Supplementary information

Biocatalytic synthesis of a novel atorvastatin catechol derivative as an anti-hyperlipidemic drug candidate using bacterial tyrosinase

Hae Chan Jeong,[†a] Yu-Jin Lee,[†a] Gun Su Cha,[b] Fikri A. R. Hardiyanti Oktavia,[a] Chan Mi Park,[b] and Chul-Ho Yun [*a,c,d]

[a] *School of Biological Sciences and Biotechnology, Graduate School, Chonnam National University, 77 Yongbongro, Gwangju 61186, Republic of Korea*

[b] *Institute of Natural BIO Industry of Namwon, 43 Simyogil, Namwonsi, Jeonbukdo 55801, Republic of Korea*

[c] *School of Biological Sciences and Technology, Chonnam National University, 77 Yongbongro, Gwangju 61186, Republic of Korea.*

[d] *Institute of Synthetic Biology for Carbon Neutralization, Chonnam National University, 77 Yongbongro, Gwangju 61186, Republic of Korea*

[†] These authors contributed equally to this work

*Corresponding author at: School of Biological Sciences and Technology, Chonnam National University, 77 Yongbongro, Gwangju 61186, Republic of Korea. E-mail address: chyun@chonnam.ac.kr (C.-H. Yun)

Materials and methods

Materials

L-Ascorbic acid (LAA), atorvastatin, hydrogen peroxide (H_2O_2), isopropyl β-D-1-thiogalactopyranoside (IPTG), and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Hydroxy atorvastatin was purchased from Cayman Chemical (Ann Arbor, MI, USA). All other chemicals and solvents were analytical grade.

Protein expression and preparation

CYP102A1 H1120 mutant

The heme domain gene of CYP102A1 H1120 mutant (D23G/R47L/F77Y/F81V/F87V/F107L/D136G/E143G/L188Q/E267V/T463I) in pCW vector was obtained from random mutagenesis by error-prone PCR. To express H1120 mutant, the plasmid was transformed into *Escherichia coli* DH5αF'IQ. The recombinant cell was cultivated in Terric Broth and the gene was induced by adding 0.50 mM of IPTG. The harvested cells were suspended with sonication buffer (0.25 M sucrose, 50 mM Tris-acetate buffer, and 0.25 mM ethylenediaminetetraacetic acid, pH 7.6). After sonication of resuspended cells, the lysate was centrifuged at 100,000 xg for 90 min. The supernatant fractions were collected and used for enzymatic assay. The details of H1120 mutant preparation and quantification were described in our previous study.[1]

Tyrosinase

The tyrosinase gene (GenBank: PV891361) from *Priestia megaterium* GJ804 (GJ804 Ty) was cloned in pET28a vector. *E. coli* C2566 was used as an expression host and cultured in Luria-Bertani media. The gene of GJ804 Ty was induced by 0.50 mM IPTG. After cell harvest, cell

pellet was lysed through sonication and centrifuged. The soluble fraction was obtained and used for the purification of tyrosinase using HisPur™ Ni-NTA column (Thermo Fisher Scientific, Waltham, MA, USA). Purification procedure and buffers were followed by the manufacturer's protocol. More details have been described in our previous study.[2]

Enzyme Assay

All assays were performed with a volume of 0.25 mL for each sample. To optimize H1120 mutant activity toward atorvastatin, reaction mixtures were prepared in 0.10 M potassium phosphate buffer. Reactions were started and stopped by adding H_2O_2 and ethyl acetate, respectively. For optimization of GJ804 Ty activity toward 4-hydroxy atorvastatin, the reaction solutions were prepared in 0.10 M potassium phosphate buffer. The reaction was initiated by adding GJ804 Ty and stopped by adding ethyl acetate. Atorvastatin and 4-hydroxy atorvastatin were dissolved in water and sonicated prior to use. At the optimal conditions, the concentration-dependent hydroxylation of atorvastatin or 4-hydroxy atorvastatin was catalysed by H1120 mutant or GJ804 Ty, respectively. For one-pot reaction, 0.20 μM of H1120 mutant reacted with 0.50 mM atorvastatin and 1 mM H_2O_2 in 0.10 M potassium phosphate buffer (pH 6.0) at 30 ℃ for 30 min. Then, 80 μg/mL of GJ804 Ty and 20 mM LAA were added to the solution and reacted at 50 ℃ for 1 h.

HPLC analysis (atorvastatin hydroxylation assay)

The reactions were quenched by adding ethyl acetate and vortexing vigorously. The samples were centrifuged at 3000 xg for 10 min. The upper layer was transferred to clear tube and evaporated by blowing nitrogen gas. Then, the samples were dissolved in mobile phase solution (A:B=6:4) and analysed by HPLC (Shimadzu, Kyoto, Japan) equipped UV-vis detector and Gemini C18 column (150 x 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA). Mobile phase

A was 0.1% formic acid in water and mobile phase B was 100% acetonitrile. Atorvastatin and its products were separated using programmed gradient at a flow rate of 1 mL/min as follows: 0–4 min (B: 35%), 4–11 min (B: up to 70%), 11–13 min (B: 70%), 13–14 min (B: up to 35%), and 14–25 min (B: 35%). The samples were detected at 260 nm.

Identification of product catalyzed by tyrosinase

LC-MS

The mass analysis of atorvastatin derivatives was performed using a Xevo TQ-S micro mass spectrometer coupled to an ACQUITY UPLC H-Class PLUS system (Waters, Milford, MA, USA). The analytes were separated on an ACQUITY UPLC BEH C18 column (100 x 2.1 mm, 1.7 μm, Waters) maintained at 35 ℃. The mobile phases were 0.1% formic acid in water (A) and 100% acetonitrile (B), with a flow rate 0.2 mL/min and an injection volume was 1 μL. The programmed gradient was as follows: 0–2.0 min (B: 35%), 2.0–5.5 min (B: up to 70%), 5.5–6.5 min (B: 70 %), 6.5–7.0 min (B: up to 35%), and 7.0–10.0 min (B: 35%). The mass spectra recorded by electrospray ionization in negative mode (ESI^-). The capillary voltage was set at 3.2 kV, cone voltage at 30 V, source temperature at 120 ℃, desolvation temperature at 350 ℃, and desolvation gas flow at 800 L/h. Data were acquired in full scan mode over the *m/z* range of 500–700, and processed using MassLynx 4.2 software (Waters).

Nuclear magnetic resonance (NMR) spectroscopy

The samples were separated using HPLC as described above. The NMR analysis was performed at 25 ℃ on 600 MHz NMR spectrometer using DMSO-d_6 as the solvent. Chemical shifts were acquired in parts per million (ppm) relative to residual solvent signals (^{1}H: 2.50 ppm; ^{13}C: 39.52 ppm). Structural assignments were established using 1D (^{1}H, ^{13}C) and 2D (COSY, HSQC, HMBC) spectra. The analysis was carried out at Korea Basic Institute Gwangju

Center (Gwangju, Republic of Korea).

HMG-CoA reductase inhibition assay

The inhibitory effects of atorvastatin, 4-hydroxy atorvastatin, 3,4-dihydroxy atorvastatin toward HMG-CoA reductase were determined by HPLC. The reaction mixture contained assay buffer, NADPH, and atorvastatin/its derivatives (50–1000 nM) without HMG-CoA. The mixture was pre-incubated at 37 ℃ for 5 min, and reaction was started by adding HMG-CoA. The reactions were quenched after 5 min by adding 2 M hydrochloric acid. The samples were injected into HPLC after filtration with 0.45 μm syringe filter. HMG-CoA and CoA were separated in Gemini C18 column (150 x 4.6 mm, 5 μm) and detected at 260 nm. The mobile phases were 0.1 M KH_2PO_4 (A) and methanol (B). The mobile phase flow was as followed: 0–15 min (B: 10–30%), 15–16 min (B: up to 40%), 16–17 min (B: 40%), 17–17.5 min (B: up to 10%), and 17.5–27.5 min (B: 10%) at a flow rate of 0.8 mL/min. The relative activity was compared with the negative control (absence of inhibitor). The results were analyzed by nonlinear regression fitted using a four-parameter logistic model in GraphPad Prism (GraphPad Software, San Diego, CA, USA).

NMR Result

Atorvastatin

^{1}H NMR (600 MHz, dmso) δ 9.80 (s, 1H), 7.51 (d, *J* = 7.3, 2H), 7.27 – 7.15 (m, 6H), 7.07 (d, *J* = 4.4 Hz, 4H), 7.03 – 6.95 (m, 2H), 4.77 (s, 1H), 3.95 (ddd, *J* = 14.7, 10.7, 4.9 Hz, 1H), 3.79 (ddd, *J* = 13.4, 8.7, 5.0 Hz, 2H), 3.54 (dp, *J* = 8.1, 4.4 Hz, 1H), 3.23 (hept, *J* = 7.1 Hz, 1H), 2.08 (m, 1H), 1.97 (dd, *J* = 15.4, 8.2 Hz, 1H), 1.66 – 1.49 (m, 2H), 1.45 – 1.39 (m, 1H), 1.37 (dd, *J* = 7.1, 2.0 Hz 6H), 1.25 (dt, *J* = 13.6, 4.8 Hz, 1H). **^{13}C NMR** (151 MHz, dmso) δ 178.37, 166.20, 162.42, 160.79, 139.47, 135.97, 134.93, 133.40 (d, *J* = 8.3 Hz), 129.16, 128.75, 128.46, 127.65, 127.32, 125.38, 123.00, 120.59, 119.43, 117.51, 115.40 (d, *J* = 21.3 Hz), 66.29 (d, *J* = 2.9 Hz), 43.96, 43.62, 40.89, 39.03, 25.67, 22.33 (d, *J* = 3.7 Hz).

4-Hydroxy atorvastatin

^{1}H NMR (600 MHz, dmso) δ 9.57 (s, 1H), 9.27 (s, 1H), 7.28 – 7.26 (m, 2H), 7.26 – 7.22 (m, 2H), 7.20 – 7.16 (m, 2H), 7.10 – 7.04 (m, 4H), 7.03 – 6.97 (m, 1H), 6.62 (dd, 2H), 4.83 (s, 1H), 3.92 (ddd, *J* = 16.0, 11.3, 5.0 Hz, 1H), 3.75 (ddd, *J* = 14.9, 11.1, 5.1 Hz, 1H), 3.68 – 3.57 (m, 1H), 3.51 (tt, *J* = 8.1, 4.3 Hz, 1H), 3.20 (h, *J* = 7.0 Hz, 1H), 1.97 (dd, *J* = 15.0, 4.2 Hz, 1H), 1.83 – 1.73 (m, 1H), 1.63 – 1.54 (m, 1H), 1.50 (ddd, *J* = 18.8, 12.8, 7.5 Hz, 1H), 1.39 – 1.30 (m, 7H), 1.21 – 1.02 (m, 1H). **^{13}C NMR** (151 MHz, dmso) δ 175.65, 165.57, 162.37, 160.75, 153.29, 135.63, 135.08, 133.37 (d, *J* = 8.2 Hz), 131.18, 129.17, 128.86, 127.59, 127.28, 125.26, 121.22, 120.48, 117.72, 115.35 (d, *J* = 21.3 Hz), 114.80, 66.49, 66.32, 44.13, 43.62, 40.78, 38.97, 25.66, 22.30(d, *J* = 8.5 Hz).

3,4-Dihydroxy atorvastatin

^{1}H NMR (600 MHz, dmso) δ 9.46 (s, 1H), 8.84 (s, 1H), 8.51 (s, 1H), 7.27 – 7.20 (m, 2H), 7.20 – 7.15 (m, 2H), 7.13 (d, *J* = 2.5 Hz, 1H), 7.12 – 7.02 (m, 4H), 7.01 (ddt, *J* = 7.3, 5.9, 1.9 Hz, 1H), 6.66 (dd, *J* = 8.6, 2.5 Hz, 1H), 6.55 (dd, *J* = 8.4, 3.5 Hz, 1H), 3.92 (m, 1H), 3.82 (tt, *J* = 8.4, 4.7 Hz, 1H), 3.77 – 3.71 (m, 1H), 3.53 (m, 1H), 3.20 (dq, *J* = 13.9, 6.9 Hz, 1H), 2.25 (dd, *J* = 15.0, 4.8 Hz, 1H), 2.20 – 2.13 (m, 1H), 1.62 (m, 1H), 1.52 (dd, *J* = 11.9, 5.4 Hz, 1H), 1.42 (dd, *J* = 14.0, 7.4 Hz, 1H), 1.37 (dd, *J* = 12.3, 7.0 Hz, 6H), 1.30 (dt, *J* = 13.8, 5.1 Hz, 1H).**^{13}C NMR** (151 MHz, dmso) δ 173.11, 165.46, 162.39, 160.76, 144.69, 141.23, 135.53, 135.15, 133.36 (d, *J* = 8.4 Hz), 131.59, 129.14, 128.84, 127.62, 127.14, 125.29, 120.44, 117.87, 115.38 (d, *J* = 21.5 Hz), 115.03, 110.83, 108.35, 65.90, 65.45, 43.70, 42.62, 40.72, 38.83, 25.67, 22.32(d, *J* = 4.1 Hz).

Table S1. Chemical shifts of 3,4-dihydroxy atorvastatin.

Atom[a]	δ_H (mult, *J* in Hz)	δ_C	HMBC(H→C)
1	-	131.6	-
2	7.13 (d, *J*=2.4)	108.3	1, 3, 6
3	-	144.7	-
4	-	141.2	-
5	6.55 (dd, *J* = 8.4, 3.4)	115.0	1, 3
6	6.66 (dd, *J* = 8.5, 2.5)	110.8	2, 3, 5
1’	-	135.1	-
2’,6’	7.07	129.1	4’
3’,5’	7.08	127.6	1’
4’	7.01	125.3	2’, 6’
1’’	-	128.8	-
2’’,6’’	7.19/7.23	133.4	
3’’,5’’	7.18/7.22	115.4	
4’’	-	162.4/160.8	-
1’’’	-	127.1	-
2’’’	-	120.4	-
3’’’	-	117.9	-
3’’’a (carbonyl)	-	165.5	-
3’’’b	9.46	-	2, 6, 3’’’a
4’’’	-	135.5	-
5’’’	3.21	25.7	3’’’, 4’’’ ,6’’’
6’’’	1.36	22.3	4’’’, 5’’’
1’’’’	3.74/3.92	40.7	
2’’’’	1.52/1.62	38.8	
3’’’’	3.53	65.9	
4’’’’	1.30/1.42	43.7	2’’’’,3’’’’,5’’’’,6’’’’
5’’’’	3.82	65.4	
6’’’’	2.17/2.25	42.6	4’’’’,5’’’’,7’’’’
7’’’’ (carbonyl)	-	173.1	-

[a]Assignments correspond to Figure S13.

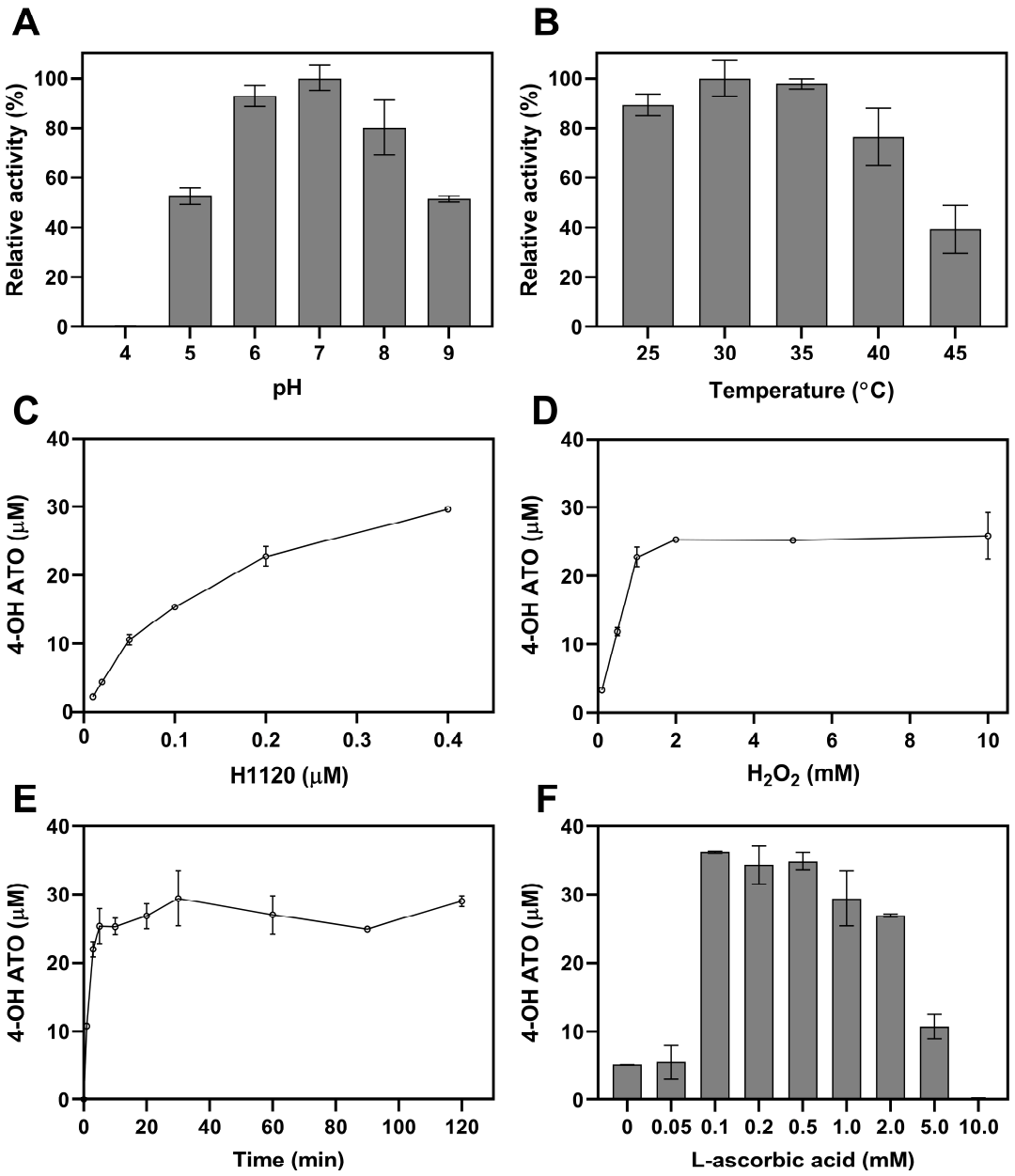

Figure S1. Effect of reaction parameters on production of 4-hydroxy atorvastatin from atorvastatin by CYP102A1 H1120. (A) pH, (B) temperature, (C) enzyme concentration, (D) H_2O_2 concentration, (E) reaction time, (F) L-ascorbic acid (LAA) concentration. Unless otherwise stated, reactions were conducted with 100 μM atorvastatin, 1 mM H_2O_2, and 1 mM LAA in 0.10 M potassium phosphate buffer (pH 7.0) at 30 ℃ for 10 min. The enzyme concentration was 0.050 μM for (A–B) and 0.20 μM for (D–F). For (E–F), the H_2O_2 concentration was 2 mM, and the reaction time for (F) was 30 min.

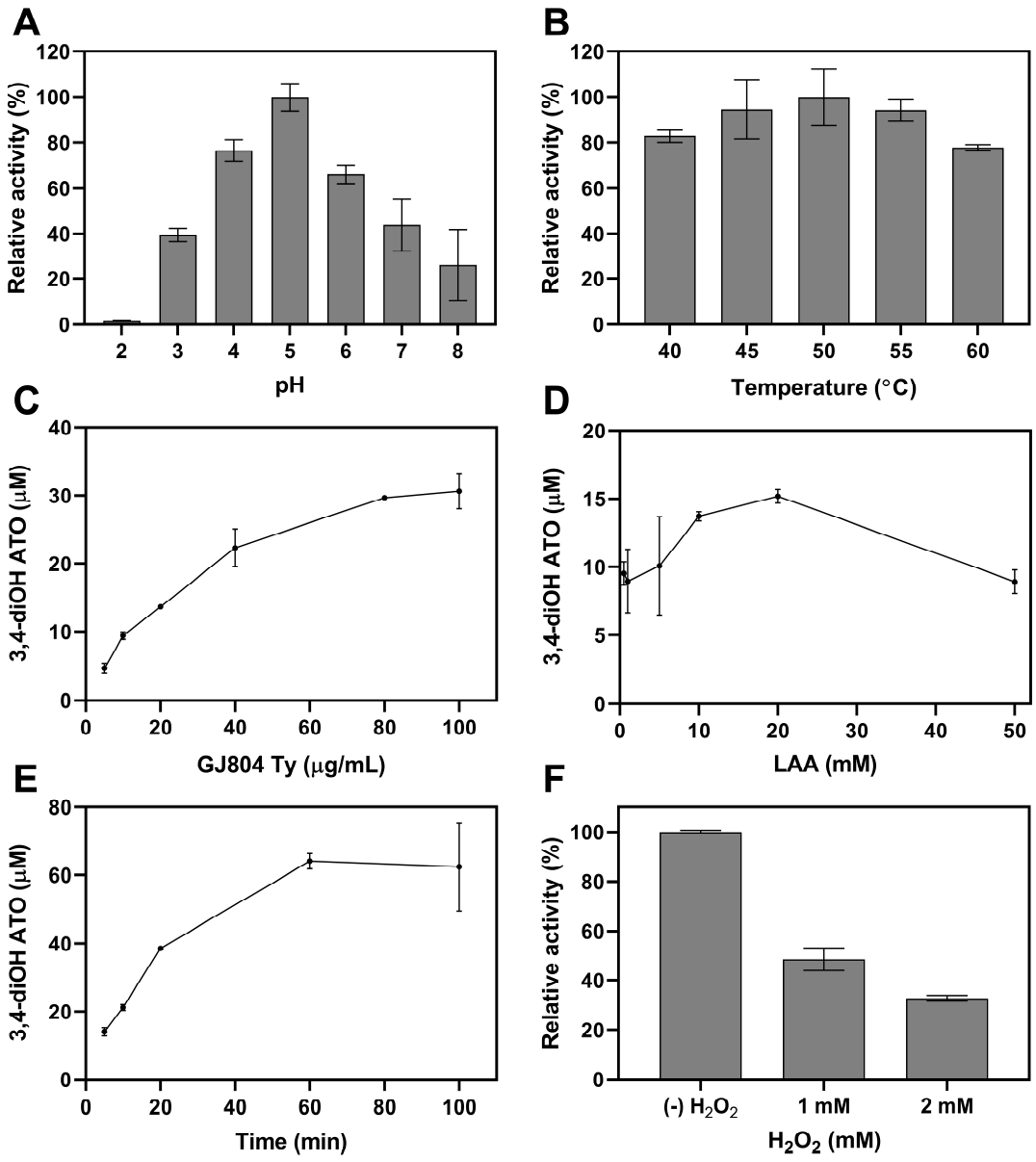

Figure S2. Effect of reaction parameters on production of 3,4-dihydroxy atorvastatin (3,4-diOH ATO) from 4-hydroxy atorvastatin (4-OH ATO) by tyrosinase (GJ804 Ty). (A) pH, (B) temperature, (C) enzyme concentration, (D) L-ascorbic acid (LAA) concentration, (E) reaction time, (F) H_2O_2 concentration. Unless otherwise stated, reactions were carried out with 20 μg/mL GJ804 Ty, 25 μM 4-OH ATO, and 10 mM LAA in 0.10 M potassium phosphate (pH 5.0) at 50 °C for 30 min. The buffer pH was 7.0 for (B). For (C–D), 50 μM 4-OH ATO was used. For (E), reactions were performed with 80 μg/mL GJ804 Ty and 100 μM 4-OH ATO. For (F), reactions were carried out with 80 μg/mL GJ804 Ty, 100 μM 4-OH ATO, and 20 mM LAA for 60 min.

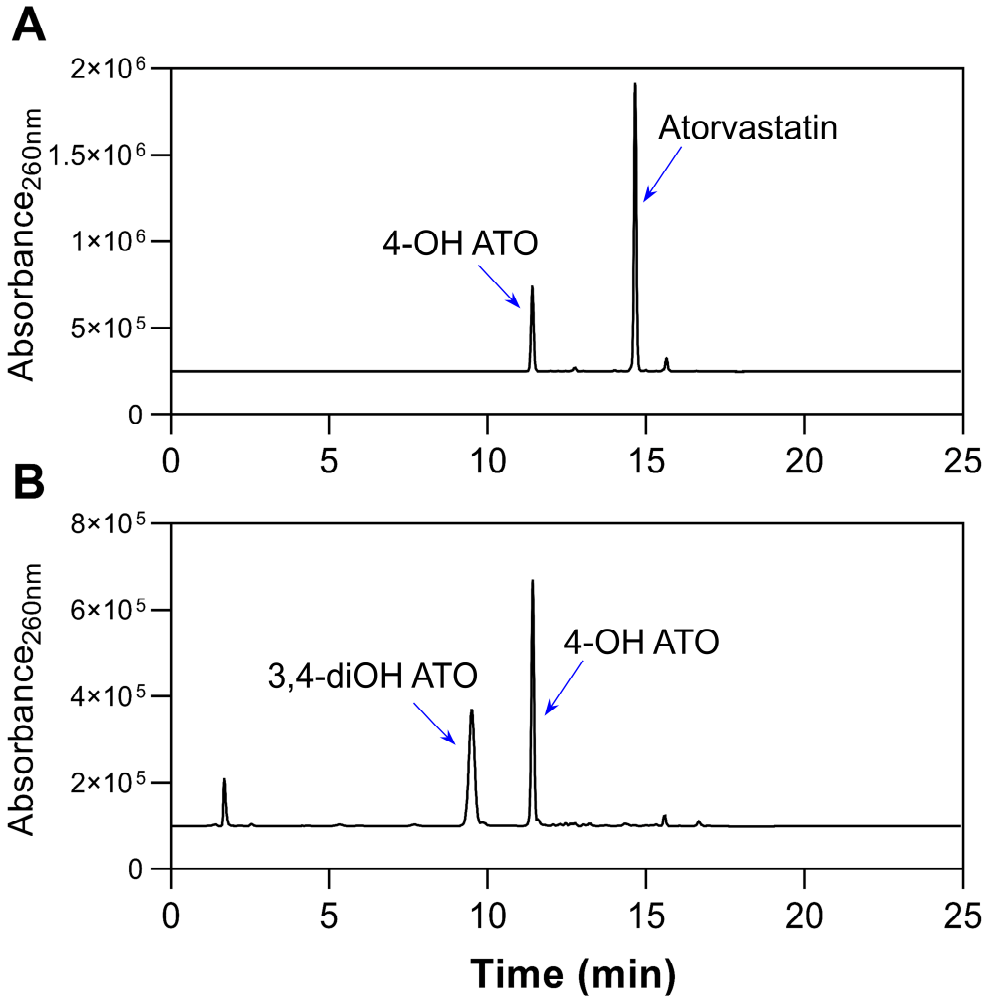

Figure S3. High- performance liquid chromatography (HPLC) trace. (A) Hydroxylation of atorvastatin catalyzed by H1120 mutant. (B) Hydroxylation of 4-hydroxy atorvastatin catalyzed by GJ804 Ty.

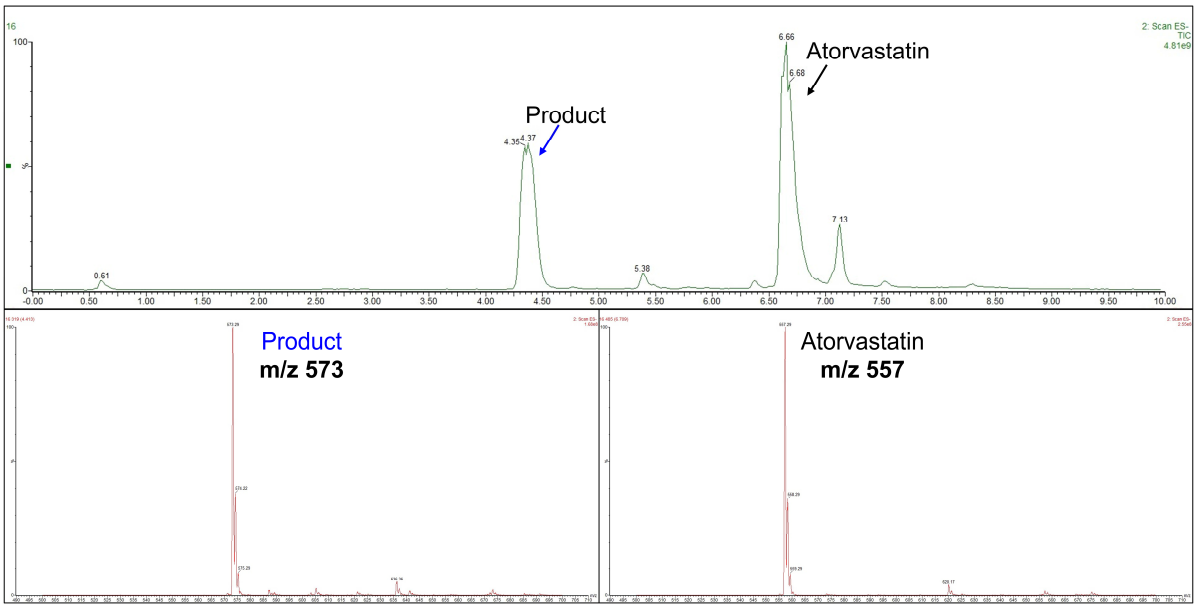

Figure S4. LC-MS analysis of enzymatic conversion of atorvastatin by H1120 mutant. Total ion chromatogram (TIC) and MS spectra of atorvastatin and corresponding major product were obtained from the reaction with H1120 mutant.

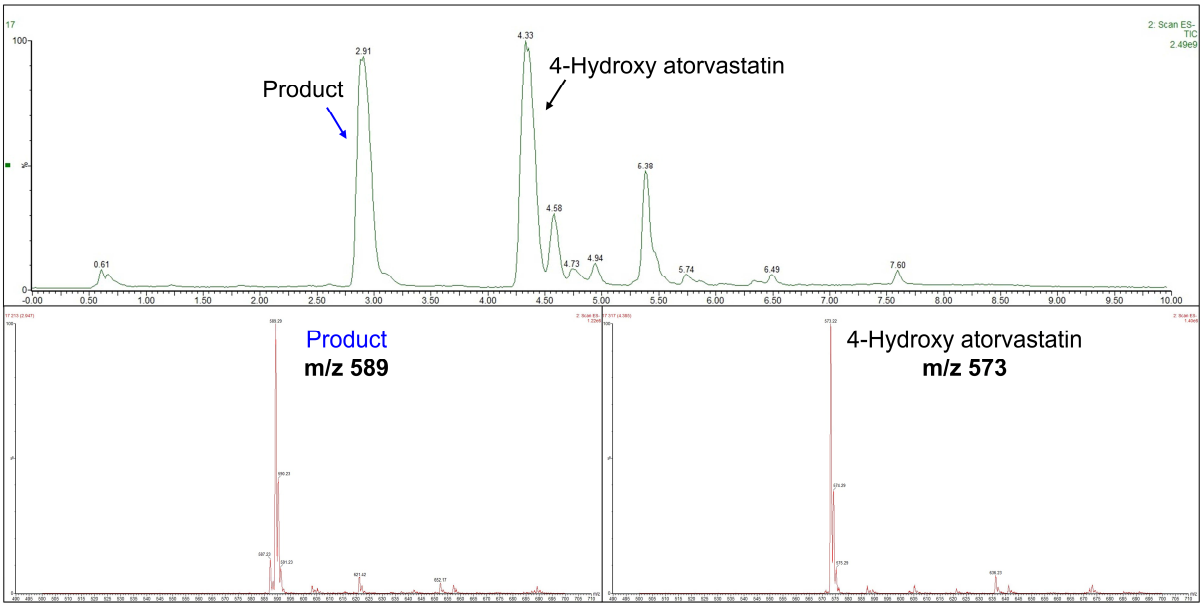

Figure S5. LC-MS analysis of enzymatic conversion of 4-hydroxy atorvastatin by GJ804 Ty. TIC and MS spectra of 4-hydroxy atorvastatin and corresponding major product were obtained from the reaction with H1120 mutant.

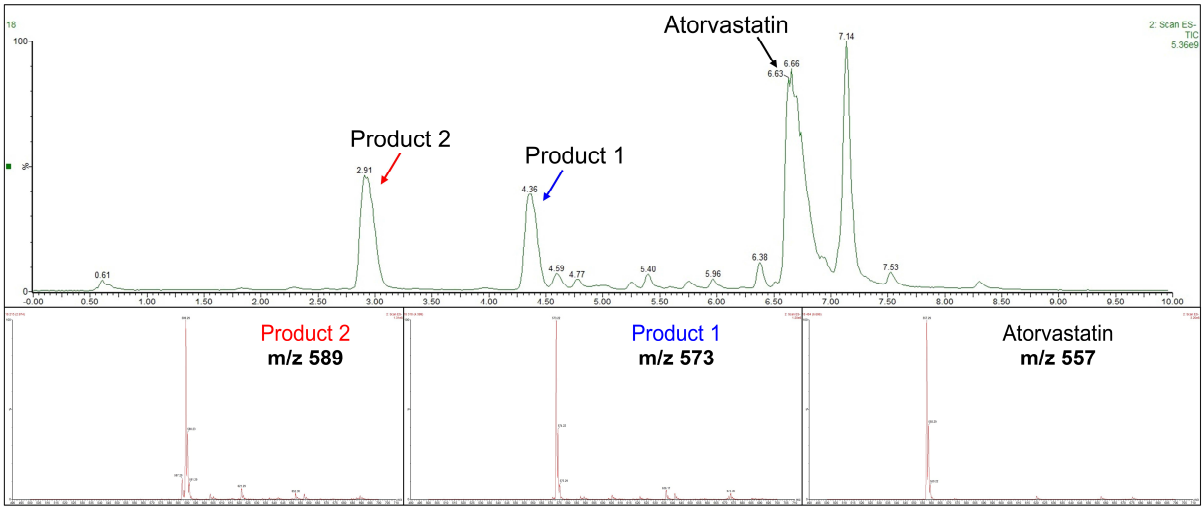

Figure S6. LC-MS analysis of enzymatic conversion of atorvastatin by H1120 mutant and GJ804 Ty. TIC and MS spectra of atorvastatin and corresponding major products were obtained from the one-pot reaction with H1120 mutant and GJ804 Ty.

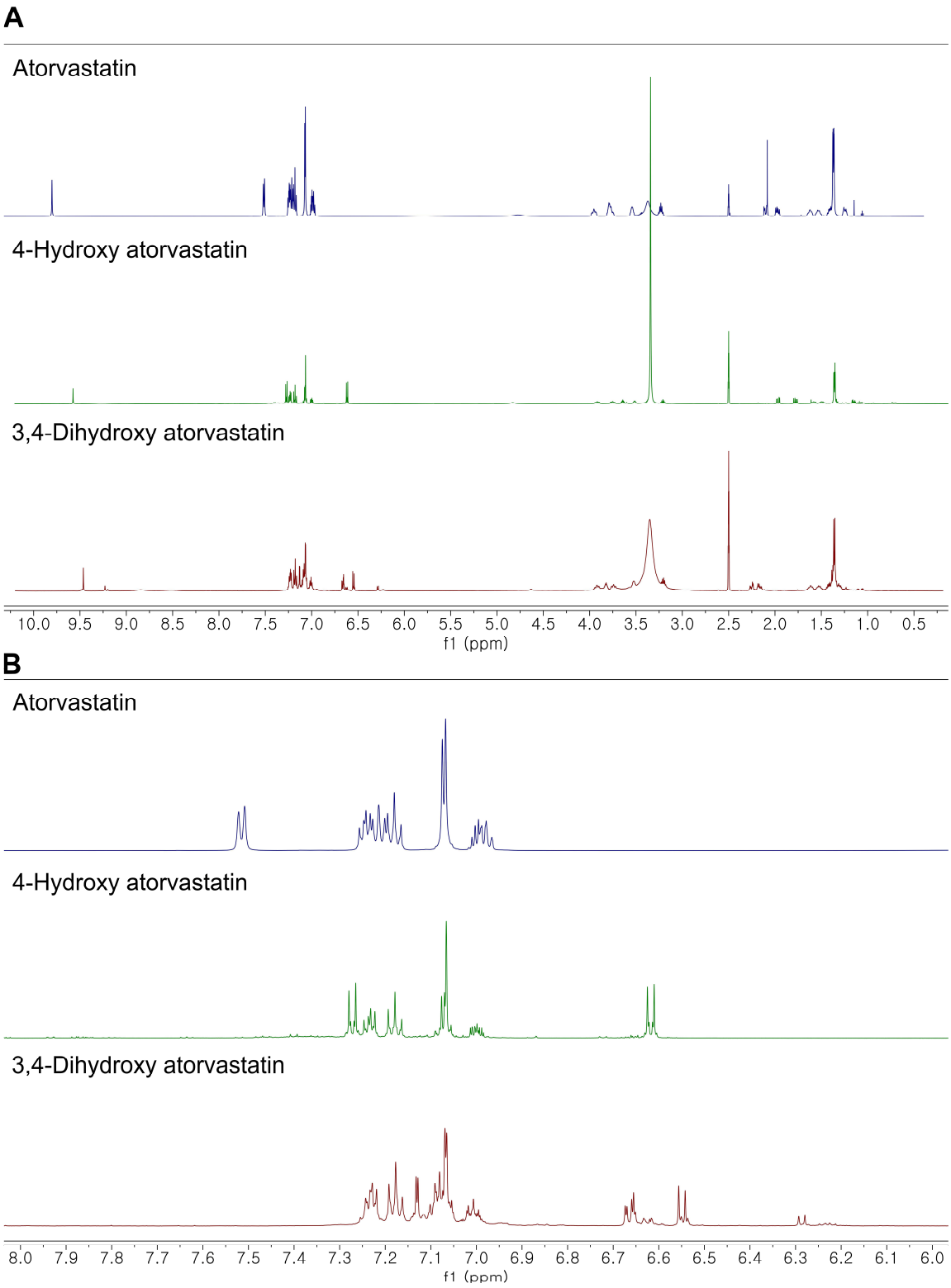

Figure S7. ^{1}H NMR spectra of atorvastatin, 4-hydroxy atorvastatin, and 3,4-dihydroxy atorvastatin. (A) Full spectra. (B) Expanded aromatic region.

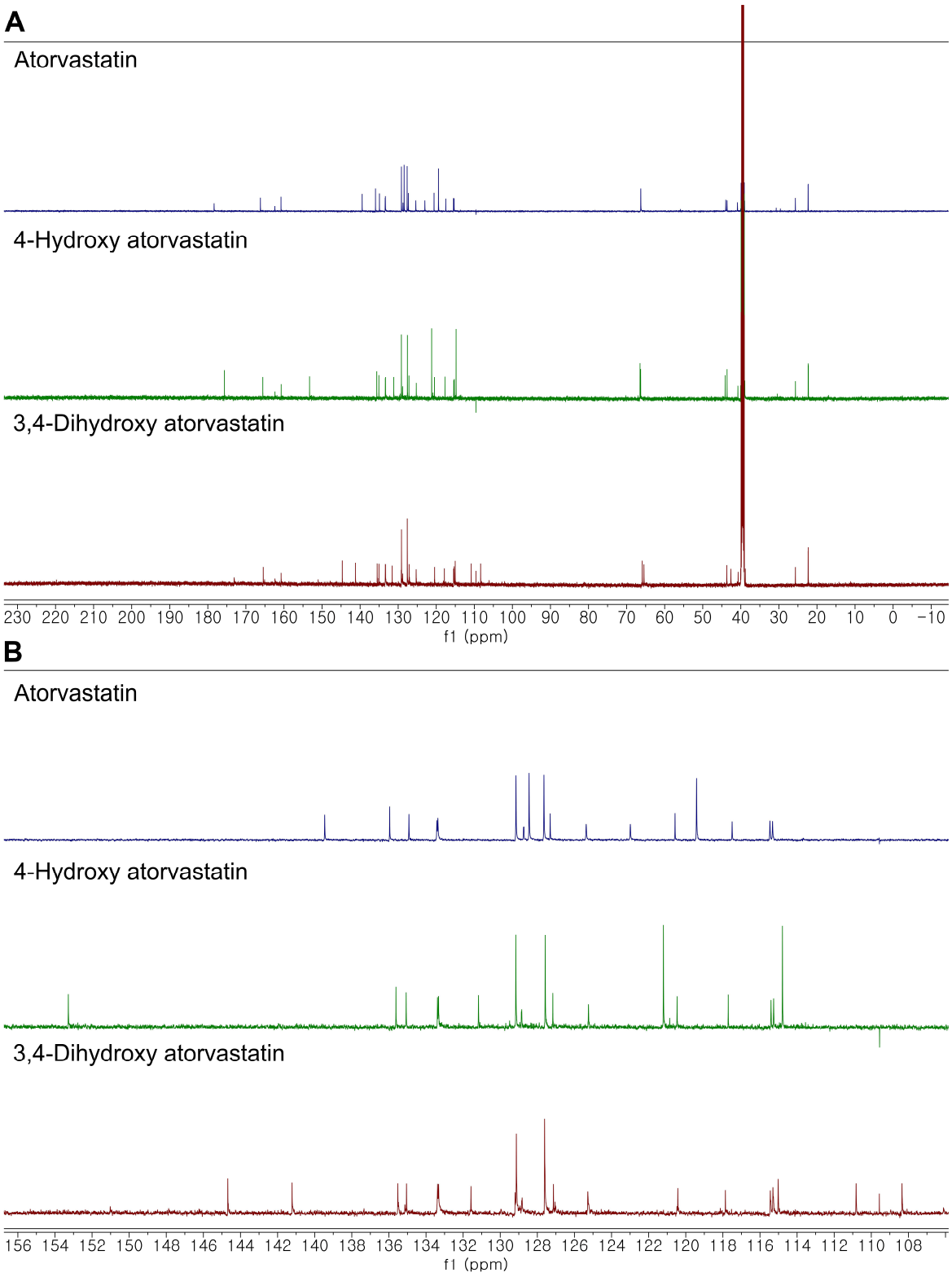

Figure S8. ^{13}C NMR of atorvastatin, 4-hydroxy atorvastatin, and 3,4-dihydroxy atorvastatin. (A) Full spectra. (B) Expanded aromatic region.

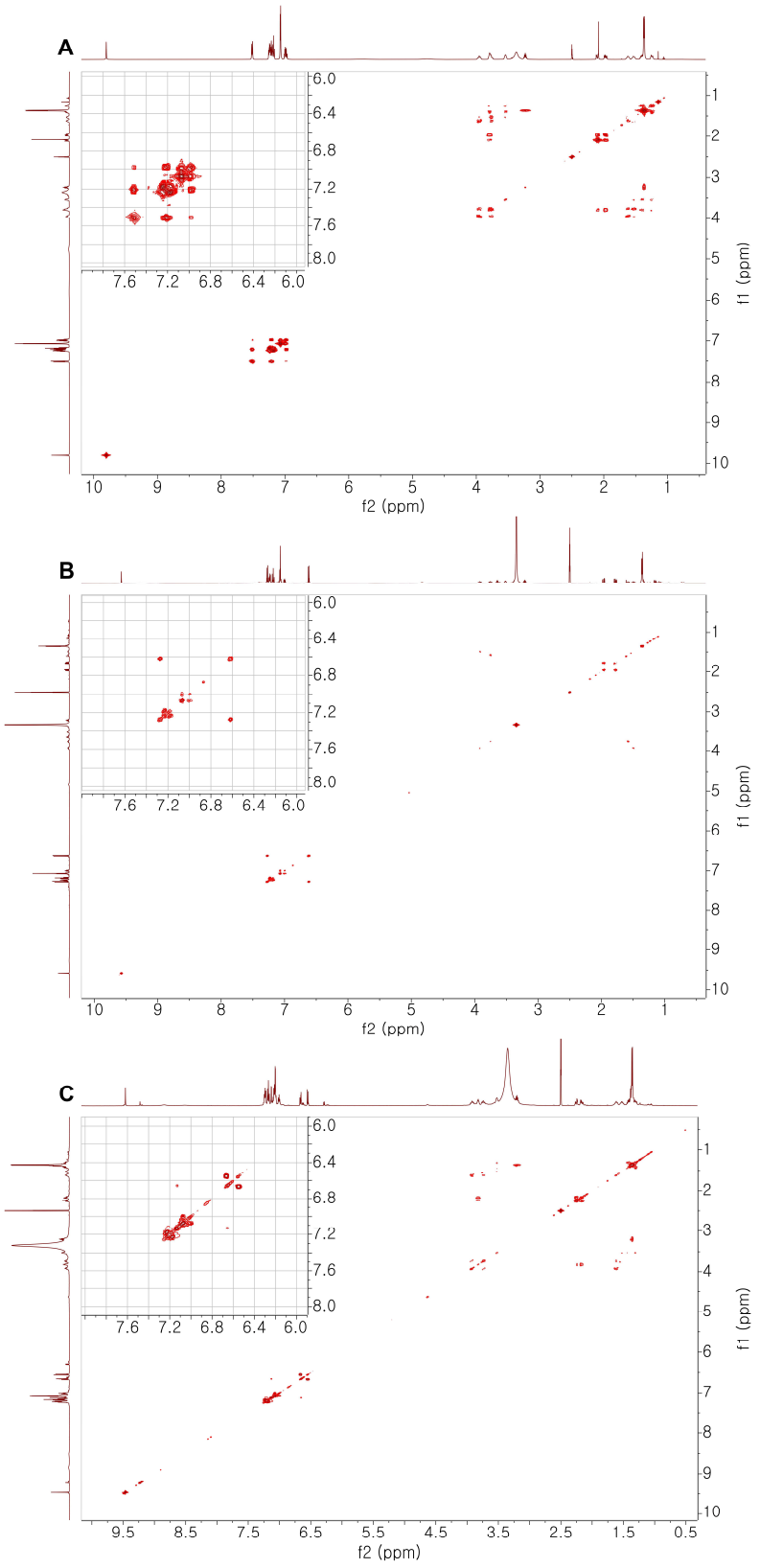

Figure S9. COSY spectra of atorvastatin and its derivatives with expanded aromatic regions. (A) Atorvastatin. (B) 4-Hydroxy atorvastatin. (C) 3,4-Dihydroxy atorvastatin.

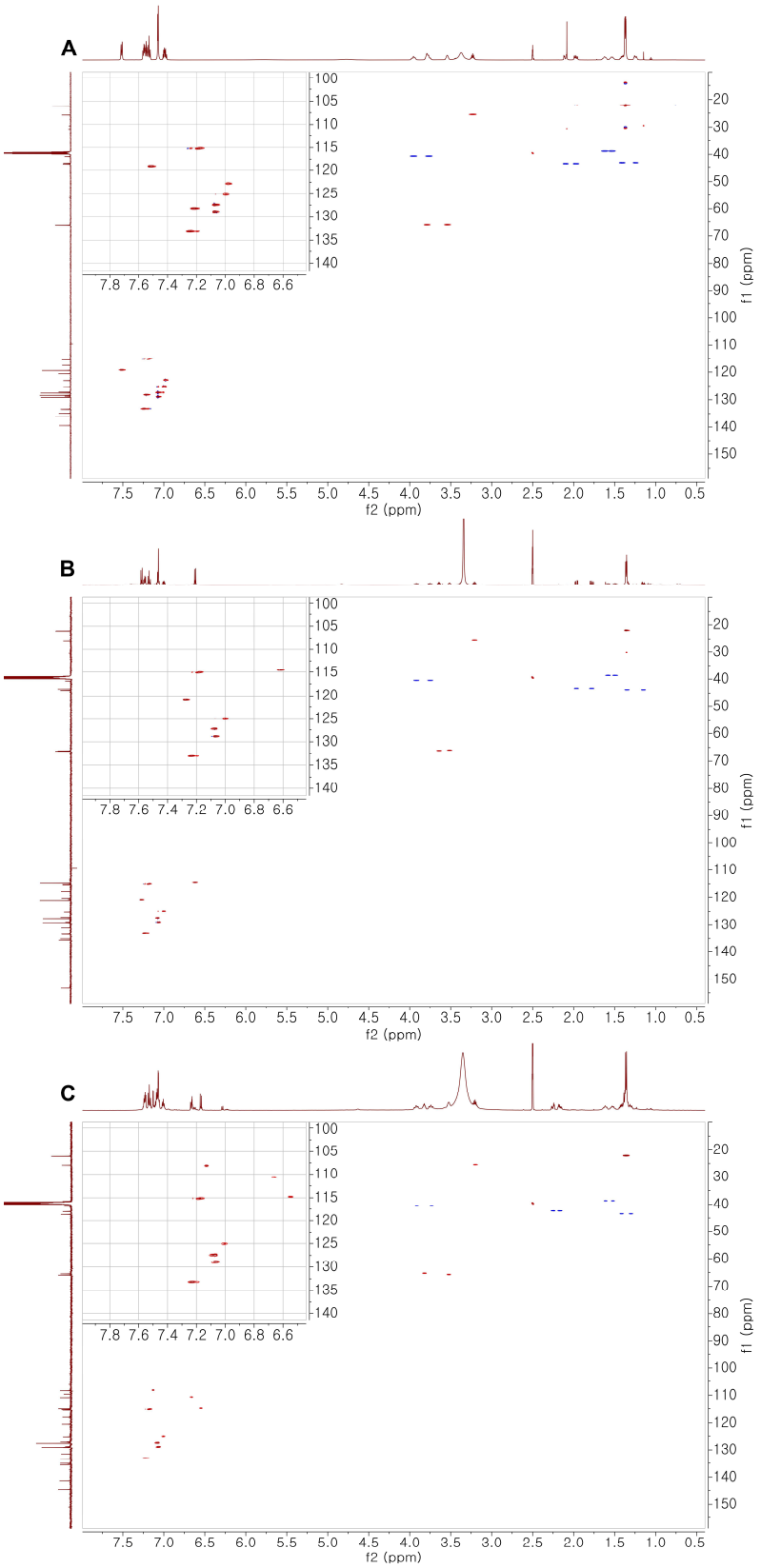

Figure S10. HSQC spectra of atorvastatin and its derivatives with expanded aromatic regions. (A) Atorvastatin. (B) 4-Hydroxy atorvastatin. (C) 3,4-Dihydroxy atorvastatin.

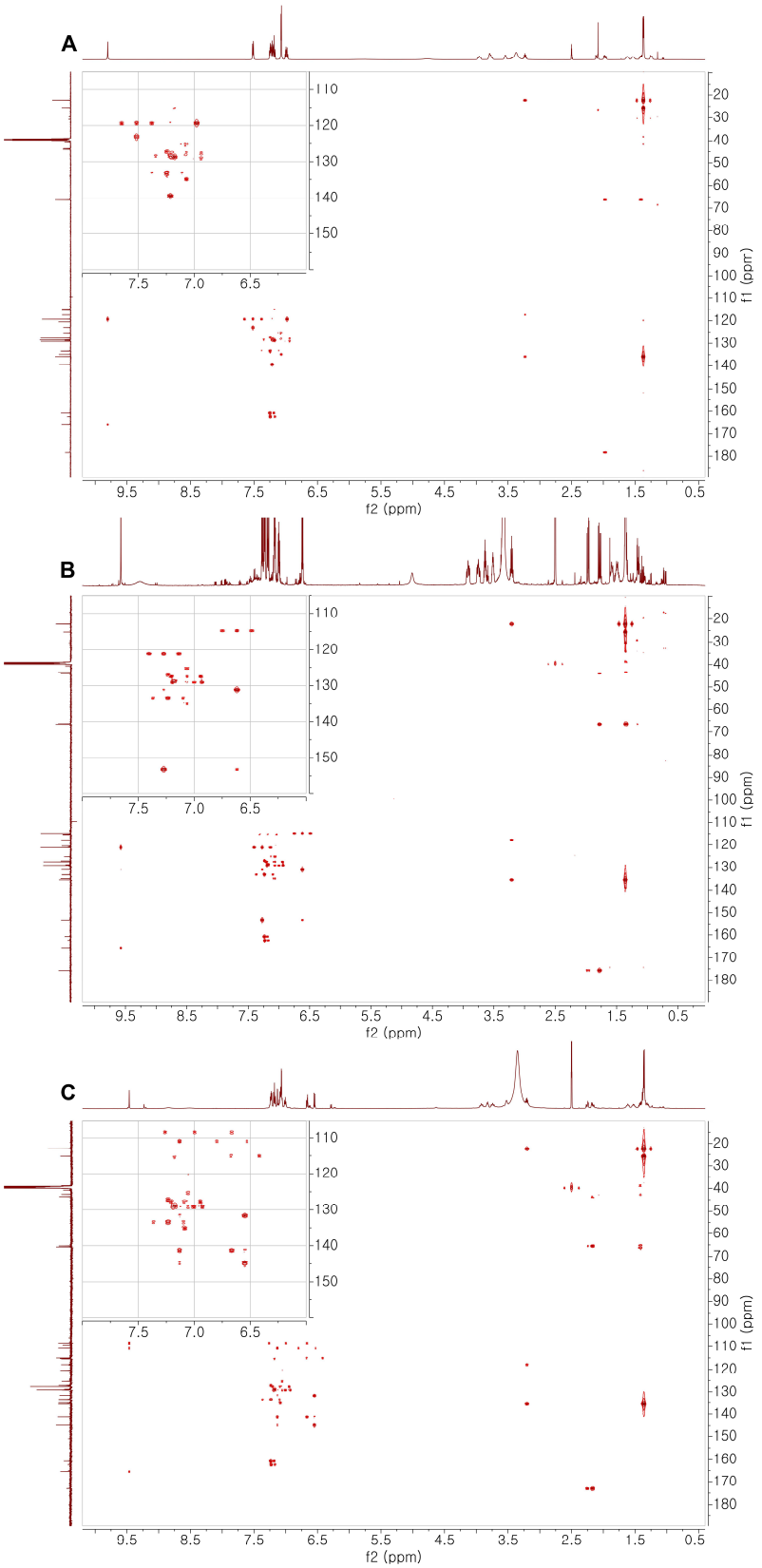

Figure S11. HMBC spectra of atorvastatin and its derivatives with expanded aromatic regions. (A) Atorvastatin. (B) 4-Hydroxy atorvastatin. (C) 3,4-Dihydroxy atorvastatin.

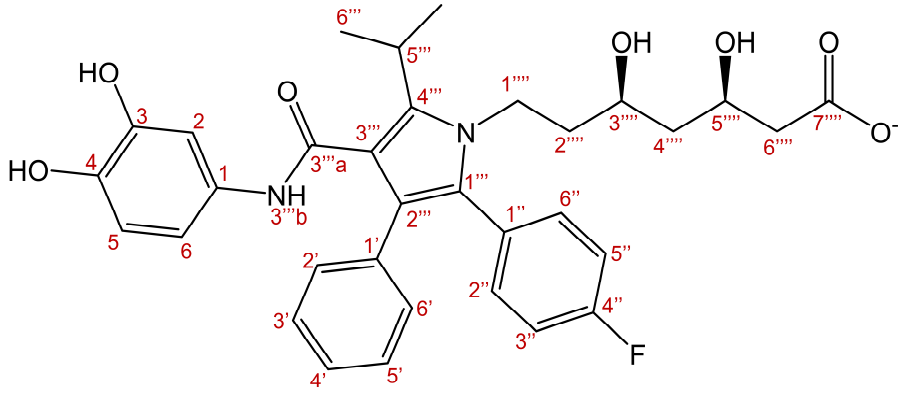

Figure S12. Chemical structure of 3,4-dihydroxy atorvastatin with atom numbering for NMR assignments.

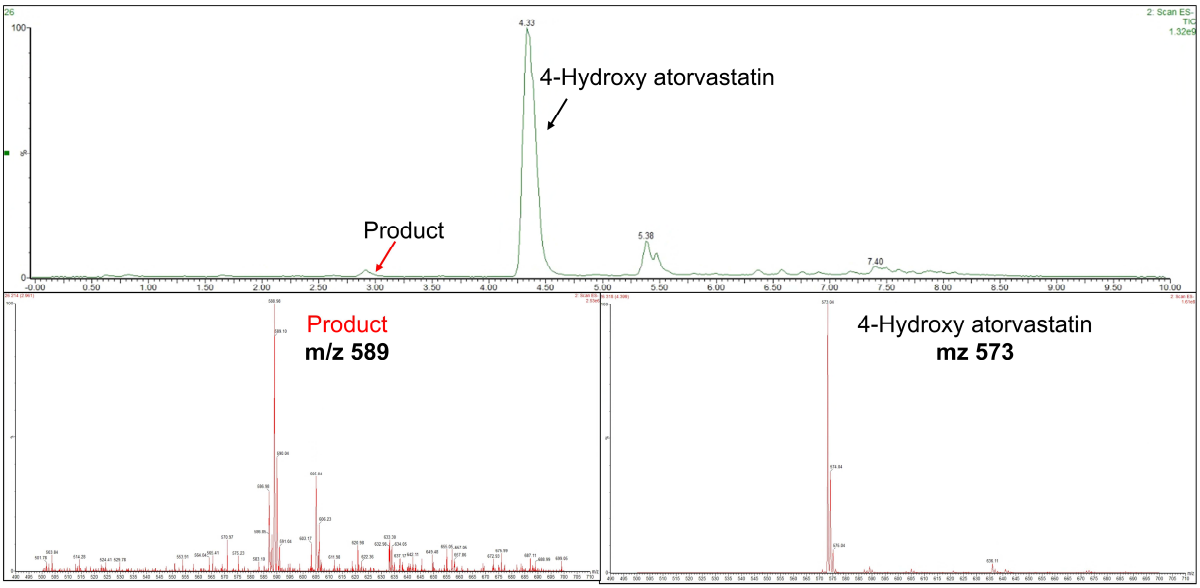

Figure S13. LC-MS analysis of enzymatic conversion of atorvastatin by human liver microsomes (HLMs). TIC and MS spectra of 4-hydroxy atorvastatin and corresponding product were obtained from the reaction with HLMs.

References

1. F. A. R. H. Oktavia, N. A. Nguyen, C. M. Park, G. S. Cha, T. H. H. Nguyen and C.-H. Yun, *J. Inorg. Biochem.*, 2023, **242**, 112165.
2. H. C. Jeong, Y.-J. Lee, N. A. Nguyen, G. S. Cha, C. M. Park and C.-H. Yun., *Int. J. Biol. Macromol.*, 2025, **321**, 146401.