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Supplemental information

Glycosyltransferase engineering and multi-glycosylation routes development facilitating synthesis of high-intensity sweetener mogrosides

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Supporting information

Glycosyltransferases engineering and multi-glycosylation routes development facilitating biosynthesis of high-intensity sweetener mogrosides

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1. Supporting tables

Table S1. Kinetic parameters of glycosyltransferases towards mogrosides. Related to Table1.

Substrate	Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
IIE	MS1	34.31 ± 0.5	0.014 ± 0.002	0.41 ± 0.01
	MS2	193.20 ± 3.2	0.026 ± 0.002	0.14 ± 0.006

Table S2. Enzymatic activity and product composition for MS1 and mutant M7 under different mogroside substrates. Related to Figure 5. To detect the product types and composition glycosylated by MS1 and its mutants, the glycosylation processes were controlled, in which 10-100 μg of the purified enzyme at 40°C for 15-30 min (depending on the activity of mutants) and one glucose residue was transferred to substrates.

Substrates	IIE			
Product types and composition	Conversion (%)		mU/mg	fold
	IIIA	IIIE		
WT	20	0	0.0085	1
M7	30	0	2.97	351

Substrates	IIIA			
Product types and composition	Conversion (%)		mU/mg	fold
	IVA	IVX		
WT	18	0	0.0074	1
M7	26	9	1.91	259

Substrates	IIIE			
Product types and composition	Conversion (%)		mU/mg	fold
	IVE	SiaI		
WT	6	17	0.0096	1
M7	3	32	3.09	322

Substrates	IVA		
Product types and composition	Conversion (%)	mU/mg	fold
	VA		
WT	-	0	-
M7	25	1.25	-

Substrates	IVE			
Product types and composition	Conversion (%)		mU/mg	fold
	V	VE		
WT	18	-	0.0072	1
M7	1	36	2.72	378

Substrates	Sia I		
Product types and composition	Conversion (%)	mU/mg	fold

	V	VX		
WT	15	-	0.0075	1
M7	14	11	1.04	139

Substrates	V		
Product types and composition	Conversion (%)	mU/mg	fold
	VI		
WT	-	0	-
M7	27	1.08	-

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Table S3. Kinetic parameters of glycosyltransferase MG1 towards mogrol. Related to Table1.

Substrate	Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
Mogrol	MG1	10.10 ± 0.3	0.86 ± 0.04	86.00 ± 6.6
IE	MG1	61.27 ± 0.7	0.04 ± 0.005	0.63 ± 0.04

Table S4. Primers used in this study. Related to STAR Methods.

Primer name	Sequence (5' to 3')
MS1-F	GGTACCGACGACGACGACAAGATGGATGCTGCCCAACAAG
MS1-R	CAGTGGTGGTGGTGGTGGTGTCATATTTTAAGCAAGAGAG
T11H-F	TGACACCACACATATTTTGATGCTTCCATGGCTCGGCTA
T11H-R	GCATCAAAATATGTGTGGTGTCACCTTGTTGGGCAGCAT
L13V-F	CACAACCATTGTGATGCTTCCATGGCTCGGCTATGGCCA
L13V-R	ATGGAAGCATCACAATGGTTGTGGTGTCACCTTGTTGGG
P16A-F	TTTGATGCTTGCGTGGCTCGGCTATGGCCATCTTTCAGC
P16A-R	AGCCGAGCCACGCAAGCATCAAAATGGTTGTGGTGTCAC
W17A-F	GATGCTTCCAGCGCTCGGCTATGGCCATCTTTCAGCTTT
W17A-R	CATAGCCGAGCGCTGGAAGCATCAAAATGGTTGTGGTGT
L18A-F	GCTTCCATGGGCGGGCTATGGCCATCTTTCAGCTTTTCT
L18A-R	GGCCATAGCCCGCCCATGGAAGCATCAAAATGGTTGTGG
G19A-F	TCCATGGCTCGCGTATGGCCATCTTTCAGCTTTTCTCGA
G19A-R	GATGGCCATACGCGAGCCATGGAAGCATCAAAATGGTTG
Y20A-F	ATGGCTCGGCGCGGGCCATCTTTCAGCTTTTCTCGAGCT
Y20A-R	AAAGATGGCCCGCGCCGAGCCATGGAAGCATCAAAATGG
G21A-F	GCTCGGCTATGCGCATCTTTCAGCTTTTCTCGAGCTGGC
G21A-R	CTGAAAGATGCGCATAGCCGAGCCATGGAAGCATCAAAA
L23A-F	CTATGGCCATGCGTCAGCTTTTCTCGAGCTGGCCAAAAG
L23A-R	GAAAAGCTGACGCATGGCCATAGCCGAGCCATGGAAGCA
S24A-F	TGGCCATCTTGCGGCTTTTCTCGAGCTGGCCAAAAGCCT
S24A-R	CGAGAAAAGCCGCAAGATGGCCATAGCCGAGCCATGGAA
A25P-F	CCATCTTTCACCGTTTCTCGAGCTGGCCAAAAGCCTCTC
A25P-R	GCTCGAGAAACGGTGAAAGATGGCCATAGCCGAGCCATG
F26A-F	TCTTTCAGCTGCGCTCGAGCTGGCCAAAAGCCTCTCAAG
F26A-R	CCAGCTCGAGCGCAGCTGAAAGATGGCCATAGCCGAGCC
S34A-F	CAAAAGCCTCGCGAGGAGGAACTTCCATATCTACTTCTG
S34A-R	AGTTCCTCCTCGCGAGGCTTTTGGCCAGCTCGAGAAAAG
N37G-F	CTCAAGGAGGGGCTTCCATATCTACTTCTGTTCAACCTC
N37G-R	AGATATGGAAGCCCCTCCTTGAGAGGCTTTTGGCCAGCT
F38H-F	AAGGAGGAACCATCATATCTACTTCTGTTCAACCTCTGT
F38H-R	AGTAGATATGATGGTTCCTCCTTGAGAGGCTTTTGGCCA
I40V-F	GAACTTCCATGTGTACTTCTGTTCAACCTCTGTTAATCT
I40V-R	AACAGAAGTACACATGGAAGTTCCTCCTTGAGAGGCTTT
S46P-F	CTGTTCAACCCCGGTTAATCTTGACGCCATTAAACCAAA
S46P-R	CAAGATTAACCGGGGTTGAACAGAAGTACACATGGAAGT
D62P-F	TTCTTTCTCTCCGTCCATTCAATTTGTGGAGCTCCATCT
D62P-R	ATTGAATGGACGGAGAGAAAGAAGAAGGAAGCTTTGGTT
F77L-F	TTCTCCTGAGCTGCCTCCTCATCTTCACACAACCAACGG
F77L-R	GATGAGGAGGCAGCTCAGGAGAAGAAGGGAGATGGAGCT
H82E-F	TCCTCATCTTGAAACAACCAACGGCCTTCCCCCTACCCT

H82E-R	CGTTGGTTGTTTCAAGATGAGGAGGGAACTCAGGAGAAG
A102L-F	CTTCTCCATGCTGGCCCAGCACTTTGAGTCCATTTTACA
A102L-R	AGTGCTGGGCCAGCATGGAGAAGGCTTGGTGGAGAGCGG
E107S-F	CCAGCACTTTAGCTCCATTTTACAAACACTTGCCCCGCA
E107S-R	GTAAAATGGAGCTAAAGTGCTGGGCAGCCATGGAGAAGG
S108E-F	GCACTTTGAGGAAATTTTACAAACACTTGCCCCGCACCT
S108E-R	TTTGTAAAATTTCCTCAAAGTGCTGGGCAGCCATGGAGA
H116D-F	ACTTGCCCCGGATCTTCTCATTTATGACTCTCTTCAACC
H116D-R	AAATGAGAAGATCCGGGGCAAGTGTTTGTAAAATGGACT
I139V-F	AATTCCGGCCGTGAACTTCAATACTACGGGAGTTTTCGT
I139V-R	TATTGAAGTTCACGGCCGGAATTTTGAGGGATGAAGCTA
N142L-F	CATCAACTTCCTGACTACGGGAGTTTTCGTCATTTCTCA
N142L-R	CTCCCGTAGTCAGGAAGTTGATGGCCGGAATTTTGAGGG
V146A-F	TACTACGGGAGCGTTCGTCATTTCTCAAGGGCTTCACCC
V146A-R	AAATGACGAACGCTCCCGTAGTATTGAAGTTGATGGCCG
N354A-F	CTGTGGATGGGCGTCGGTGATGGAGAGCATGATGTTTGG
N354A-R	CCATCACCGACGCCCATCCACAGTGGCTCACGAATCCCC

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Table S5. Strains and plasmids used in this study. Related to STAR Methods.

Strains and plasmids	description	Ref.
plasmids		
pET32a (+)	pBR322 ori with P_{T7}; Amp^R	Novagen
pET32a-MG1	pET32 carrying MG1	This study
pET32a-MS1	pET32 carrying MS1	This study
pET32a-MS2	pET32 carrying MS2	This study
pYZ291	2MICRON, URA3	Novagen
pYZ291-MS1	TEF1p-MG1-CYC1t	This study
pYZ291-MS1-MS1	GPD1p-MS1-adh1t-TEF1p-MS1-CYC1t	This study
pYZ291-MS1-MG1	GPD1p-MS1-adh1t-TEF1p-MG1-CYC1t	This study
Strains		This study
CEN.PK2-1C	Saccharomyces cerevisiae strain CEN.PK2-1C (MATaleu2-3, 112 ura3-52 trp1-289 his3-Δ1 MAL2-8cSUC2)	Novagen
Mog1	CEN.PK2-1C containing pYZ291-MS1	This study
Mog2	CEN.PK2-1C containing pYZ291-MS1, with gene Exg1 deleted	This study
Mog3	CEN.PK2-1C containing pYZ291-MS1-MS1, with gene Exg1 deleted	This study
Mog4	CEN.PK2-1C containing pYZ291-MS1-MG1, with gene Exg1 deleted	This study

83 **Table S6**. ^{13}C and ^{1}H NMR data for **VI** (600 MHz). Related to Figure 5.

C	δ ^{13}C	δ ^{1}H
1	27.2	CH_2, 1.49(m), 2.24(m)
2	29.3	CH_2, 1.91(m)
3	88.6	CH, 3.49(br, s)
4	42.9	
5	144.7	
6	119.9	CH, 5.47(d, J=5.4Hz)
7	25.2	CH_2, 1.79(m), 2.44(m)
8	44.7	CH, 1.67(m)
9	41.0	
10	37.1	CH, 2.50(d, J=12.4Hz)
11	79.6	CH, 3.85(m)
12	41.2	CH_2, 1.81(m)
13	48.5	
14	50.4	
15	35.4	CH_2, 1.14(m), 1.21(m)
16	29.5	CH_2, 1.32(m), 1.96(m)
17	51.9	CH, 1.62(dd)
18	17.3	CH_3, 0.92(s)
19	26.3	CH_3, 1.15(s)
20	37.5	CH, 1.50(m)
21	19.3	CH_3, 0.97(d)
22	34.1	CH_2, 1.48(m), 1.56(m)
23	29.9	CH_2, 1.40(m), 1.55(m)
24	93.2	CH, 3.40(m)
25	73.8	
26	26.8	CH_3, 1.12(s)
27	24.2	CH_3, 1.15(s)
28	28.1	CH_3, 1.07(s)
29	26.6	CH_3, 1.21(s)
30	20.0	CH_3, 0.89(s)
3-O-Glc-1		
C-1	104.8	CH, 4.40(d, J=7.80Hz)
C-2	80.2	CH, 3.63(m)
C-3	78.2	CH, 3.54(m)
C-4	71.6	CH, 3.19(m)
C-5	77.1	CH, 3.44(m)
C-6	69.8	CH_2, 3.80(dd, J1=5.8Hz, J2=11.4Hz), 4.06(d, J=11.4Hz)
3-O-Glc-2-Glc		
C-1	104.0	CH, 4.67(d, J=7.80Hz)
C-2	77.9	CH, 3.25(m)
C-3	78.6	CH, 3.63(m)

C-4	71.8	CH, 3.29(m)
C-5	75.7	CH, 3.27(m)
C-6	63.1	CH_2, 3.67(dd), 3.85(d)
3-O-Glc-6-Glc		
C-1	104.8	CH, 4.42(d, J=7.80Hz)
C-2	77.9	CH, 3.19(m)
C-3	78.1	CH, 3.28(m)
C-4	71.6	CH, 3.32(m)
C-5	72.4	CH, 3.22(m)
C-6	62.7	CH_2, 3.67(dd), 3.85(d)
24-O-Glc-1		
C-1	104.1	CH, 4.44(d, J=7.80Hz)
C-2	81.3	CH, 3.61(m)
C-3	78.0	CH, 3.37(m)
C-4	71.7	CH, 3.27(m)
C-5	76.5	CH, 3.51(m)
C-6	70.2	CH_2, 3.63(dd, J1=5.8Hz, J2=11.4Hz), 4.24(d, J=11.4Hz)
24-O-1Glc-2-Glc		
C-1	104.6	CH, 4.77(d, J=7.80Hz)
C-2	78.3	CH, 3.28(m)
C-3	77.7	CH, 3.37(m)
C-4	75.2	CH, 3.20(m)
C-5	78.0	CH, 3.26(m)
C-6	63.6	CH_2, 3.64(dd), 3.87(d)
24-O-1Glc-6-Glc		
C-1	104.4	CH, 4.29(d, J=7.80Hz)
C-2	75.2	CH, 3.23(m)
C-3	77.7	CH, 3.36(m)
C-4	71.5	CH, 3.27(m)
C-5	76.2	CH, 3.26(m)
C-6	62.8	CH_2, 3.67(dd), 3.85(d)

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2. Supporting figures

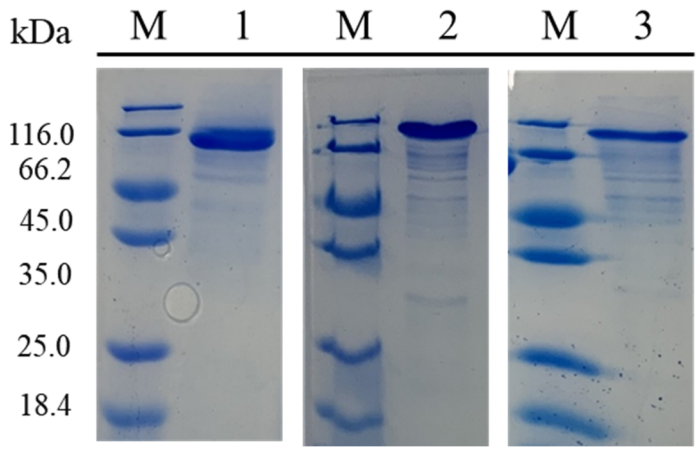


Figure S1. SDS-PAGE of recombinant protein purification fractions. Related to Figures 2 and 3. Lane M: protein marker; lane 1: purified MG1 (54 kDa); lane 2: MS1 (72 kDa); lane 3: MS2 (71 kDa).

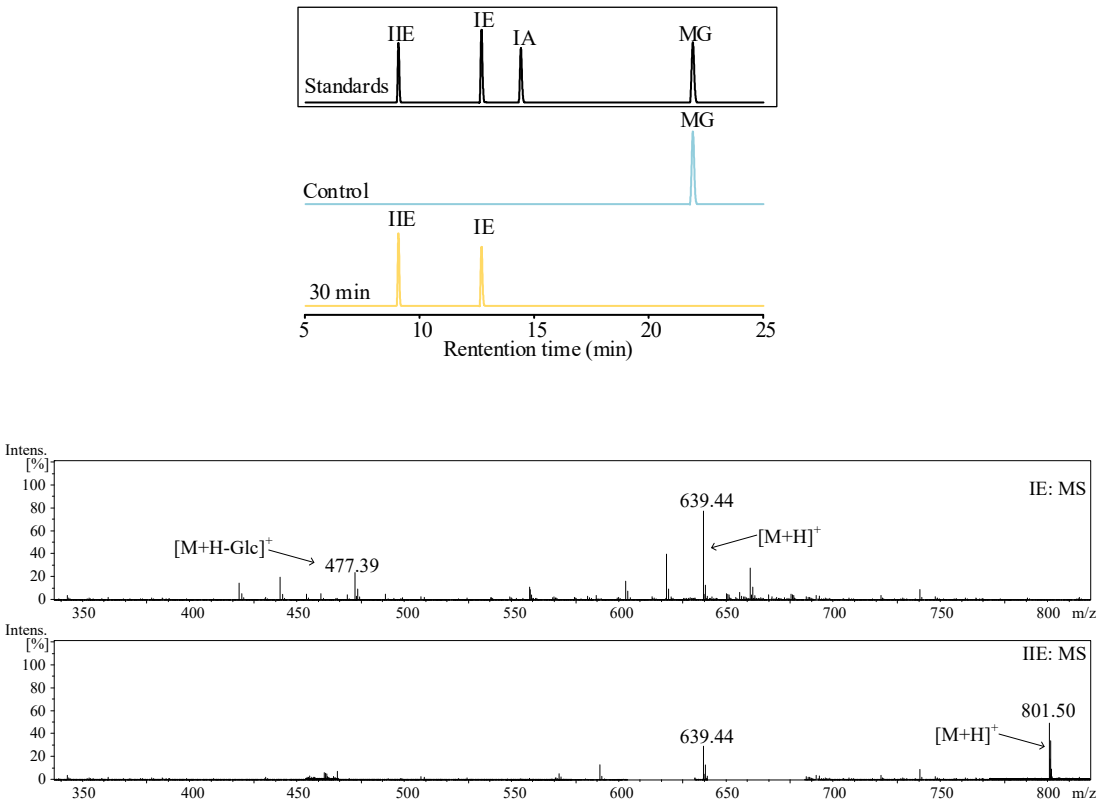


Figure S2. HPLC and MS analysis of MG1 enzymatic reaction products using mogrol as substrate. Related to Figure 2.

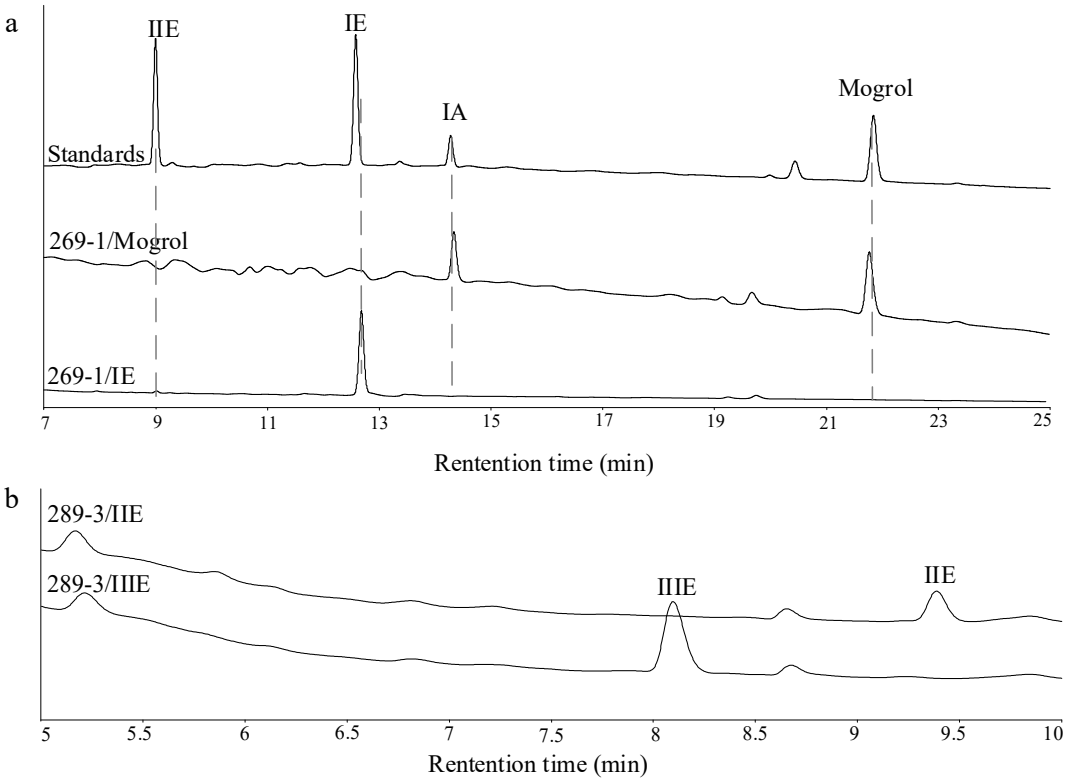


Figure S3. HPLC analysis of UGT72-269-1 and UGT94-289-3 enzymatic reaction products using mogrosides as substrates. Related to Figure 3.

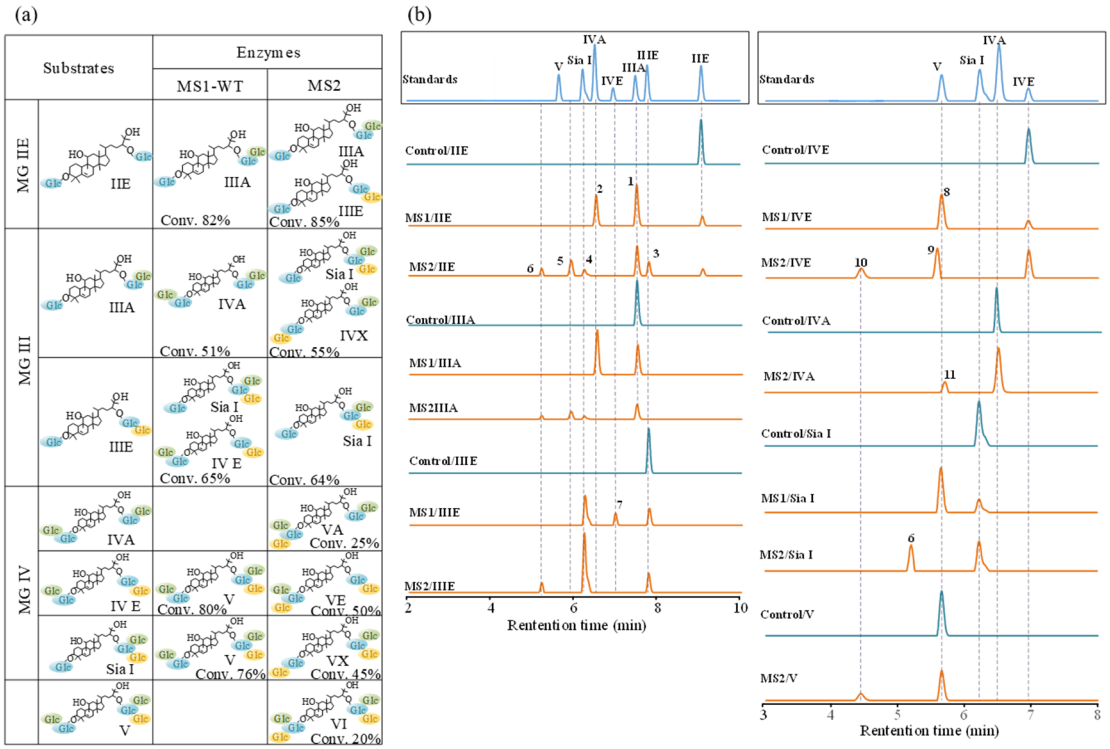


Figure S4. Glycosylations catalyzed by enzymes MS1 and MS2 using various substrates. Related to Figure 5. (a) Schematic summary of glycosylations by enzymes MS1 and MS2 using various substrates. (b) HPLC analysis of MS1 and MS2 enzymatic reaction products using mogrosides as substrates. The HPLC methods as follow: Mobile phase A was ddH_2O with 0.1% formic acid, and mobile phase B contained CH_3CN and 0.1% formic acid. The gradient are as follows: 0-25 min, 25%-80% pump B for mogrol, mogroside IE, mogroside IIE, and mogroside III; 0-25 min, 25%-55% pump B for mogroside IV, V, and VI, and the flow rate was 1 mL/min.

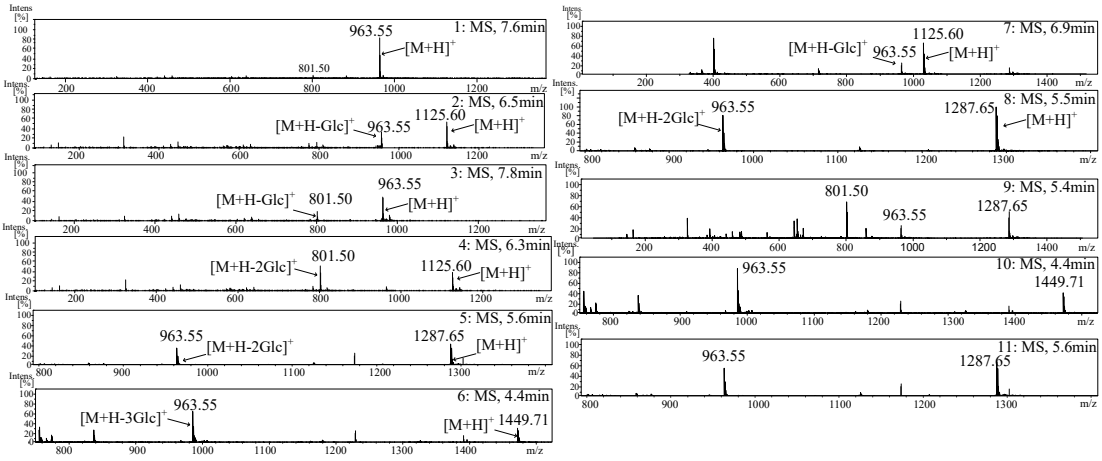


Figure S5. MS analysis of MS1 and MS2 enzymatic reaction products using mogrosides as substrates. Related to Figure 5. Flight-high-resolution electrospray ionization-mass spectrometry (ESI-MS) parameters were as follows: the scan range was 100–1,500 m/z in positive ion mode, spray voltage was 4,500 V, capillary temperature was 400°C, dry gas was 6 ml/min, dry temperature was 180°C and nebulizer pressure was 1 bar.

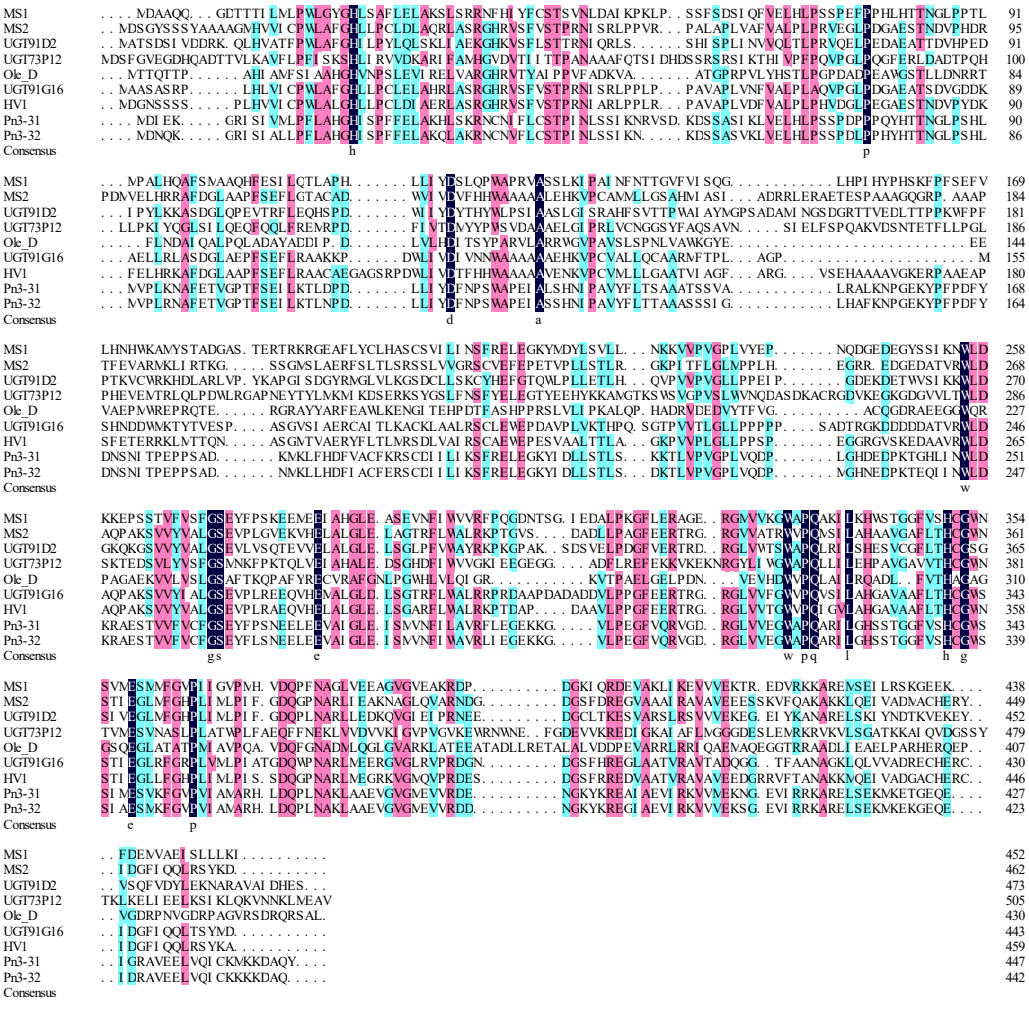
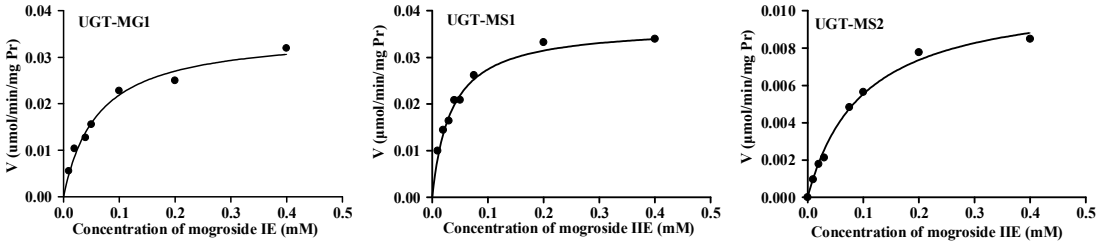


Figure S6. Multiple sequence alignment of 9 UGTs that show branched glycosylation activity to triterpenes. Related to Figure 4. The following UGTs were used in this analysis: MS1, MS2 (GenBank: XP_015629141), UGT91D2 (GenBank: ACE87855.1), UGT73P12 (GenBank: BBN60799.1), OleD (GenBank: ABA42119.2), UGT91G16 (GenBank: QHG10987.1), HV1 (GenBank: BAJ98242.1), Pn3-31 (GenBank: QOJ43868.1) and Pn3-32 (GenBank: QOJ43866.1).



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122 **Figure S7**. Determination of kinetic parameters for recombinant glycosyltransferase

123 MG1 (on IE) and MS1 and MS2 (on mogroside IIE). Related to Table 1. The catalytic

124 constants were obtained using GraphPad Prism 5 software.

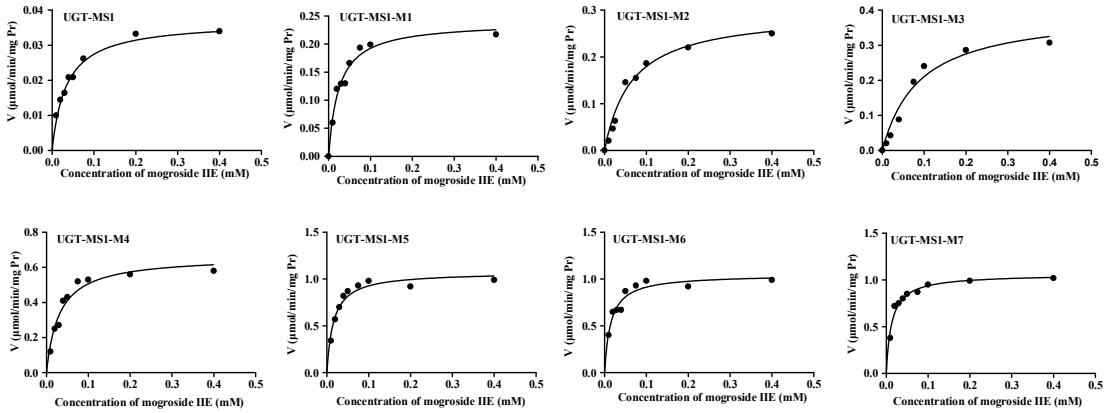


Figure S8. Determination of kinetic parameters for recombinant glycosyltransferase MS1, and its mutants on mogroside IIE. Related to Table 1.

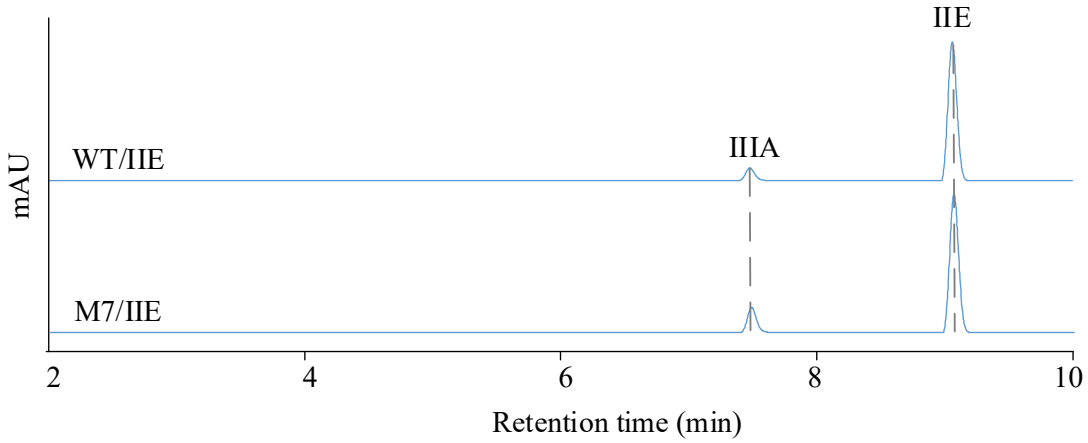
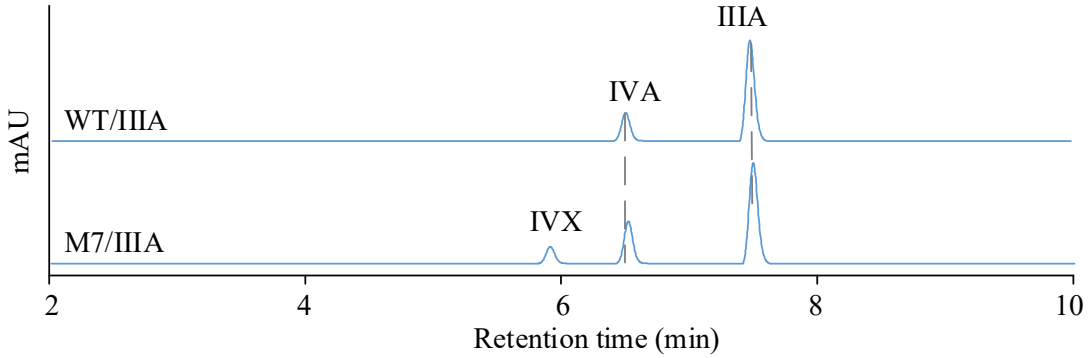


Figure S9. HPLC analysis of MS1 WT and mutant M7 enzymatic reaction products using mogroside IIE as substrate. Related to Figure 5.



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132 **Figure S10**. HPLC analysis of MS1 WT and mutant M7 enzymatic reaction products
133 using mogroside IIIA as substrate. Related to Figure 5.

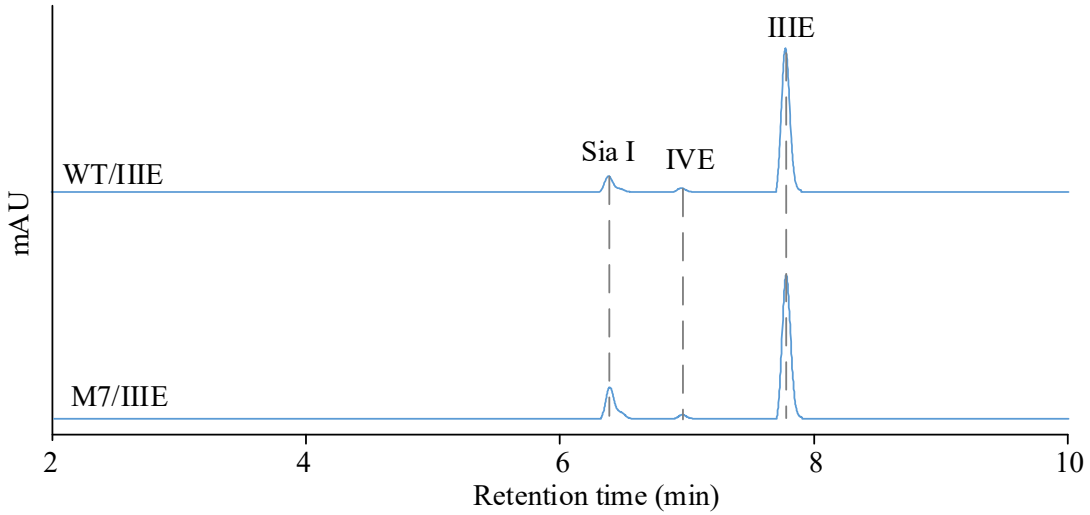


Figure S11. HPLC analysis of MS1 WT and mutant M7 enzymatic reaction products using mogroside IIIE as substrate. Related to Figure 5.

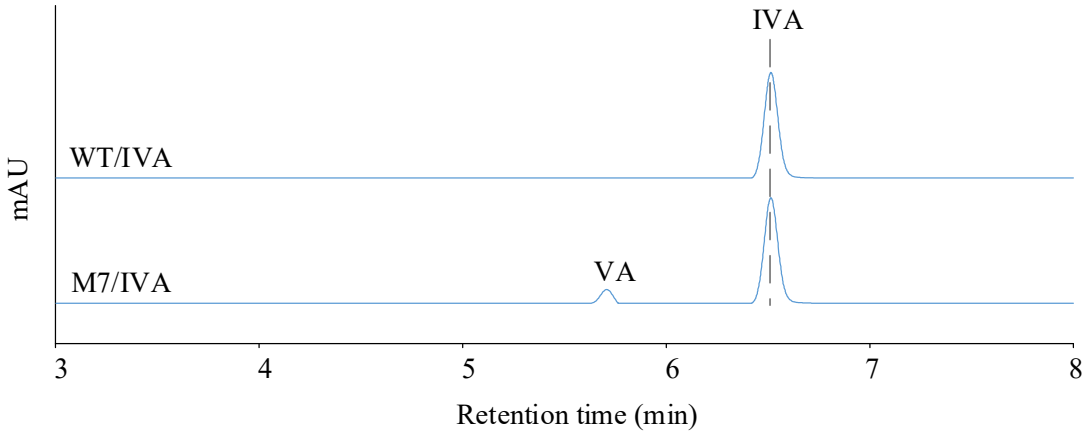


Figure S12. HPLC analysis of MS1 WT and mutant M7 enzymatic reaction products using mogroside IVA as substrate. Related to Figure 5.

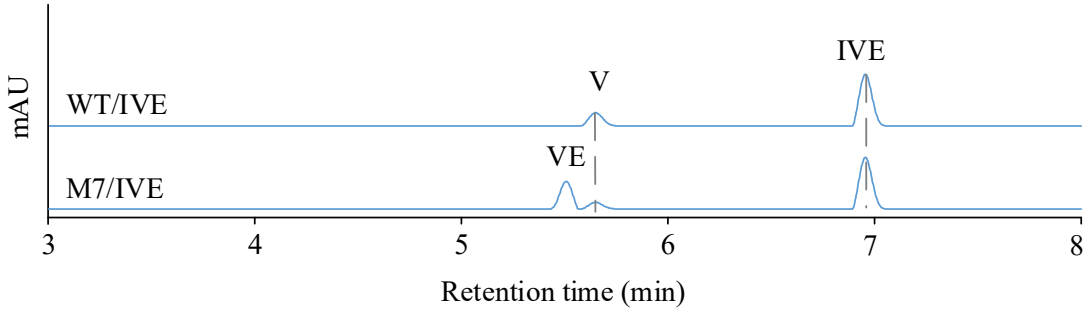
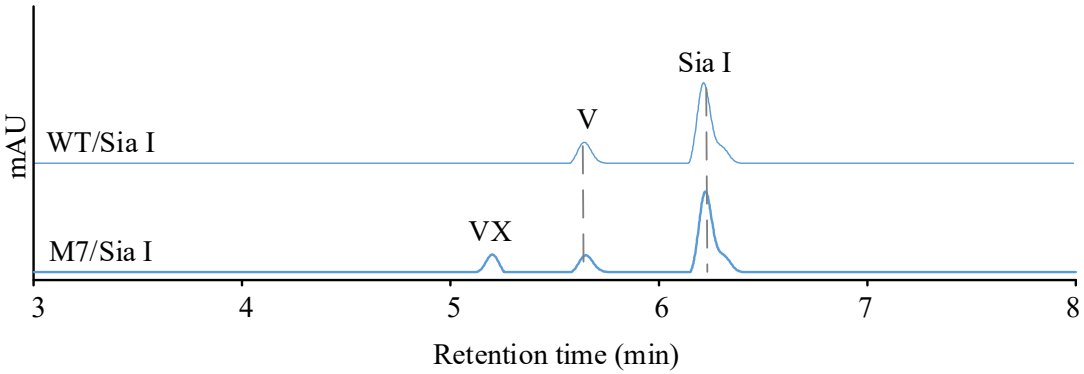


Figure S13. HPLC analysis of MS1 WT and mutant M7 enzymatic reaction products using mogroside IVE as substrate. Related to Figure 5.



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144 **Figure S14**. HPLC analysis of MS1 WT and mutant M7 enzymatic reaction products
145 using mogroside Sia I as substrate. Related to Figure 5.

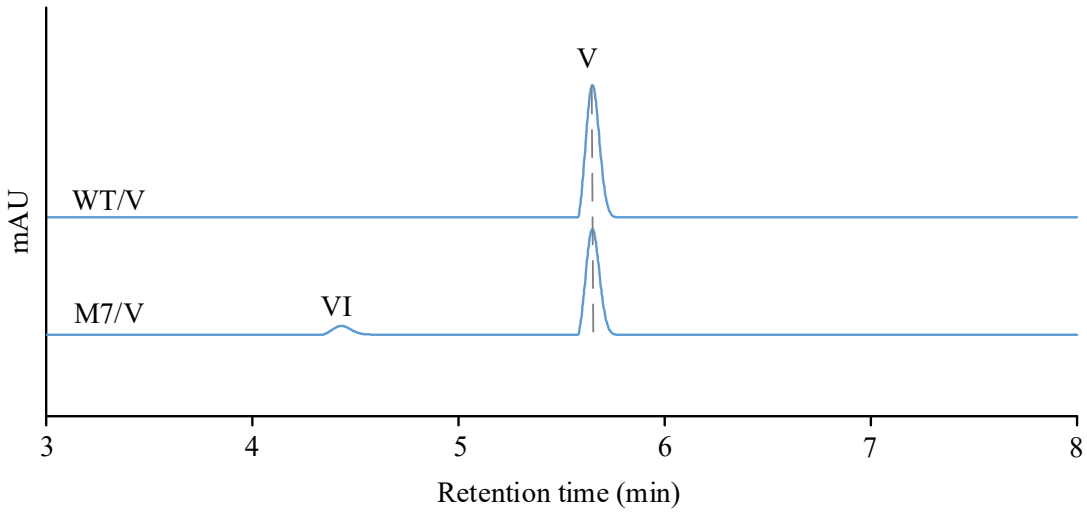


Figure S15. HPLC analysis of MS1 WT and mutant M7 enzymatic reaction products using mogroside V as substrate. Related to Figure 5.

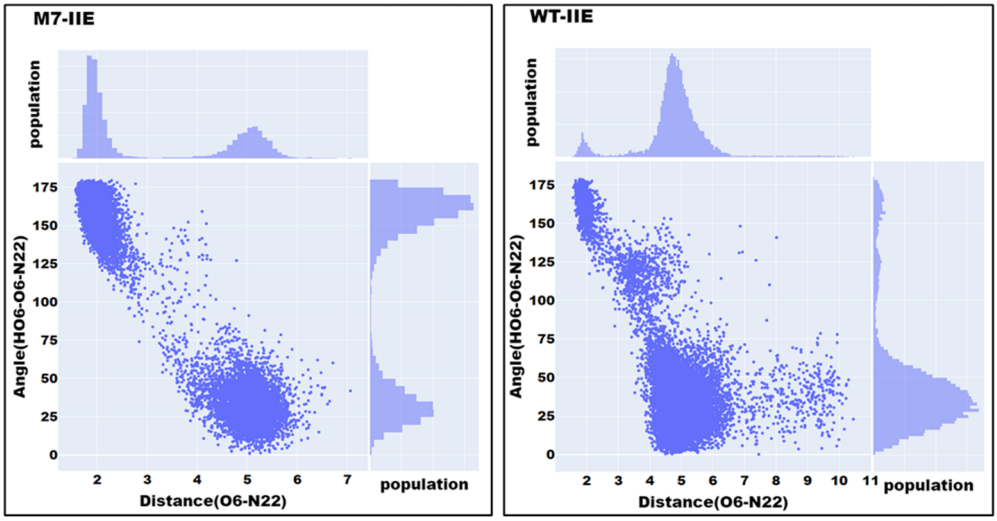
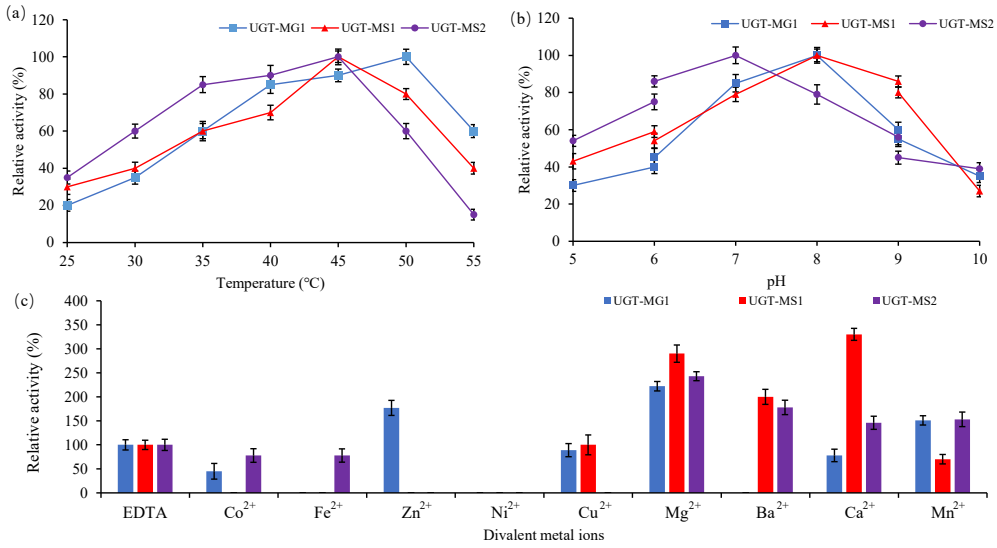


Figure S16. Conformation maps of M7-IIE and WT-IIE complexes during the 3*50 ns MD simulations. Related to Figure 6. According to reaction mechanism, a catalytic conformation, which supports catalysis, occurs when the distance between the O_6 atom of C24-sugar and the N_{22} nitrogen of the catalytic residue His22 is less than 3.6 Å and the angle of H_{O6}-O_6-N_{22} is larger than 135°



156

157 **Figure S17**. Optimization of reaction temperature, pH and divalent metal ions of MS1
158 and MS2. Related to Figure 7. (**a**) Relative activity of MS1 and MS2 at various
159 temperatures (25−60°C) at pH 8.0. (**b**) Relative activity of MS1 and MS2 at different
160 pH conditions (pH 5.0−10.0). (**c**) Relative activity of MS1 and MS2 with divalent metal
161 ions.

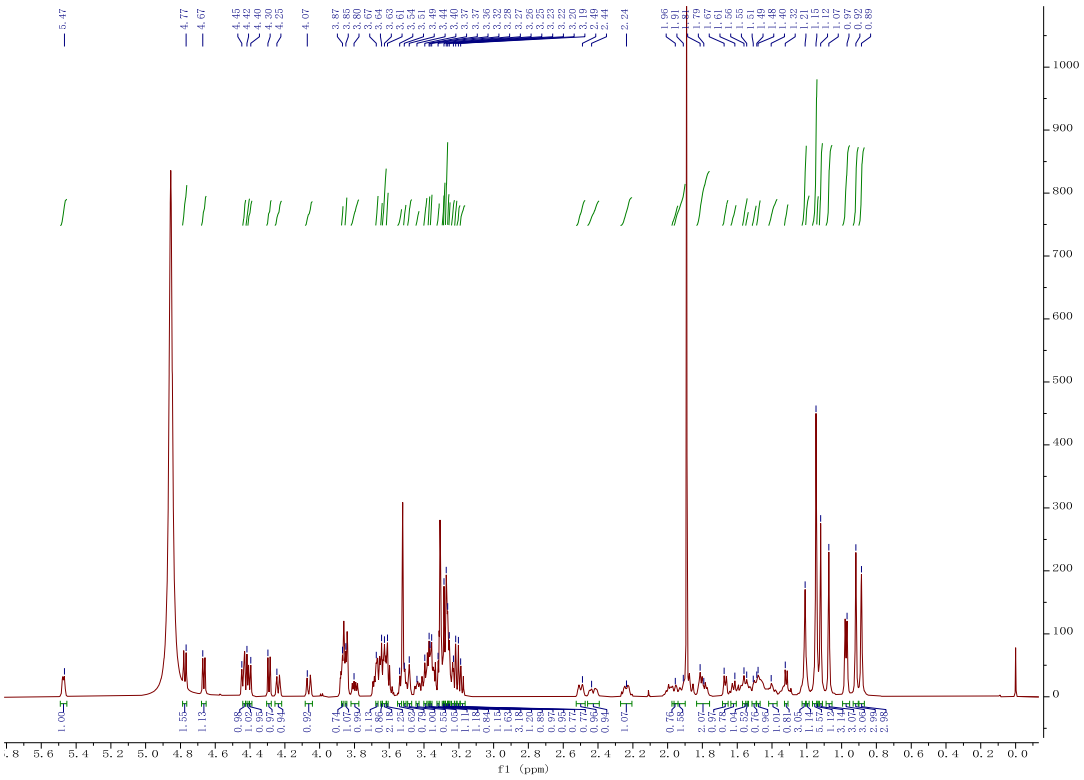
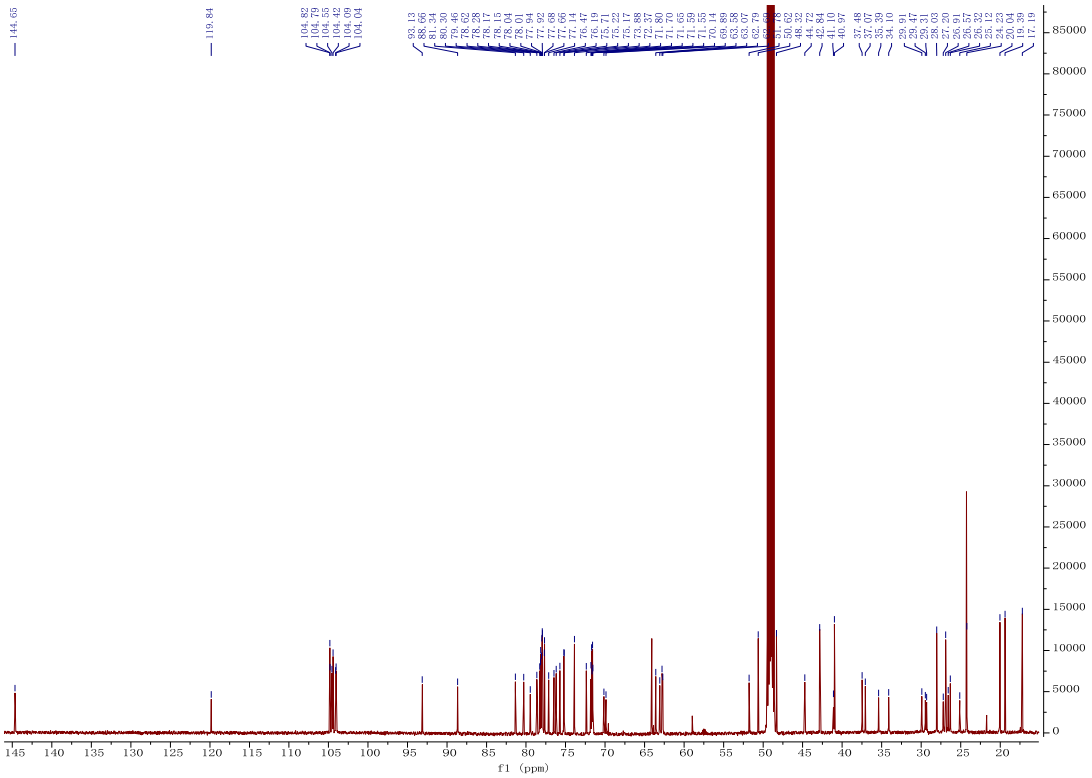


Figure S18. 1H NMR spectrum of VI in Dimethyl sulfoxide-d6 (600 MHz). Related to Figure 5. Glycosides VI was was purified by an Agilent 1260 preparative HPLC system with semi-preparative C18 reverse-phase column (21.2 × 250 mm, 5 μm particles, Welch, Shanghai, China). The fractions with the same mogroside glucoside were collected and concentrated on rotary evaporator. The purified product was dissolved in deuterated methanol, after vacuum freeze drying. 1D NMR and 2D NMR spectroscopies of the product, including ^{1}H NMR, ^{13}C NMR, correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC), were recorded on an Advance DMX-600 NMR spectrometer (Bruker, Karlsruhe, Germany), to identify the chemical structure of the compound.



174

175 **Figure S19**. ^{13}C NMR spectrum of **VI** in Dimethyl sulfoxide-d6 (600 MHz). Related

176 to Figure 5.

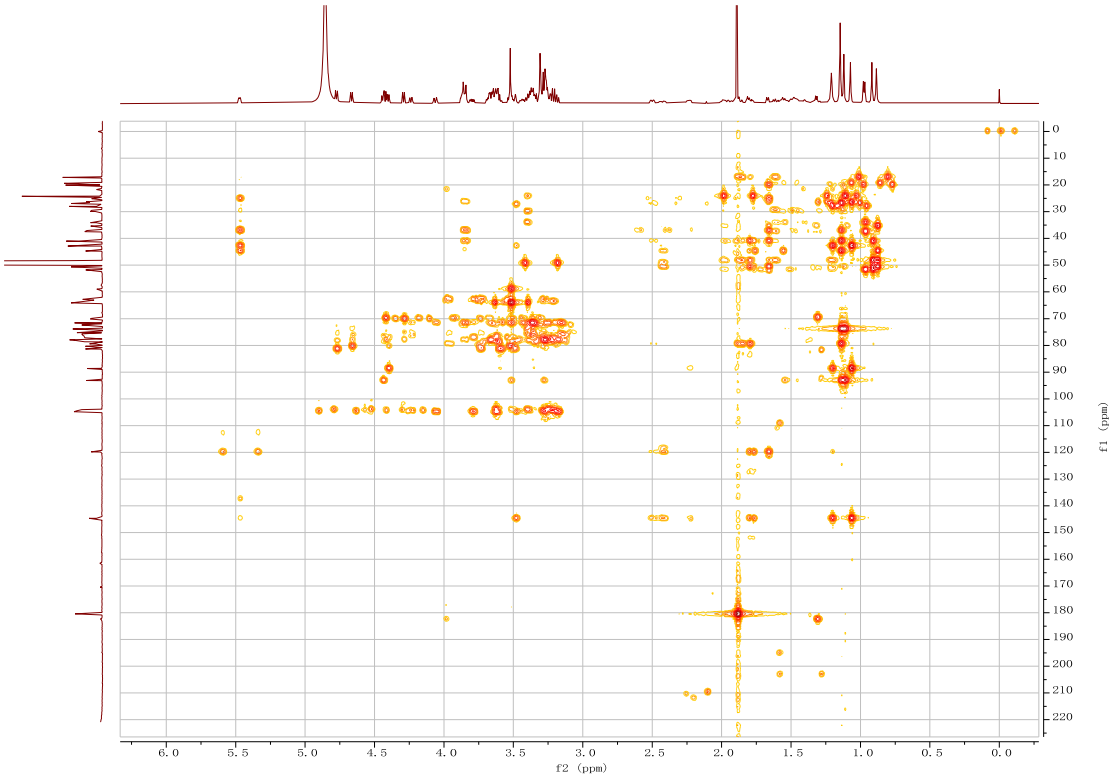


Figure S20. HMBC spectrum of **VI** in Dimethyl sulfoxide-d6 (600 MHz). Related to Figure 5.

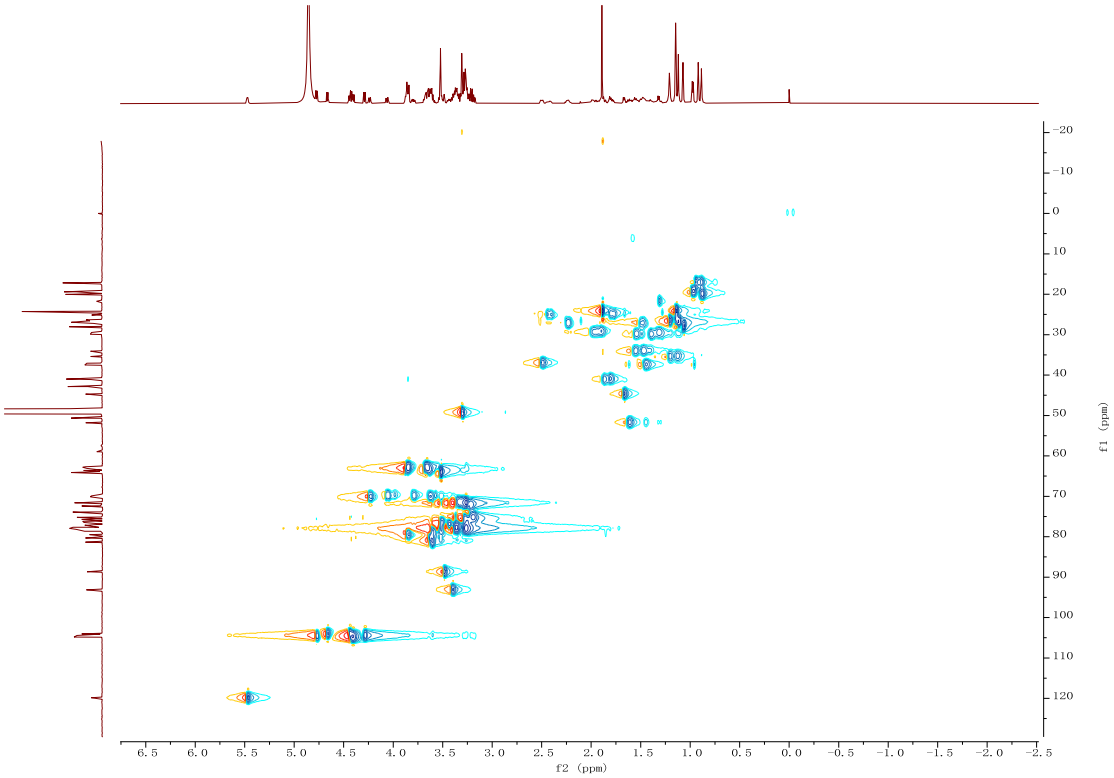
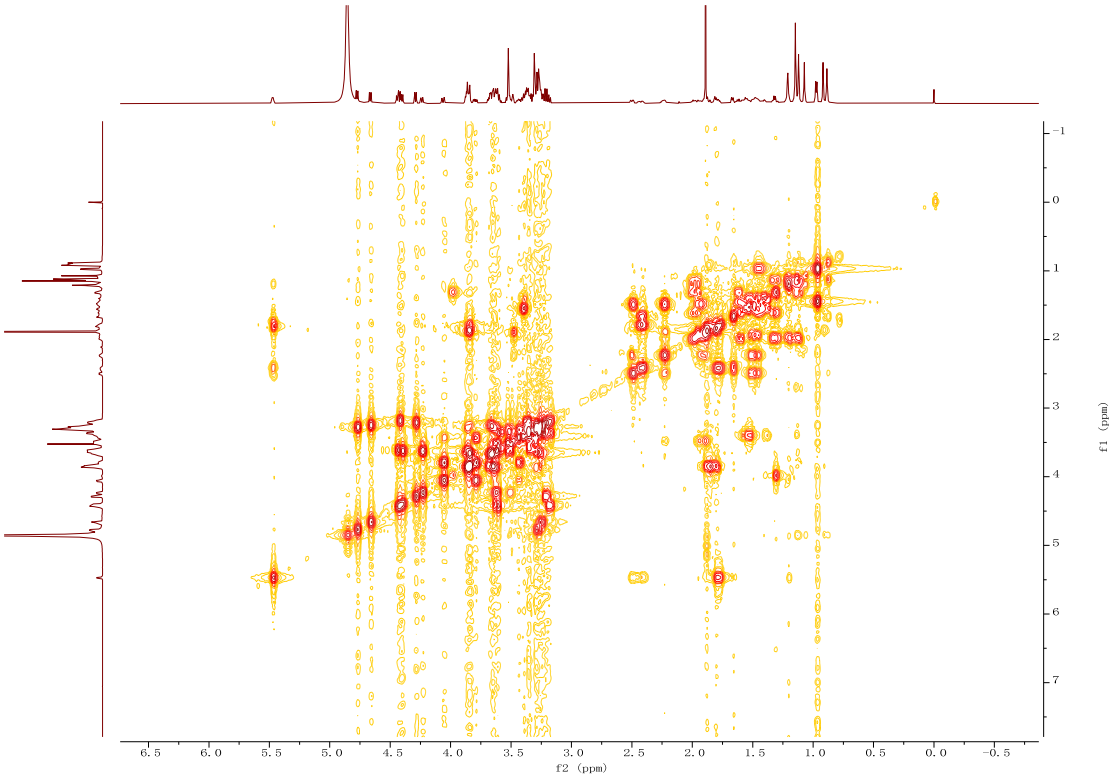


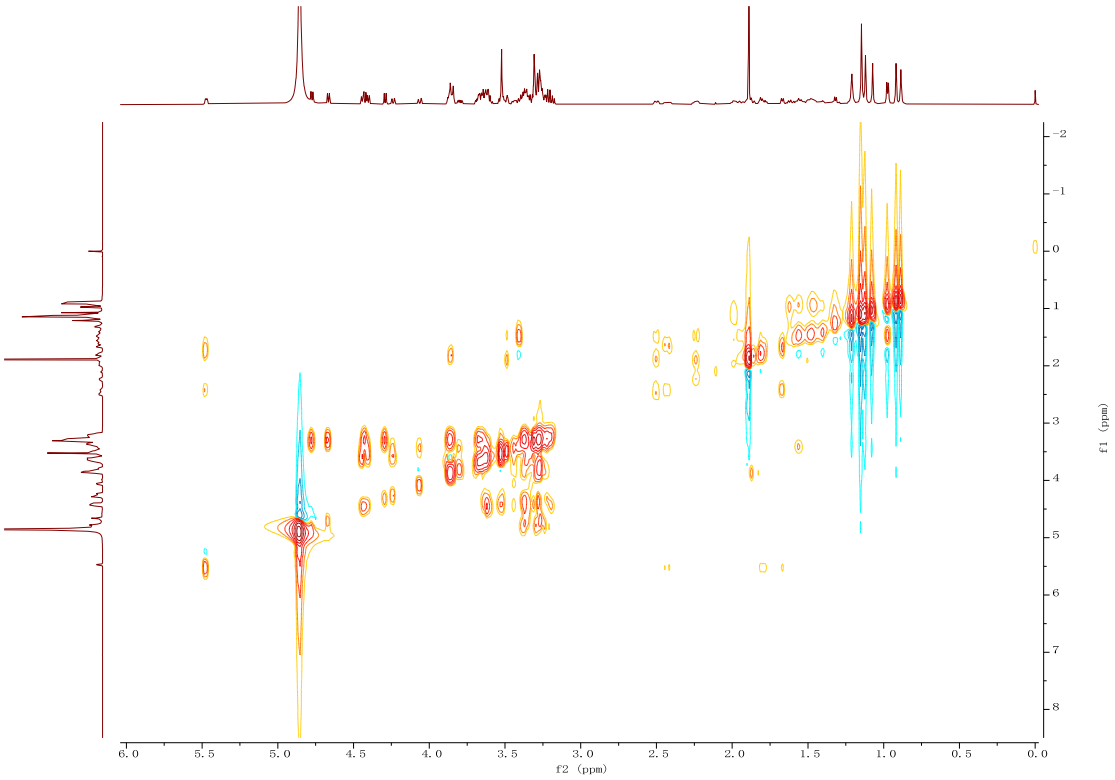
Figure S21. HSQC spectrum of **VI** in Dimethyl sulfoxide-d6 (600 MHz). Related to Figure 5.



183

184 **Figure S22**. COSY spectrum of **VI** in Dimethyl sulfoxide-d6 (600 MHz). Related to

185 Figure 5.



186

187 **Figure S23**. TOCSY spectrum of **VI** in Dimethyl sulfoxide-d6 (600 MHz). Related to

188 Figure 5.

>MG1
GGATCCATGGAAAAGGGTGACACTCACATCTTGGTTTTCCCATTCCCAGCTCAAGGTCACATTAACCCATTGTTGCAATTGTCTAAGCAC
TTGATCGCTAAGGGTATTAAGGTTTCTTTGGTCACTACCTTGCACGTCTCTAACAGAATGCAATTGCAAGGTGCTTACTCTAACTCTGTT
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CAATTGAAGTTGCCACCAGAAACTCCAACCATTTCTTTGCCATCTATGCCATTGTTAAGACCATCTGACTTGCCAGCTTACGACTTCGAC
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AGAGTTAAGAGAAACGAACAAAGATTGGCTTCTAAGGAAGAAGTTAGATCTTGTATCTGGGAAGTCATGGAAGGTGAAAGAGCTTCTGAA
TTCAAGTCTAACTCTATGGAATGGAAGAAGTGGGCTAAGGAAGCTGTTGACGAAGGTGGTTCTTCTGACAAGAACATTGAAGAATTCGTC
GCTATGTTGAAGCAAACCtaaCTCGAG
> MS1
GGATCCATGGACGCTGCTCAACAAGGTGACACTACCACTATTTTGATGTTGCCATGGTTGGGTTACGGTCACTTGTCTGCTTTCTTGGAA
TTGGCTAAGTCTTTGTCTAGAAGAAACTTCCACATCTACTTCTGTTCTACCTCTGTTAACTTGGACGCTATTAAGCCAAAGTTGCCATCT
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ATCTCTCAAGGTTTGCACCCAATTCACTACCCACACTCTAAGTTCCCATTCTCTGAATTCGTCTTGCACAACCACTGGAAGGCTATGTAC
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TACGAACCAAACCAAGACGGTGAAGACGAAGGTTACTCTTCTATTAAGAACTGGTTGGACAAGAAGGAACCATCTTCTACTGTCTTCGTT
TCTTTCGGTTCTGAATACTTCCCATCTAAGGAAGAAATGGAAGAAATTGCTCACGGTTTGGAAGCTTCTGAAGTTAACTTCATCTGGGTT
GTCAGATTCCCACAAGGTGACAACACCTCTGGTATTGAAGACGCTTTGCCAAAGGGTTTCTTGGAAAGAGCTGGTGAAAGAGGTATGGTT
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GTTGAAGCTAAGAGAGATCCAGACGGTAAAATCCAAAGAGACGAAGTTGCTAAGTTGATTAAGGAAGTTGTCGTTGAAAAGACTAGAGAA
GACGTCAGAAAGAAGGCTAGAGAAATGTCTGAAATCTTGAGATCTAAGGGTGAAGAAAAGTTCGACGAAATGGTTGCTGAAATCTCTTTG
TTGTTGAAGATTtaaCTCGAG

Figure S24. The gene sequences of MG1 and MS1, which were synthesized by GenScript (Nanjing, China) with codon optimization according to *S. cerevisiae*. Related to Figures 1 and 2.