A Bio-inspired Environmentally Friendly and Cost-effective Chemo-enzymatic Synthesis of (−)-Ambrox from *trans*-Nerolidol

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Supplementary Table 1. Plasmids used in this study.

Plasmids	Characteristics	Sources
pET28A(+)-SUMO	*Kan*^{R}	Lab
pET28A(+)	*Kan*^{R}	Lab
pET28A(+)-SUMO-BVMO1	*Kan*^{R}, expressing *BVMO1* gene using T7 promoter	This study
pET28A(+)-SUMO-BVMO2	*Kan*^{R}, expressing *BVMO2* gene using T7 promoter	This study
pET28A(+)-SUMO-BVMO3	*Kan*^{R}, expressing *BVMO3* gene using T7 promoter	This study
pET28A(+)-SUMO-Esterase1	*Kan*^{R}, expressing *Esterase1* gene using T7 promoter	This study
pET28A(+)-SUMO-Esterase2	*Kan*^{R}, expressing *Esterase2* gene using T7 promoter	This study
pET28A(+)-SUMO-Esterase3	*Kan*^{R}, expressing *Esterase3* gene using T7 promoter	This study
pET28A(+)-SHC(WT)	*Kan*^{R}, expressing *SHC(WT)* gene using T7 promoter	This study
pET28A(+)-SUMO-ADH	*Kan*^{R}, expressing *ADH* gene using T7 promoter	This study
pG-KJE8	*Cm*^{R}, expressing *dnaK-dnaJ-grpE* and *groES-groEL* gene using araB and Pzt-1 promoter	Takara
pGro7	*Cm*^{R}, expressing *groES-groEL* gene using araB promoter	Takara
pKJE7	*Cm*^{R}, expressing *dnaK-dnaJ-grpE* gene using araB promoter	Takara
pG-Tf2	*Cm*^{R}, expressing *groES-groEL-tig* gene using Pzt-1 promoter	Takara
pTf16	*Cm*^{R}, expressing *tig* gene using araB promoter	Takara
pWS172-Cas9	Containing cas9 cassette	Lab

Supplementary Table 2. Plasmids used in this study.

Primer	Sequence(5' to 3')
pET-F	tgacctgcagtctcgagcaccac
pET-R	catggcgccctgaaaatacaggttttcg
bvmo1-F	tgtattttcagggcgccatgatgacgactccaactatgacaatgc
bvmo1-R	gtgctcgagactgcaggtcattaatggttgctgagccacgac
bvmo2-F	tgtattttcagggcgccatgatgccttccgcaatcaccccg
bvmo2-R	gtgctcgagactgcaggtcactaggcctctacagcaacatcacgc
bvmo3-F	tgtattttcagggcgccatgatgggttcaattgaacaaattgaagagc
bvmo3-R	gtgctcgagactgcaggtcattatgcctttgccgttgagtaagtc
pET-F-CX	gataacgatattattgaggctcacag
pET-R-CX	ctttcgggctttgttagcagc
esterase1-F	tgtattttcagggcgccatgatgaagtctttcgccaatgcac
esterase1-R	gtggtgctcgagactgcaggtcaacccaaattccccaacacagta
esterase2-F	tgtattttcagggcgccatgatggttcatttatcttgggctctcg
esterase2-R	gtggtgctcgagactgcaggtcaaatatggaagttagcaatatgagcattc
esterase3-F	tgtattttcagggcgccatgatgccttccgatcttccccgac
esterase3-R	gtggtgctcgagactgcaggtcatattgtgccaacagcttcatccactg
pET28a-F	aagccatcgagcgcaggtgaaagcttgcggccgcactcgag
pET28a-R	tccaccaactgctcagccatatggctgccgcgcggcaccag
215G2-F	tacccggacgtggacgacacg
215G2-R	cgtccgggtagtacacgttgtc
HO-gRNA-F	gactttgtaaggcttcattatggaga

HO-gRNA-R	aaactctccataatgaagccttacaa
CaNES2-F	cgtcaaggagaaaaaacatggatttttcgattgctctaggtcttc
CaNES2-R	cggttagagcggatttatttgatcaaaatacttttcaaaaattcttccaacctagg
HO-D-F	tgggacgctcgaaggtatagatagaattgattgctgcttatgaggatatgg
HO-D-R	catgtcatgtccacattaacatcatthgcag
HO-U-F	aggccatgtcttctcgttaagactg
HO-U-R	aagttccaaggtgcgcgcatctgctaaaatgtg
δ-F	tgttggaatagaaatcaactatcatctactaactag
δ-R	ttggaaagtcattaggtgaggttaacatt
Gal-ura-R	ggaaagtacatagggcgacattgggtaataactgatataattaattgaa
Gal-ura-F	aggttttgggacgctcgaagcagattgtatgagagtgcacc
δ1-ADH1-R	ggtatagcatgaggtcgctcacaattctataaatattatcatcatttatatg
δ2-URA-F	aatgtcgcctatgtactttccac

Supplementary Table 3. Amino acid sequences used in this study.

Names	Sequence(5' to 3')
BVMO1	MTTPTMTMPSDLGAATNDTQCRNPKIDDDFVRAALEEASAPALRLALLQVTG DQQLENMQVHKTPIRGGVLNDYTLSDTDAKLVKEKAYKYLAHLDEVQEVPK PVSKKRAFQLMDLYSDAPMYTTPNEPSFDYEEGYEELAFEDYPRDVNWTGA QPSPTELSKWKVMIVGAGISGIAAAIPLKRLGIPFEIVERQSGIGGTWLLNTYPD CRVDTLVYLFQYKFEKKYKWKDFFSSREDLQQYIEYVATKWGVKDNITYDR EVVAATWDEKTKLWSMTLKHKNGNEEVKTCNAIISAAGLFSTPNLPDIAGIH DFKGPLFHTAQWDHSKDYHGKNVALIGTGSTGTQLTPMVAEGARHLSVYQR TPNWIASYEGYRAKVTDHMHWLCDAMPYYWNWYCYSAWFRSLQLANTQY HDPEWRAQGGLINKRNDFLRTSLTKFINEKFADRPDLIAKVTPKQAPMVRRLV VDNGFYDALKRDNVSLITDSIERITEKGIMTKDGNEIEYDMIVLGAGFKTSQYL WPVNYTGTDGMTLAKAWAKDGARSYLGMTMPNYPNLFTLYGPNHQPRGGS LYSYGEMWARYAVASIVGMIERGASSMEIKKDVFDKYQAALDKGNKRIIWES EGAAYYVNEHGRQAVNMPWTTAEYHPMIAKVNFDDYNLTYDDKKQNGVH KANGINGHQTNGHSHSHTHGHTNGHANGHKESWLSNH*
BVMO2	MPSAITPPVDHRSLPGLFKPQRKLKVICVGAGASGLLLSYKIQRHFEDFELQVF EKNPEVSGTWYENRYPGCACDVPSHNYTWSFEPKTDWSANYASSKEIFKYFK DFTRKYGLSKYIKLEHEVVGATWMEAEAQWKVDVKDLRSGNTQSSFAHILV NAGGILNAWRYPPIPGIKDFKGDLVHSAAWPEHLDLNGKVVGLIGNGSSGIQI LPAIKKDVKQLVTFIREATWVAPPLGQAYRAFSTDEQAQFAQDPRHHLETRR ATEATMNQSFGIFHSGSEEQKGVRQYMQNIMETKLNNKQLESVLIPEWSVGC RRLTPGTNYLESLSDDNVKVVYGEITQITESGVICDDGKGEYPVEVLICATGFD TTFKPRFPLIGTTQEKLSDVWKDDPRGYFGIATNNYPNYFFTLGPNCPIGNGPV LCAIEAEVEYIINMLSKFQKENIRSFDIKADAVDAFNDWKDDFMKDTIWAEQC RSWYKAGSATGKILALWPGSTLHYLEALKSPRWEDWDFKYQPGRNRFHYFG NGHSCAEQDGDLSWYIRNEDDSYIDPVLKPKPKAAVESEAHIALPGIGPMLME DPRDVAVEA*
BVMO3	MGSIEQIEELDVLLVGAGFASFTLLNKLRKQGLKTKIYEKGAASGGIWYWNC YPGARVDSDTPIYQLFDKELWEDFTFKEKFAGWKELRNYFNHVEKKWDVRK DTEFNKHVDGATFDEHRRQWLIECSDGSQVYATWFIPCIGFAAKKYTPPFKDL GNFKGDMYHTAVWPQHDVNVKGKRVGVIGCGASGIQVSQEVGPKAKSMTC YIRTPNMCLPMRQAKLDEEDQQKKKENGFYEEQFKLTRTTFSGFTYDFVDRN TFDDTEEEREAFYQKLWDMGGFRFWLATYKDMLFDSKANYEAYKFWRKKV LERVPDPQKAAILAPEKPPHPWGTKRPSLEQRYYEVLSQDNVKVVDVNADPII EVTENGLRTANGLQEFDVLVLATGFDSVTGSLAQLNIRGTTGGTIADHWKDG TRTSMGIAIPTFPNMFFLYGPQAPTAFSNGPSCTQFQAEFVEKAMKVCQEKKI NRLESTPESEEDWCKRMQEKWDITLFPQAKSWYQGANIPGRKVEPLNWAGG MIEYVESLDKSLDNDLQGWTYSTAKA*
Esterase1	MKSFANALLPLLAFSYTASATCSNYQPVATVASGVVLGTTTSFTGATATVNK

	FLGIPYAASPTRFEPPTQPTSWSTPINATAFKDACIQQFVYPLVVREFTMAIFNN PGGGPAPPESEDCLYINVFAPSSLPPLGGFPVMQWIYGGALEFGSSNVDGYDG SNFAAFQDVIVVTFNYRTNSKTNHILQAYATDEDAVFGFPSAPEIPPTKRNLGF LDQRMALEWTRENIRAFGGNPNKVTIFGESSGAESVDILLNSWQYNPPFRAGI MESGNQALQGFLTNGVNVTANWYKAASYLNCTGNATQCLQTANATQLESVI SMNNLLFQPQIDNYTVVSDPSARIANGSTANVPILVGSNAQEGRVFQYGQTNV STVVAGLFPGITAYQDLVKQTYAINSTINSGFLSNDFDAASAIYTDLVFTCVST FSHCNAYTRNLTKYSQPPTEQTSPQQPTSPHGDTTTTASPPSHLLSHPLTQITAS FPNLNFVPNLGVYHSSEISIVFGTYPNTSVTAQEYALSKTMQTAWANFAKNPK GGPGWNAVGTFNGTDLGVLGAYGQCGVDVINSSYVDDLCGVFAPVYTVLGN LG*
Esterase2	MVHLSWALGLLPLAFAAPGAKRAVGPVVQLSGGATVVGSTSGTVDSFKGVP FAQPPVGQLRLKPPQALATQTGTILAVGEPTACPQFETQVQEGSLGSALGLLL NTPIAQTVQATGEDCLTINIQRPSTATSSSKLPVLFWIYGGGFEFGSTQTYDGSE LISTSVTQGKDIIYVAVNYRLGGFGFLPGKEILADGSSNLGLLDQRLALQWVA DNIAAFGGDPTKVTIWGESAGSISVFDQMALYGGNYTYKGQPLFRAGIMDSG SIVPANPVDAPRAQAVYDSVVSAAGCSSSADTLNCLRGLSYEKYLDAANSVP GIFGYNGIALSYLPRPDGTVLPASPEVLAANGQYAPIPFIVGDQQDEGTLFSLS QNNISTSDELVTYLNSIVFQDATRAQVQGLVDTYPDNAAAGSPYNTGDLNNIY PEYKRLAALQGDFVFILTRRAFLSLTSVAMPNIPSWSYLATYFYGTPVLGTFH ASDIIPAYGIIPSFASNTIQSYYLSFVNTLNPNTGTTSSLPSWPQWSNGNELVQF GATNNTYVADTFRNSSYQYLNAHIANFHI*
Esterase3	MPSDLPRPAYDPEIEPFLSMVPLPPTINADIMKELRKAPLLSQAPDLDALLSDK PITHREVSIPGLNSQDPQITLSIFSSTLEGGPKPCIYFVHGGGMIIGCRFVGIEDYL QYVEQNDAVVVAVEYRLAPEHPDPAPVNDCYAGLLWTAANAAELGIDLERL LICGASAGGGLSAGVALMARDKKGPKLVGQLLCYPMLDDRNDSLSSQQYVD EGVWSRGSNAFGWKQLLGDRAGKEGVSIYAAPARATDLSGLPNTFIDVGSAE VFRDEDIAYASRLWAVGVQAELHVWPGGYHAAENMAPGTDYSKKVKATRL

	AWMKRVFMKAPKSTTESLPAPTVDEAVGTI*
SHC-WT	MAEQLVEAPAYARTLDRAVEYLLSCQKDEGYWWGPLLSNVTMEAEYVLLC HILDRVDRDRMEKIRRYLLHEQREDGTWALYPGGPPDLDTTIEAYVALKYIG MSRDEEPMQKALRFIQSQGGIESSRVFTRRWLALVGEYPWEKVPMVPPEIMFL GKRMPLNIYEFGSWARATVVALSIVMSRQPVFPLPERARVPELYETDVPPRRR GAKGGGGWIFDALDRVLHGYQKLSVHPFRRAAEIRALDWLLERQAGDGSWG GIQPPWFYALIALKILDMTQHPAFIKGWEGLELYGVELDYGGWMFQASISPV WDTGLAVLALRAAGLPADHDRLVKAGEWLLDRQITVPGDWAVKRPNLKPG GFAFQFDNVYYPDVDDTAVVVWALNTLRLPDERRRRDAMTKGFRWIVGMQ SSNGGWGAYDVDNTSDLPNHTPFCDFGEVTDPPSEDVTAHVLECFGSFGYDD AWKVIRRAVEYLKREQKPDGSWFGRWGVNYLYGTGAVVSALKAVGIDTREP YIQKALDWVEQHQNPDGGWGEDCRSYEDPAYAGKGASTPSQTAWALMALI AGGRAESEAARRGVQYLVETQRPDGGWDEPYYTGTGFPGDFYLGYTMYRH VFPTLALGRYKQAIERR*
CaNES	MDFFDCSRSSIIPLKASSQIVPADSTNKWGTVESNHNSAPTTPLNDRIYVEHAH MVKDFKRIMNVAGEDPSEGLAIIDAVQRLVVDHHFQEEIHSILQKHKELATST TTHGDCMTTSLRFRLLRQEGYYVSADVFEGLKDEEGKFDQNLSGDIKGLMAL YEASQFSMEGENILDEARDYSARLLNACVTQLGHDQARIVEHTLTHPHHKSL ARFMAKNFLRDFHCTNGWIDDLKKLAKADIDMAQSTFQNEVVQISQWWKEL GLAEELKFARDQPVKWYIWTMTCDEDPSFSELRINLTKPISFVYLIDDIFDVYG TLQEVTDFTEAVHRWDHDAIEQLPYHMKICFKALDDITNEISYKVYKQYGWN PLDSLRKSWGRLCNAFLTEAKWFASGHLAKAEEYLENGIISSGVPVVLLHFFF LSGEGVTQKSVETIDNTPDIVSAAAAILRLWDDLGSAKDEDQDGKDGSYLAC YTNENPGCSLEDAEKHVKSKICDEWKLLNKECLSQKNPFSGSFHRACLNVAR MVPLMYEYDKNRRLPRLEEFLKSILIK*
EtuE	MNQQDIEQVVKAVLLKMQSSDTPSAAVHEMGVFASLDDAVAAAKVAQQGL KSVAMRQLAIAAIREAGEKHARDLAELAVSETGMGRVEDKFAKNVAQARGT PGVECLSPQVLTGDNGLTLIENAPWGVVASVTPSTNPAATVINNAISLIAAGNS

VIFAPHPAAKKVSQRAITLLNQAIVAAGGPENLLVTVANPDIETAQRLFKFPGI

GLLVVTGGEAVVEAARKHTNKRLIAAGAGNPPVVVDETADLARAAQSIVKG

ASFDNNIICADEKVLIVVDSVADELMRLMEGQHAVKLTAEQAQQLQPVLLKN

IDERGKGTVSRDWVGRDAGKIAAAIGLKVPQETRLLFVETTAEHPFAVTELM

MPVLPVVRVANVADAIALAVKLEGGCHHTAAMHSRNIENMNQMANAIDTSI

FVKNGPCIAGLGLGGEGWTTMTITTPTGEGVTSARTFVRLRRCVLVDAFRIV*

Supplementary Table 4. The configurational ratio of the product obtained from enzymatic catalysis using the farnesyl acetone from *trans*-nerolidol.

Product	Ratio
Farnesyl acetone	(5*E*,9*E*)/(5*Z*,9*E*)=59.5/40.5
Homofarnesol acetate	(5*E*,9*E*)/(5*Z*,9*E*)=60.7/39.3
Homofarnesol	(3*E*,7*E*)/(3*Z*,7*E*)=58.4/41.6
Ambrox	(−)/(9-epi-)=62.3/37.7

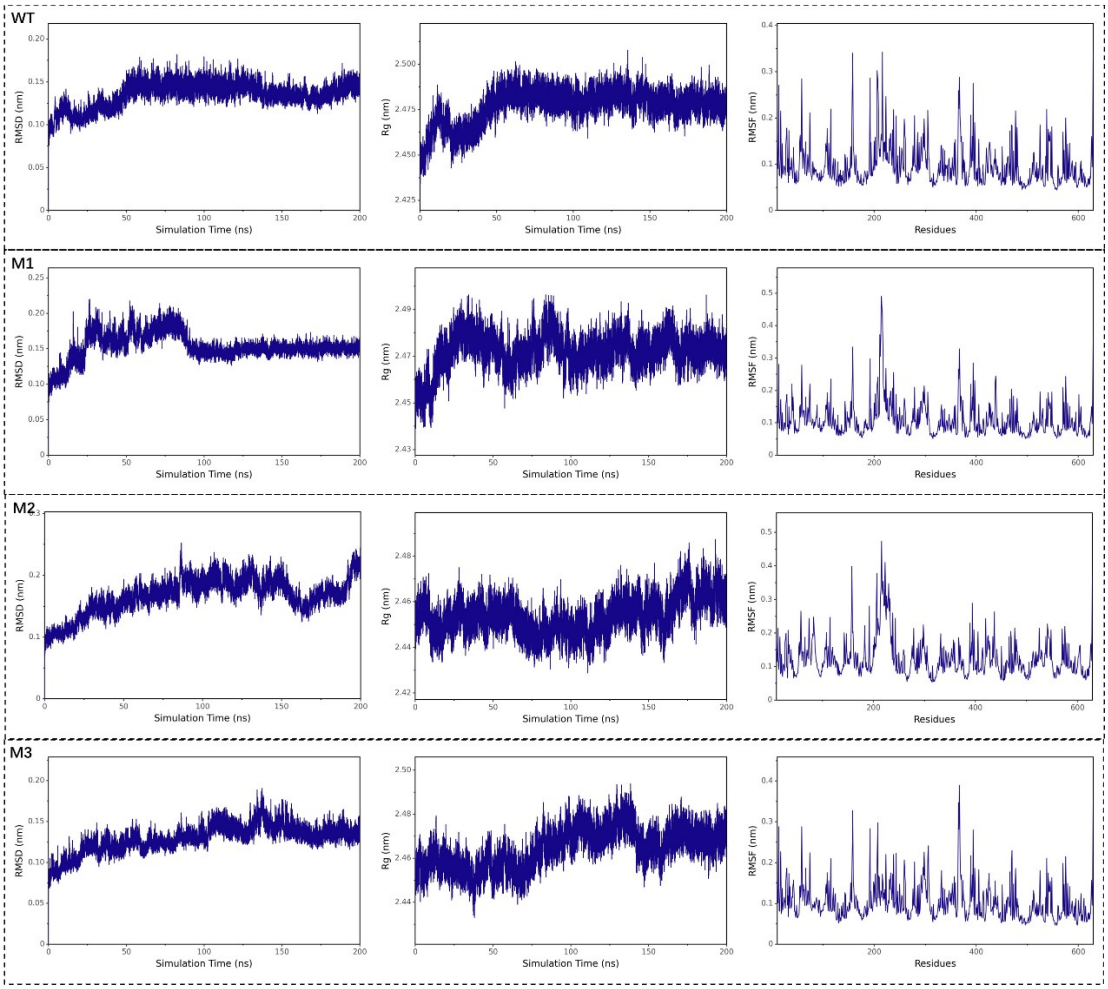

Fig. S1 RMSD, Rg, and RMSF analyses of WT, M1, M2, and M3 variants from molecular dynamics simulations

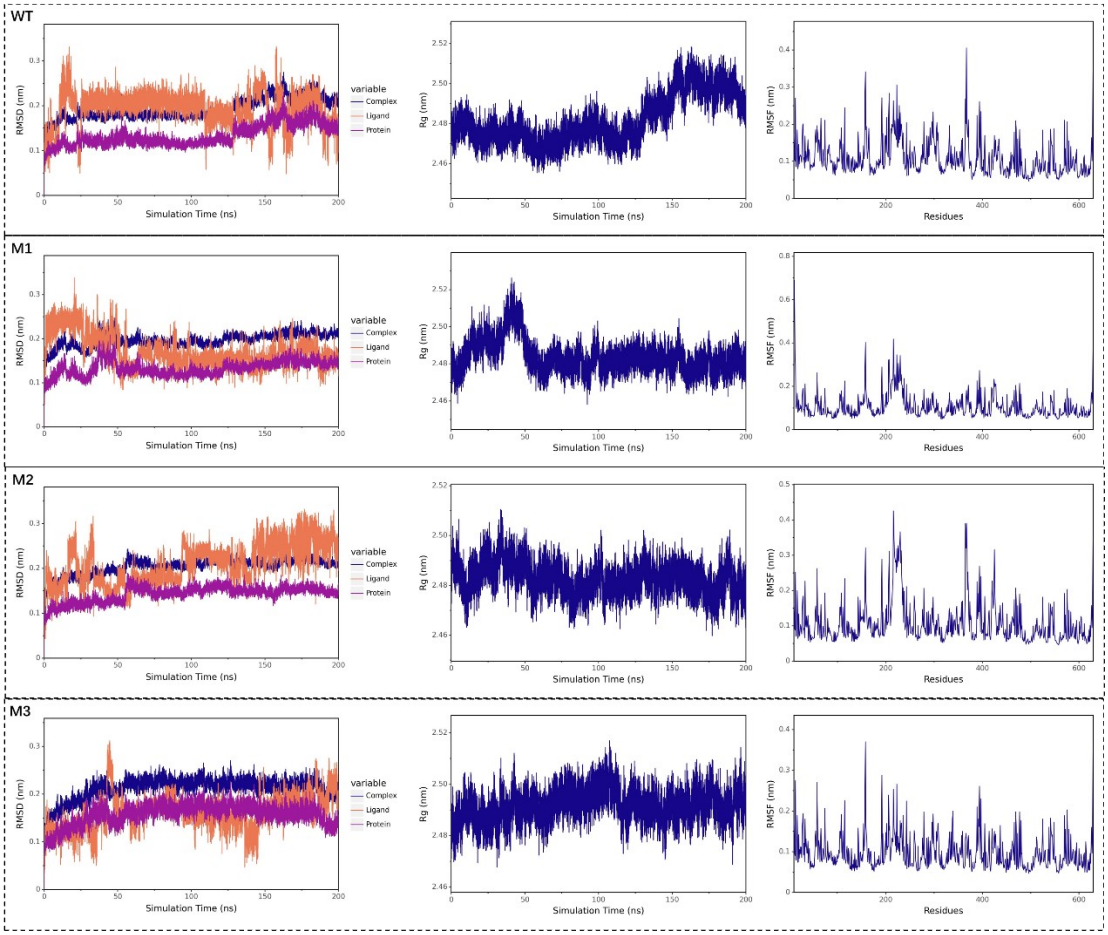

Fig. S2 RMSD, Rg, and RMSF analyses of (3*E*,7*E*)-homofarnesol–WT/M1/M2/M3 complexes from molecular dynamics simulations

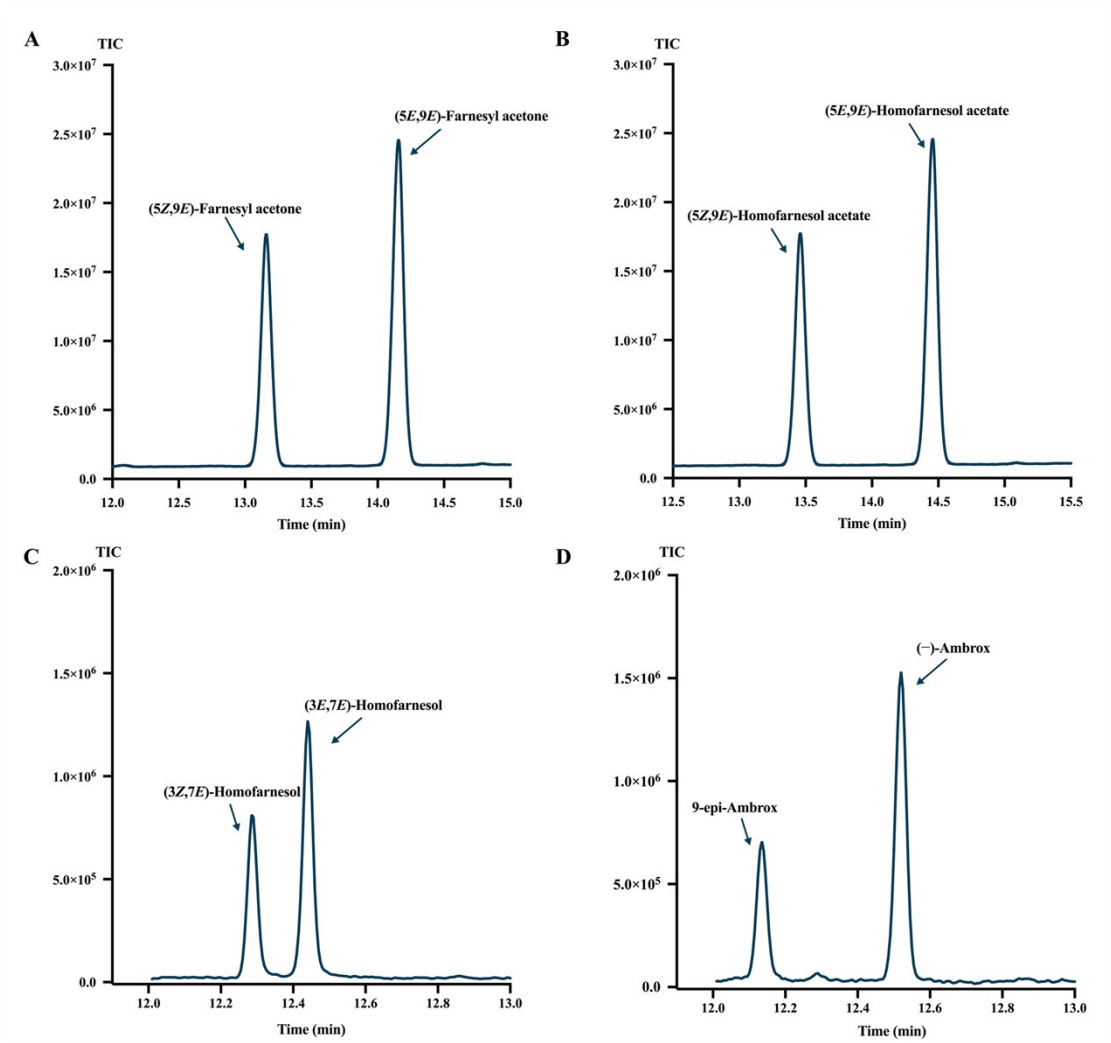

Fig. S3 Exemplary GC−MS total ion chromatograms of production obtained by (3*R*,6*E*)-nerolidol and (3*S*,6*E*)-nerolidol. (A) (5*E*,9*E*)-Farnesyl acetone and (5*Z*,9*E*)-Farnesyl acetone obtained by (3*R*,6*E*)-nerolidol and (3*S*,6*E*)-nerolidol, respectively. (B) (5*E,*9*E*)-homofarnesol acetate and (5*Z,*9*E*)-homofarnesol acetate obtained by (5*E*,9*E*)-Farnesyl acetone and (5*Z*,9*E*)-Farnesyl acetone, respectively. (C) (3*E,*7*E*)-homofarnesol and (3*Z,*7*E*)-homofarnesol obtained by (5*E,*9*E*)-homofarnesol acetate and (5*Z,*9*E*)-homofarnesol acetate, respectively. (D) (−)-Ambrox and 9-epi-ambrox obtained by (3*E,*7*E*)-homofarnesol and (3*Z,*7*E*)-homofarnesol, respectively.

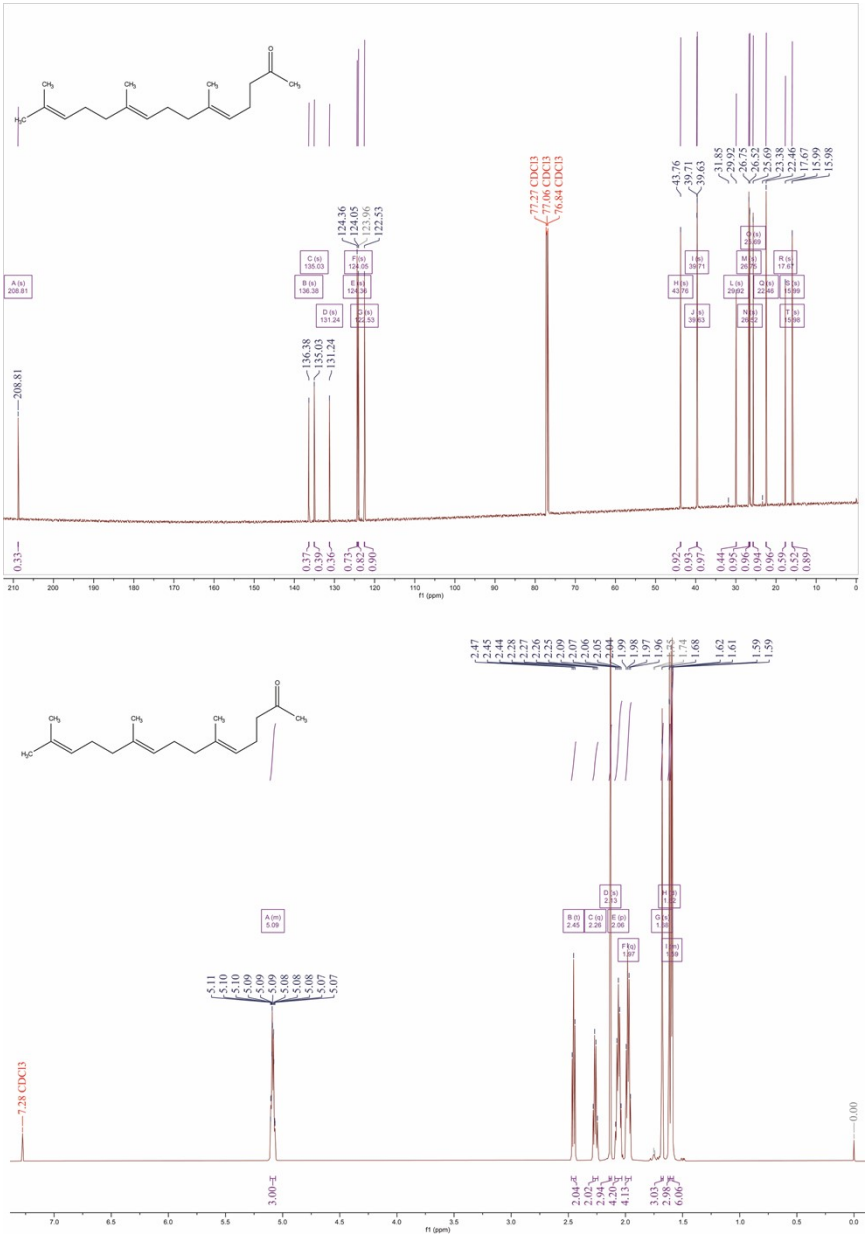

Fig. S4 ^{13}C NMR and ^{1}H NMR spectra of (5*E*,9*E*)-farnesyl acetone in $CDCl_3$. ^{13}C NMR (151 MHz, $CDCl_3$) δ 208.81, 136.38, 135.03, 131.24, 124.36, 124.05, 122.53, 43.76, 39.71, 39.63, 29.92, 26.75, 26.52, 25.69, 22.46, 17.67, 15.99, 15.98. ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.11 – 5.06 (m, 3H), 2.45 (t, *J* = 7.5 Hz, 2H), 2.26 (q, *J* = 7.4 Hz, 2H), 2.13 (s, 3H), 2.06 (p, *J* = 6.9 Hz, 4H), 1.97 (q, *J* = 7.2 Hz, 4H), 1.68 (s, 3H), 1.62 (d, *J* = 1.5 Hz, 3H), 1.61 – 1.58 (m, 6H).

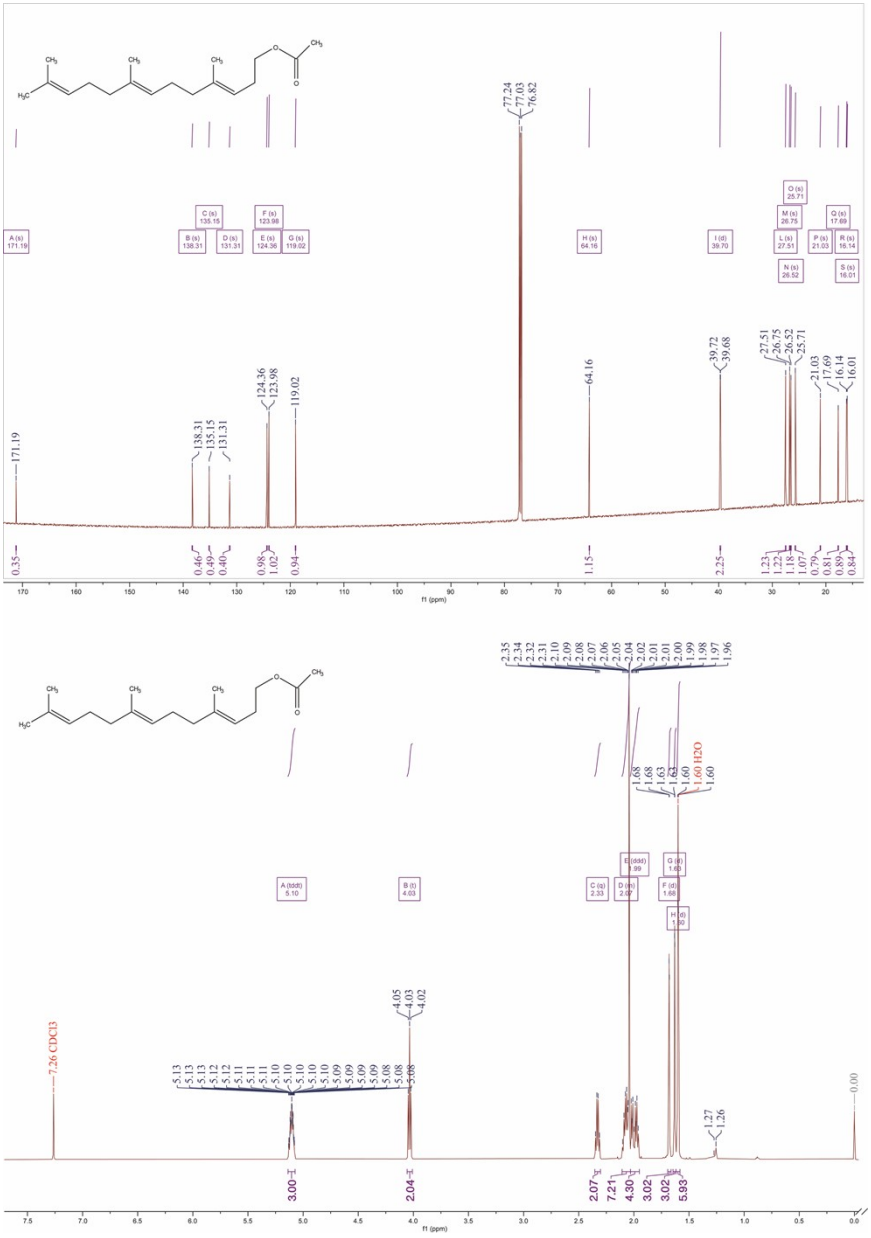

Fig S5 ^{13}C NMR and ^{1}H NMR spectra of (5*E*,9*E*)-homofarnesol acetate in $CDCl_3$. ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.19, 138.31, 135.15, 131.31, 124.36, 123.98, 119.02, 64.16, 39.70 (d, *J* = 6.2 Hz), 27.51, 26.75, 26.52, 25.71, 21.03, 17.69, 16.14, 16.01. ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.10 (tddt, *J* = 7.0, 5.6, 4.2, 1.3 Hz, 3H), 4.03 (t, *J* = 7.1 Hz, 2H), 2.33 (q, *J* = 7.1 Hz, 2H), 2.11 – 2.03 (m, 7H), 1.99 (ddd, *J* = 22.6, 9.2, 6.2 Hz, 4H), 1.68 (d, *J* = 1.7 Hz, 3H), 1.63 (d, *J* = 1.3 Hz, 3H), 1.60 (d, *J* = 3.4 Hz, 6H).

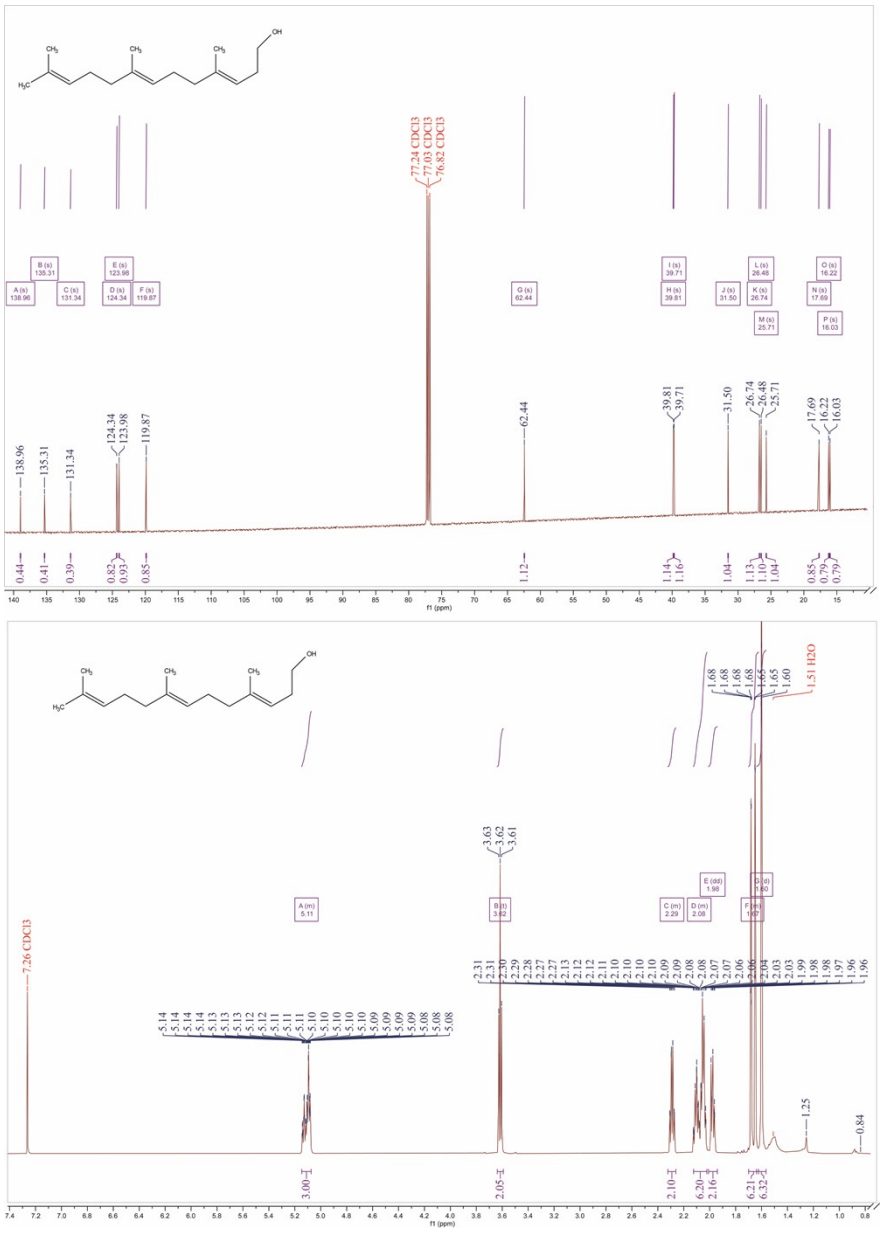

Fig S6 ^{13}C NMR and ^{1}H NMR spectra of (3*E*,7*E*)-homofarnesol in $CDCl_3$. ^{13}C NMR (151 MHz, $CDCl_3$) δ 138.96, 135.31, 131.34, 124.34, 123.98, 119.87, 62.44, 39.81, 39.71, 31.50, 26.74, 26.48, 25.71, 17.69, 16.22, 16.03. ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.15 – 5.07 (m, 3H), 3.62 (t, *J* = 6.5 Hz, 2H), 2.32 – 2.26 (m, 2H), 2.12 – 2.02 (m, 6H), 1.98 (dd, *J* = 9.2, 6.2 Hz, 2H), 1.70 – 1.62 (m, 6H), 1.60 (d, *J* = 1.4 Hz, 6H).

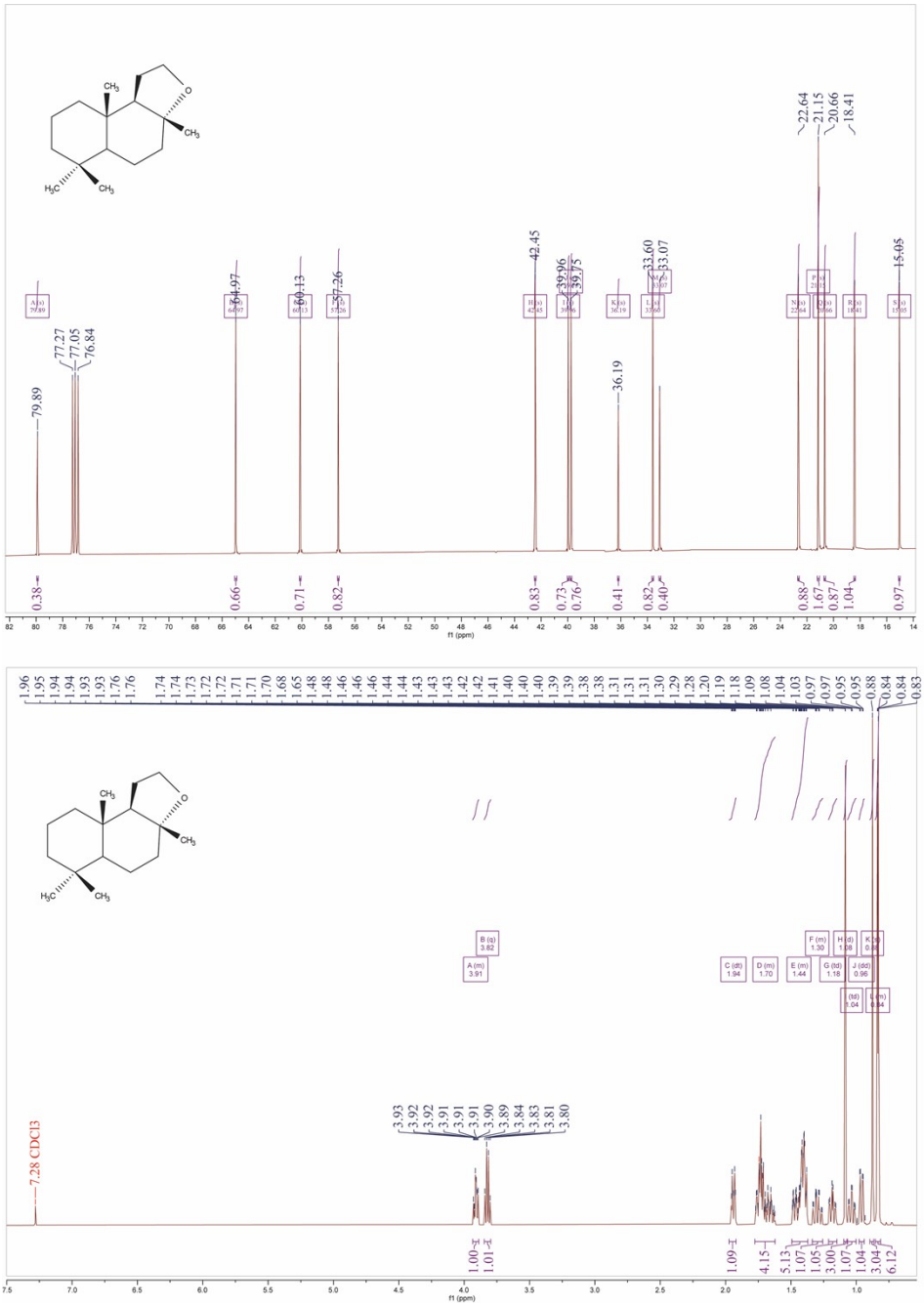

Fig. S7 ^{13}C NMR and ^{1}H NMR spectra of (−)-ambrox in $CDCl_3$. ^{13}C NMR (151 MHz, $CDCl_3$) δ 79.89, 64.97, 60.13, 57.26, 42.45, 39.96, 39.75, 36.19, 33.60, 33.07, 22.64, 21.15, 21.15, 20.66, 18.41, 15.05. ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.93 – 3.89 (m, 1H), 3.82 (q, *J* = 8.2 Hz, 1H), 1.94 (dt, *J* = 11.6, 3.2 Hz, 1H), 1.78 – 1.62 (m, 4H), 1.49 – 1.37 (m, 5H), 1.34 – 1.26 (m, 1H), 1.18 (td, *J* = 13.6, 4.4 Hz, 1H), 1.08 (d, *J* = 1.0 Hz, 3H), 1.04 (td, *J* = 12.8, 3.6 Hz, 1H), 0.96 (dd, *J* = 12.4, 2.7 Hz, 1H), 0.88 (s, 3H), 0.85 – 0.81 (m, 6H).

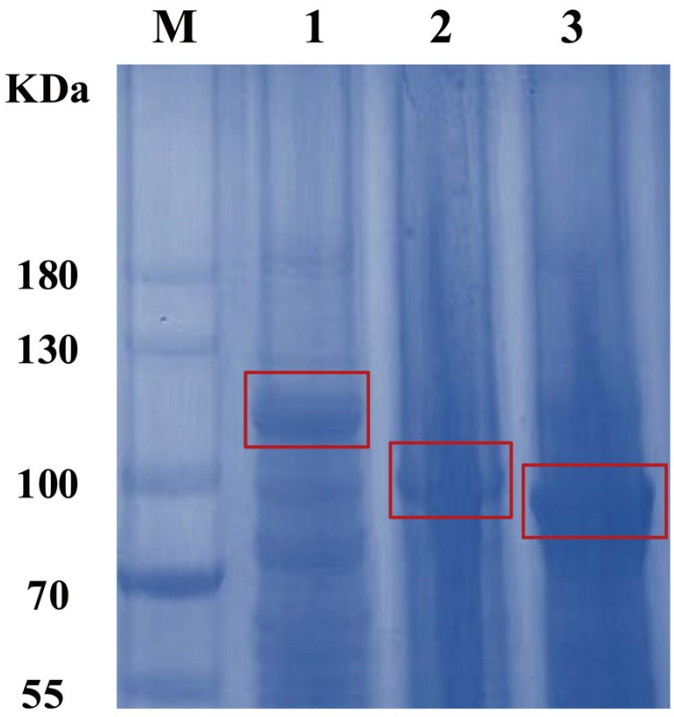

Fig. S8 SDS-PAGE analysis of the supernatants expressing BVMO1, BVMO2, and BVMO3 with SUMO-tag. Lane M represents the molecular weight marker; lanes 1–3 correspond to BVMO1, BVMO2, and BVMO3, respectively.

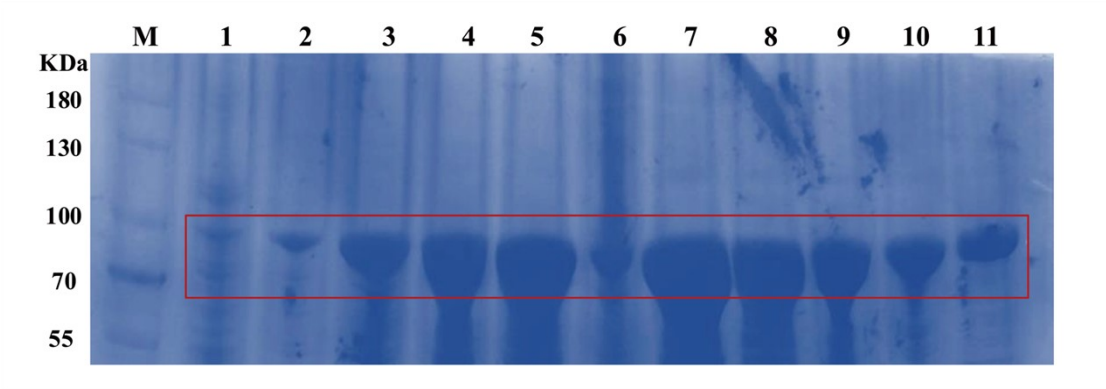

Fig. S9 SDS-PAGE analysis of purified BVMO2 with SUMO tag using Ni^{2+}-affinity chromatography on an AKTA system by the N-terminal His-tags. Lane M represents the molecular weight marker; lanes 1–11 correspond to protein fractions collected at different time points.