmentation pathway of the mono-oxidation products of GA (A) and M4 (B) in the negative ion ESI mode

Fig.S6 Typical chromatograms of blank plasma (A) and a plasma sample during 0-4 h after an 4mg/kg i.v. dose (B)

Fig.S7 The effect of condition with O_2 and without O_2 on the formation of M11. Data expressed as mean ± S.D., n=3. * $p < 0.01$.

Fig.S8 Proposed fragmentation pathway of M7 (A) and the product ion scanning spectra of M7 (B) and M6 (C) in the negative ion ESI mode. Inset: the Q1 full scan spectra of M7 and M6.

Fig.S9 Typical chromatograms for glucuronides of GA (A) and M11 (B) treated at different conditions after *in vitro* incubation, respectively.

Fig.S1

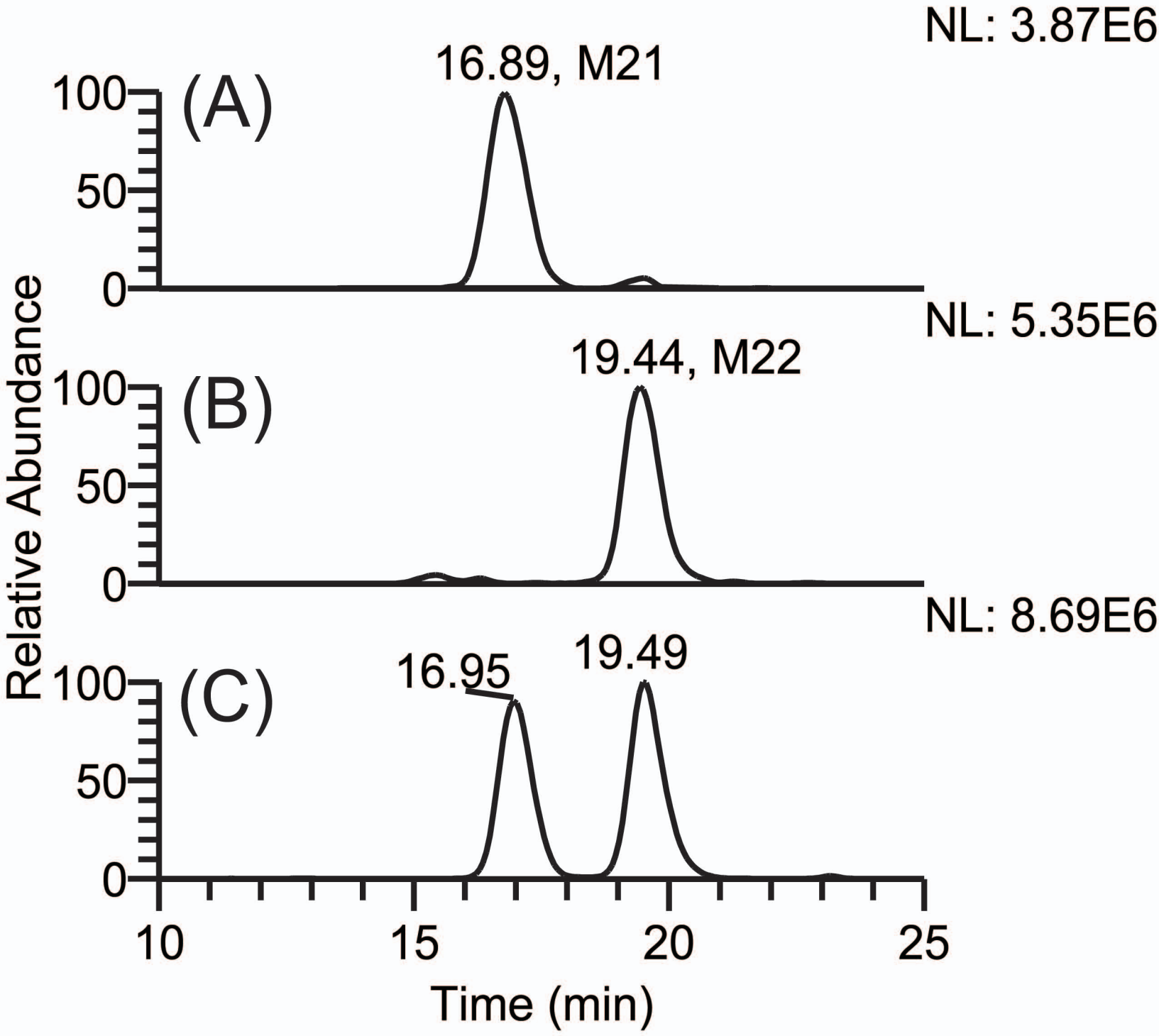

NL: 3.87E6
16.89, M21
100
(A)
50
0
NL: 5.35E6
19.44, M22
100
(B)
50
0
NL: 8.69E6
16.95
19.49
100
(C)
50
0
Relative Abundance
10
15
20
25
Time (min)

Fig.S2

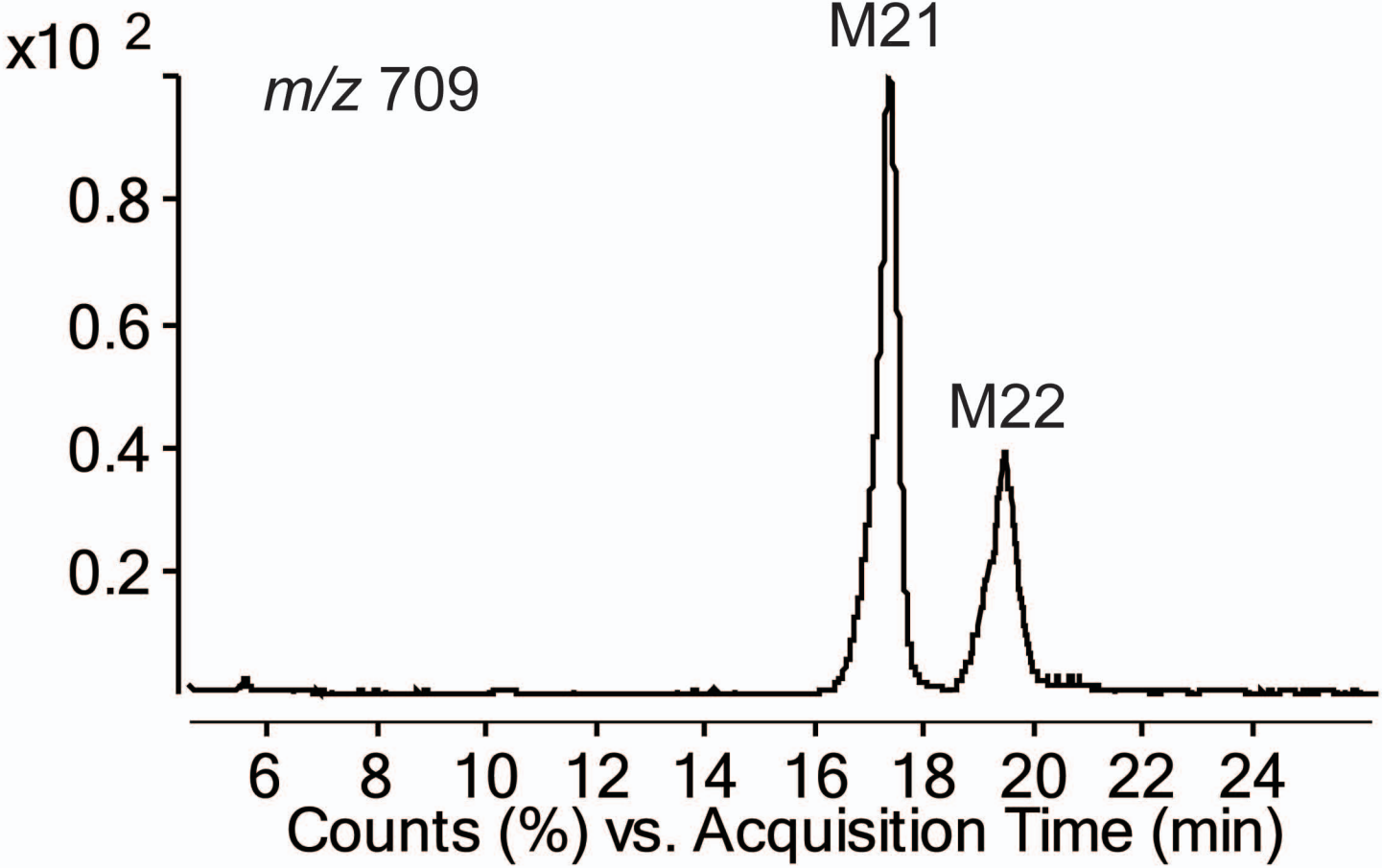
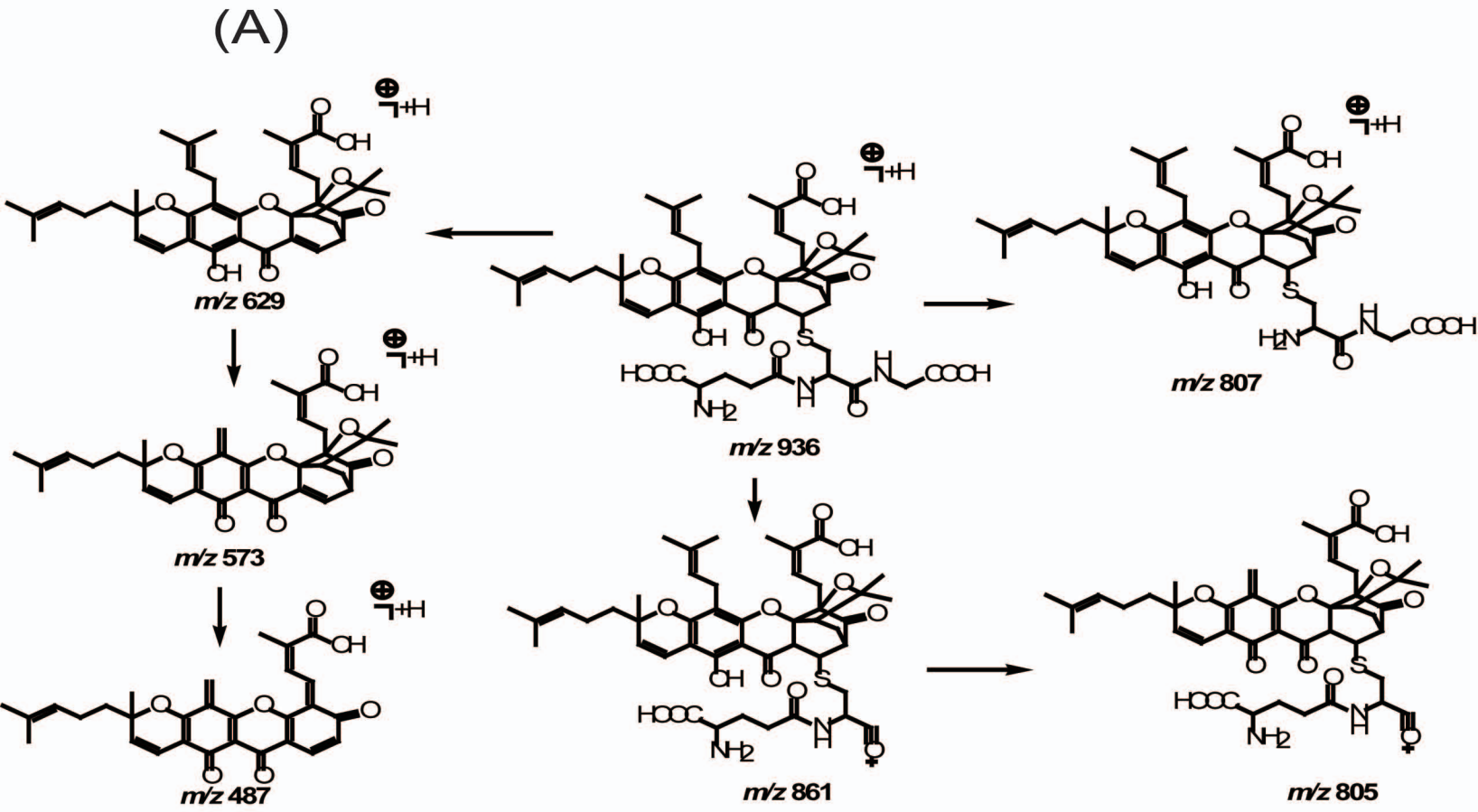
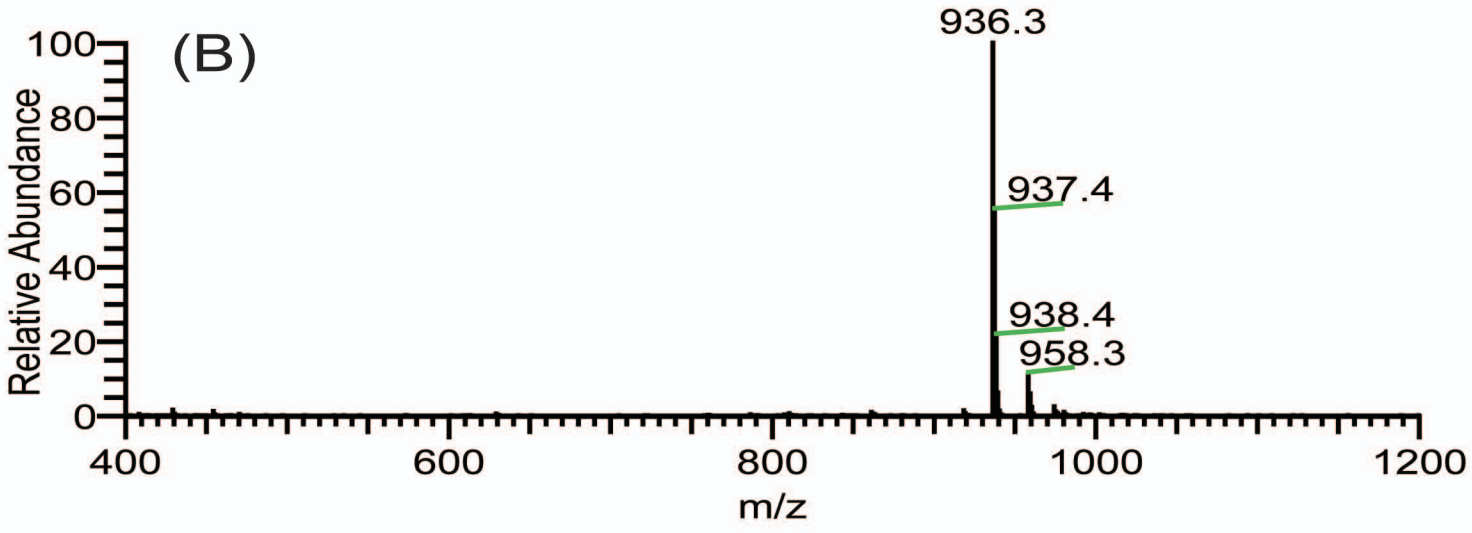

x10 2
m/z 709
M21
M22
0.8
0.6
0.4
0.2
6
8
10
12
14
16
18
20
22
24
Counts (%) vs. Acquisition Time (min)

Fig S3


(A)
m/z 629
m/z 936
m/z 807
m/z 573
m/z 487
m/z 861
m/z 805


(B)
936.3
937.4
938.4
958.3
Relative Abundance
m/z

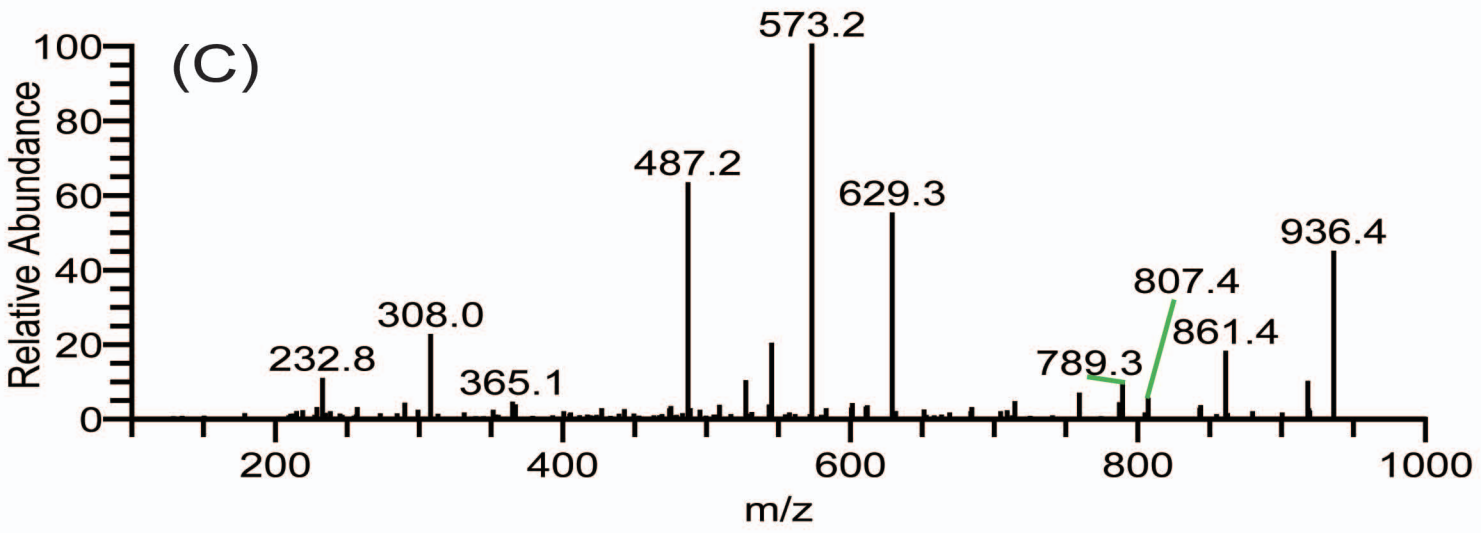

(C)
573.2
487.2
629.3
936.4
807.4
861.4
308.0
232.8
365.1
789.3
Relative Abundance
m/z

Fig.S4

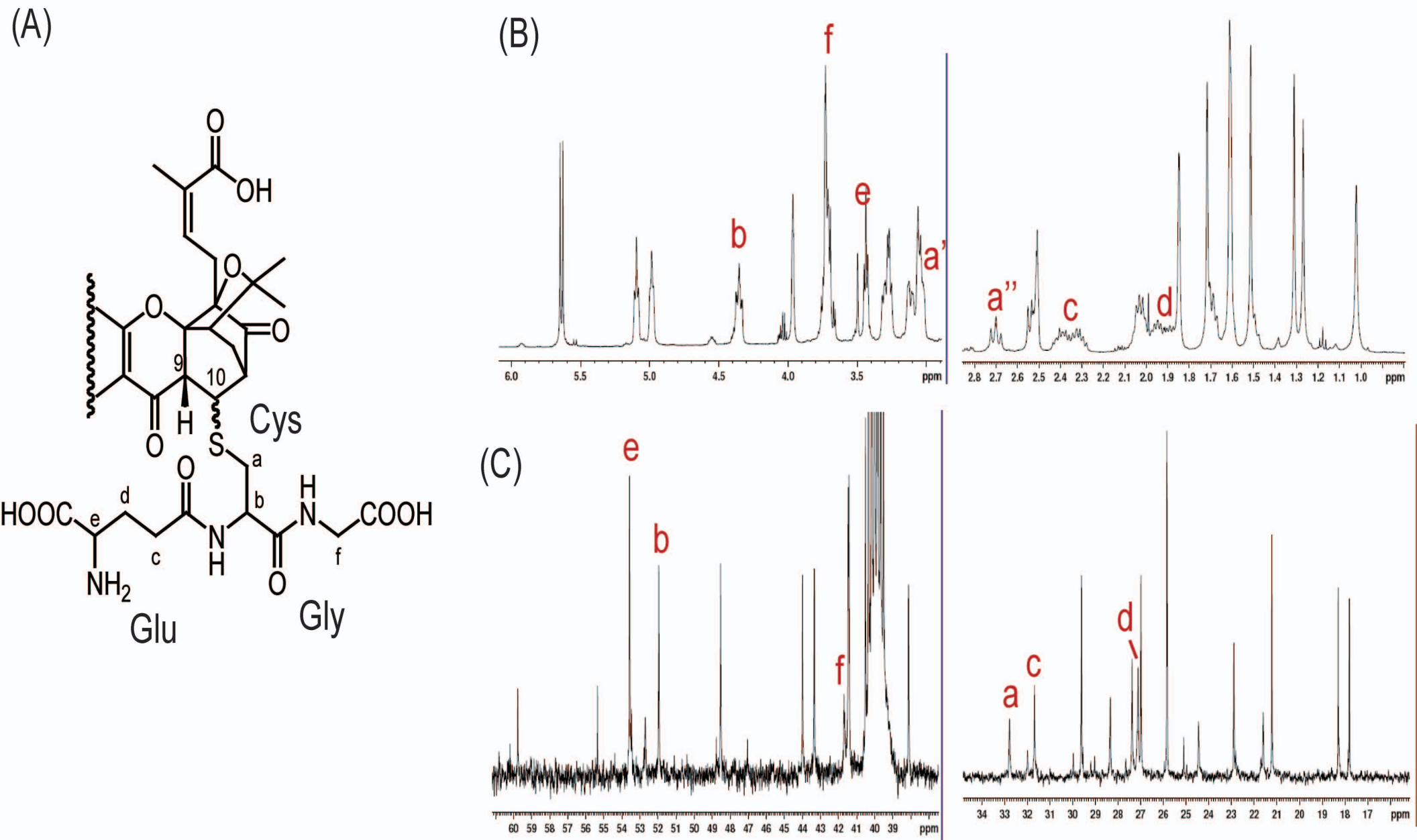

(A)
O
OH
O
O
O
9
10
H
O
Cys
S
a
HOOC
e
d
b
H
N
COOH
O
N
H
c
f
NH2
O
Glu
Gly
(B)
f
b
e
a'
a''
c
d
6.0 5.5 5.0 4.5 4.0 3.5 ppm
2.8 2.7 2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 ppm
(C)
e
b
f
a
c
d
60 59 58 57 56 55 54 53 52 51 50 49 48 47 46 45 44 43 42 41 40 39 ppm
34 33 32 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 ppm

Fig.S5

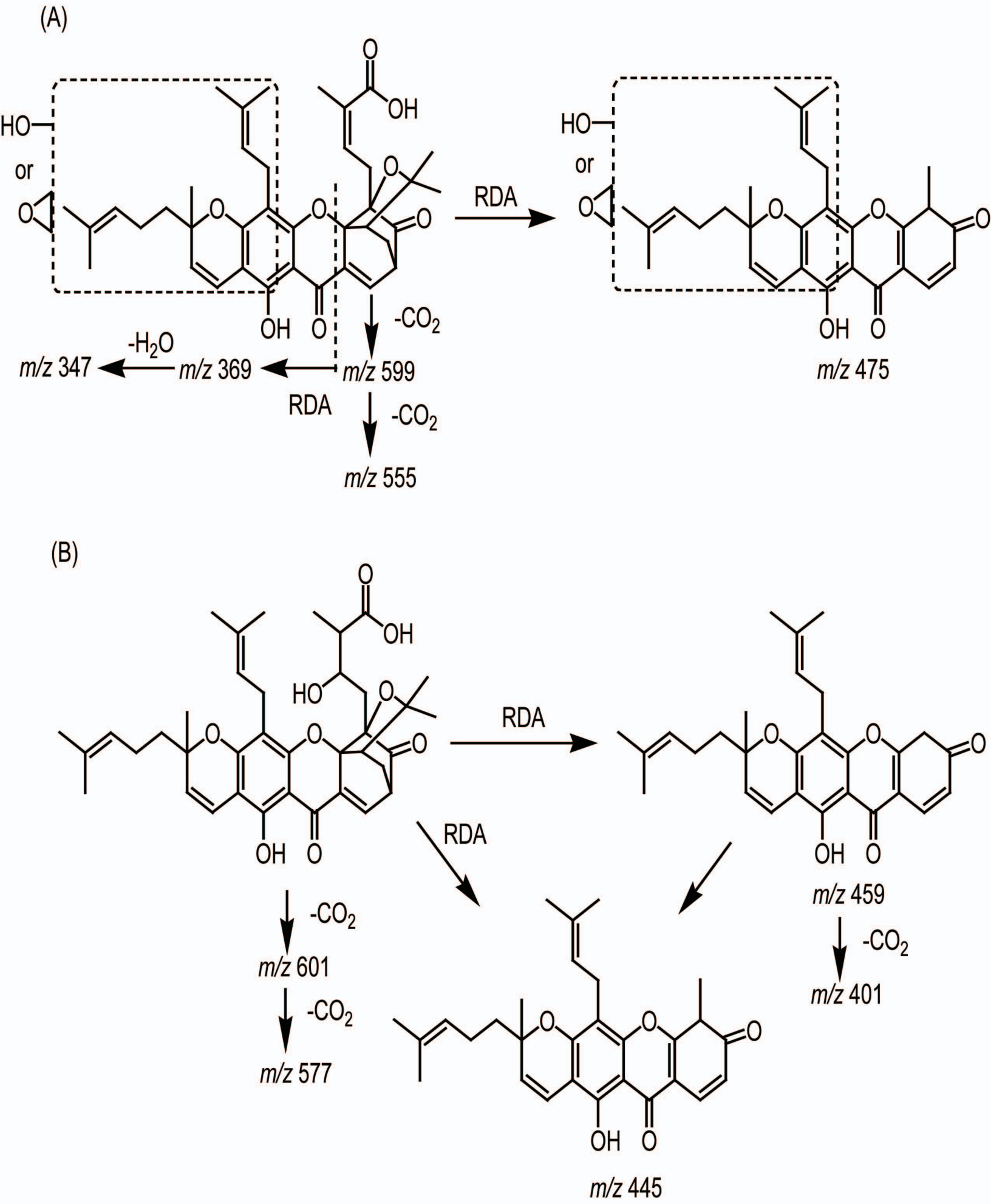

(A)
HO
or
O
OH
O
O
O
O
O
O
OH
O
RDA
HO
or
O
O
O
O
O
OH
O
-CO2
-H2O
m/z 347
m/z 369
m/z 599
RDA
-CO2
m/z 555
m/z 475
(B)
O
OH
HO
O
O
O
O
OH
O
RDA
RDA
-CO2
m/z 601
-CO2
m/z 577
m/z 459
-CO2
m/z 401
OH
O
m/z 445

Fig.S6

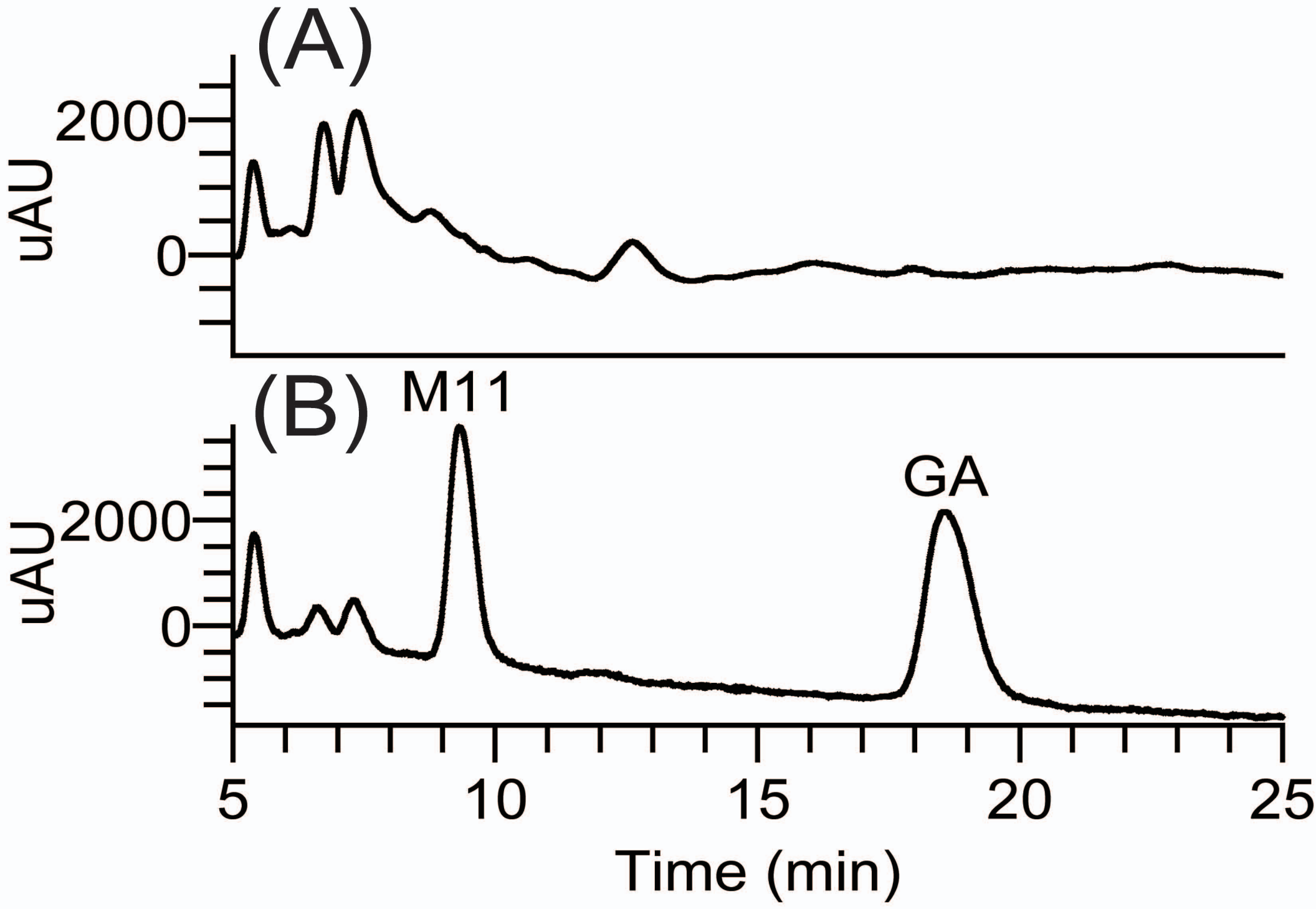


Fig.S7

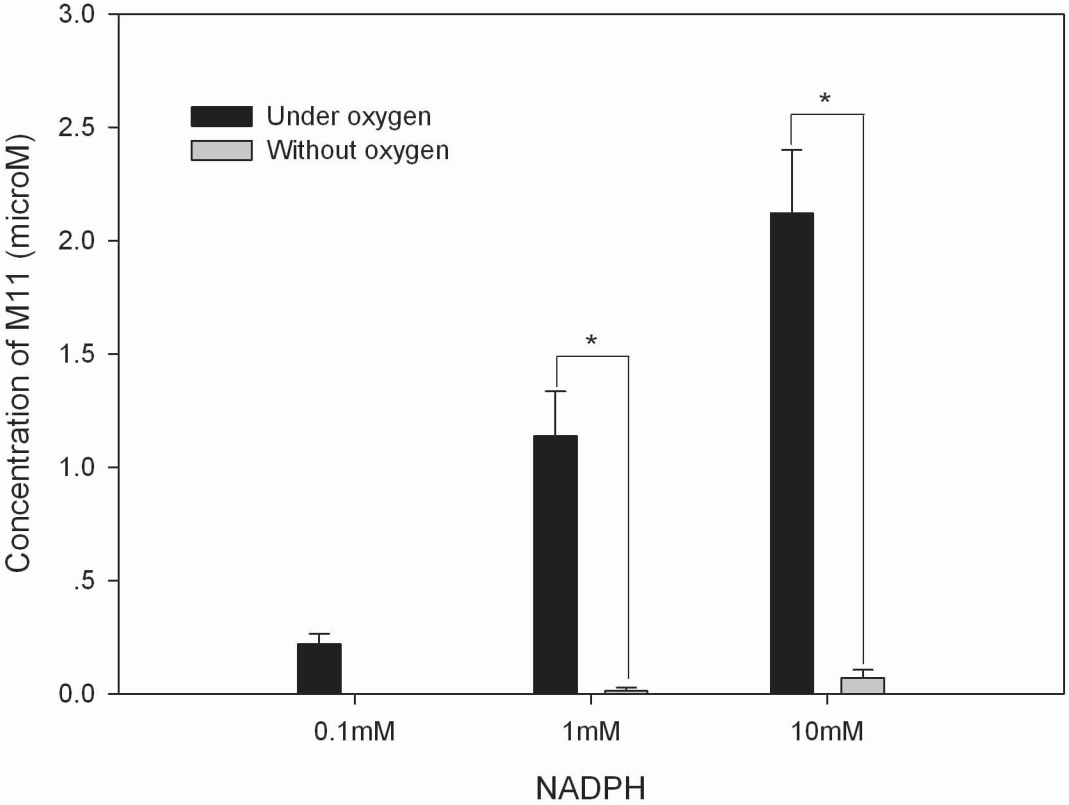

3.0
2.5
2.0
1.5
1.0
.5
0.0
Concentration of M11 (microM)
Under oxygen
Without oxygen
*
*
0.1mM
1mM
10mM
NADPH

Fig.S8

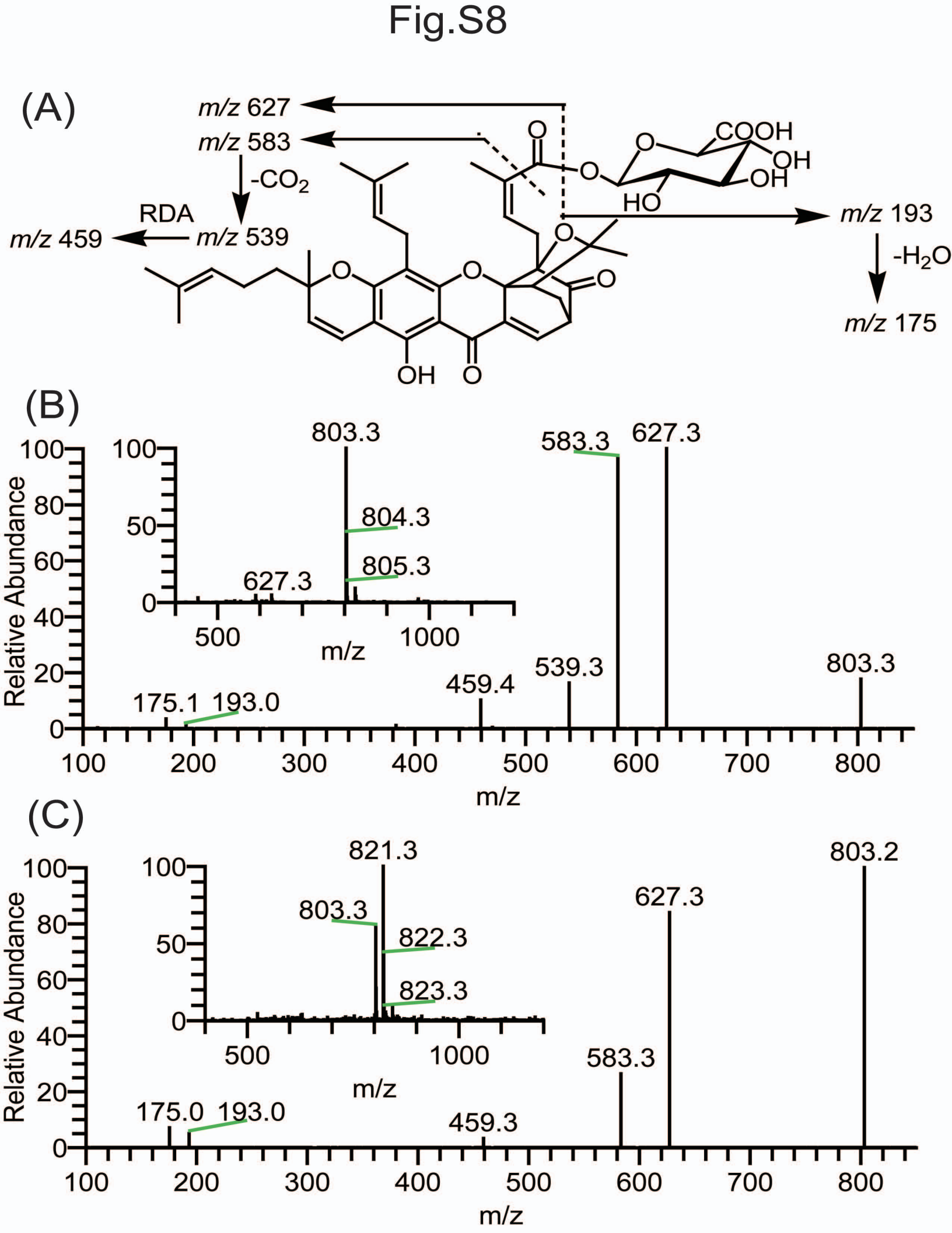
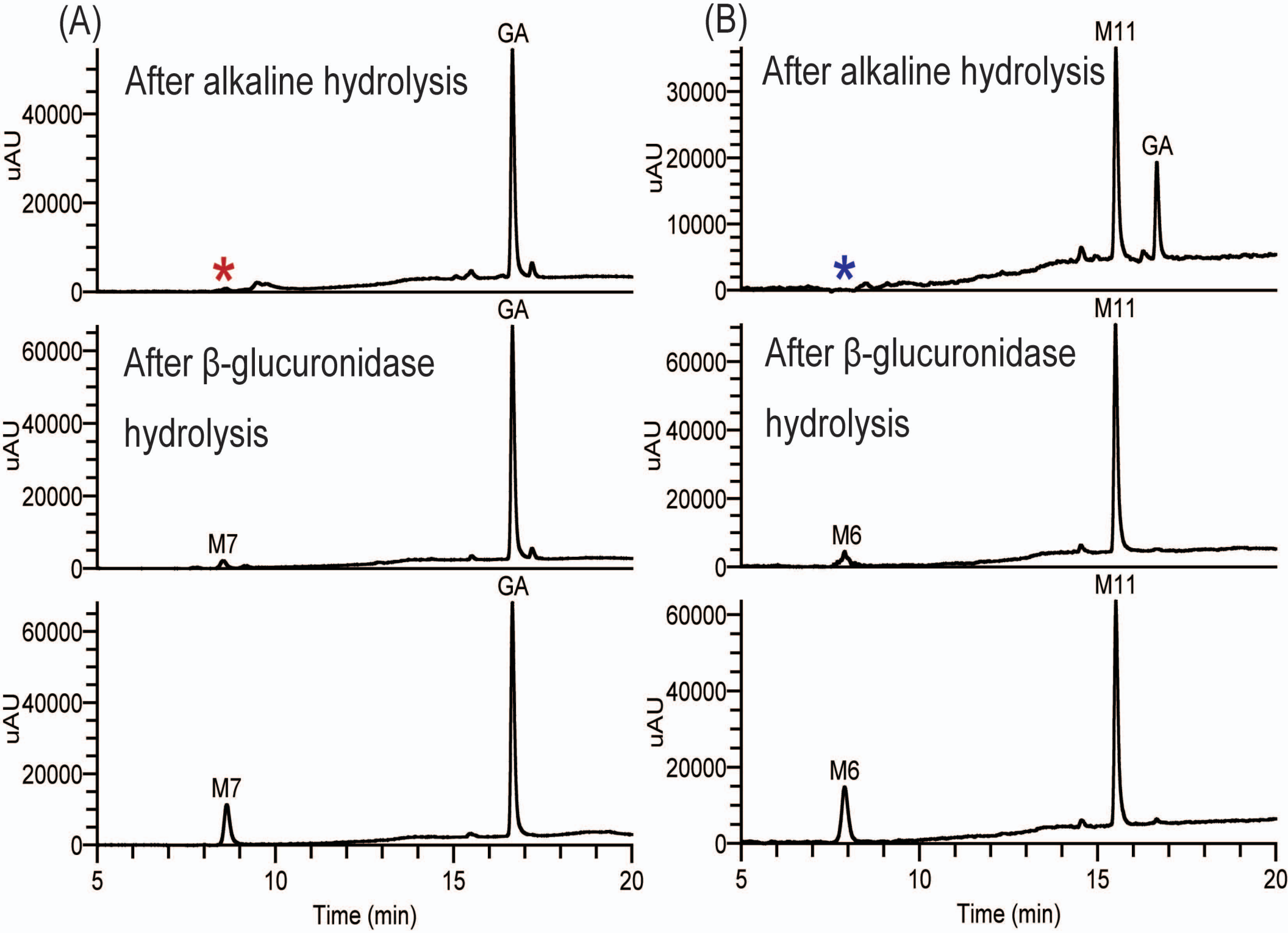

(A)
m/z 627
m/z 583
-CO2
RDA
m/z 459
m/z 539
m/z 193
-H2O
m/z 175
COOH
OH
OH
HO
O
OH
(B)
803.3
583.3
627.3
804.3
805.3
627.3
539.3
459.4
175.1
193.0
803.3
Relative Abundance
m/z
(C)
821.3
803.2
627.3
803.3
822.3
823.3
583.3
175.0
193.0
459.3
Relative Abundance
m/z

Fig.S9


(A)
After alkaline hydrolysis
GA
*
After β-glucuronidase hydrolysis
GA
M7
GA
M7
uAU
40000
20000
0
60000
Time (min)
5
10
15
20
(B)
After alkaline hydrolysis
M11
GA
*
30000
10000
After β-glucuronidase hydrolysis
M11
M6
M11
M6