Supporting Information

Stereospecific Formation of *Z*-Trisubstituted Double Bonds by the Successive Action of Ketoreductase and Dehydratase Domains from *trans*-AT Polyketide Synthases

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Materials. Ampicillin, carbenicillin, isopropylthio-β-D-galactopyranoside (IPTG), and kanamycin were purchased from Thermo Scientific. All other chemical reagents were purchased from Sigma-Aldrich and utilized without further purification. Competent *E. coli* DH5-alpha, DH10-beta, and BL21(DE3) cloning and expression strains were purchased from New England Biolabs Inc (NEB). Restriction enzymes and T4 DNA ligase were purchased from NEB and used according to the manufacturer's specifications. Pre-charged 5 mL HisTrap FF columns were purchased from GE Healthcare Life Sciences. Amicon Ultra Centrifugal Filter Units (Amicon Ultra-15 and Amicon Ultra-4, 10K, 30K, 50K) were purchased from Millipore. Ery[KS6][AT6], EryACP6, PICS TE, and Sfp, were each expressed in *E. coli* BL21 (DE3) and purified using the previously described protocols.[1-3] The expression and purification of BonKS2, BonMT2, and BonACP2 have been previously described.[4] *E. coli* S-adenosylhomocysteine (SAH) nucleosidase (SAHase) were expressed and purified following the previously described protocols.[5] NigDH1 was expressed and purified as previously described.[6] The substrates (±)-2-methyl-3-ketobutanoic acid *N*-acetylcysteamine (SNAC) thioester (**3**) and (±)-2-methyl-3-ketopentanoyl-SNAC (**4**) were prepared as previously described.[7] Reference standards of methyl (2*R*,3*S*)-2-methyl-3-hydroxypentanoate (**8-Me**), methyl (2*S*,3*R*)-2-methyl-3-hydroxypentanoate, methyl (2*R*,3*R*)-2-methyl-3-hydroxypentanoate, and methyl (2*S*,3*S*)-2-methyl-3-hydroxypentanoate, prepared as previously described, were used as standards for chiral GC-MS analysis and for comparison with the corresponding 2-methyl-3-hydroxypentanoates.[8] A reference mixture of the four diastereomers of 2-methyl-3-hydroxybutanoic acid was purchased from VDM Biochemicals and converted to the corresponding methyl esters by treatment with $TMSCHN_2$ in methanol. Authentic methyl (2*S*,3*R*)-2-methyl-3-hydroxybutanoate (**7-Me**) was prepared by reduction of (±)-2-methyl-3-ketobutyryl-SNAC (**3**) with AmpKR2, the KR from module 2 of the amphotericin PKS, and NADPH, as previously described,[7] followed by hydrolysis and methylation with $TMSCHN_2$. The configurations of each of the three remaining diastereomers of methyl 2-methyl-3-hydroxybutanoate were assigned based on their order of elution on chiral GC-MS and comparison with the elution order and known configuration of the corresponding methyl 2-methyl-3-hydroxypentanoates. The acetyl-SNAC and propionyl-SNAC were synthesized as previously described.[7, 8] (*E*)-2-Methyl-2-butenoic acid (tiglic acid) and (*Z*)-2-methyl-2-butenoic acid (angelic acid) were purchased from TCI America.

Methods. General methods were as previously described.[9] All DNA manipulations were performed following standard procedures.[9] Growth media and conditions used for *E. coli* strains and standard methods for handling *E. coli* in vivo and in vitro were those described previously, unless otherwise noted.[8, 9] Genes were synthesized by DNA 2.0 (ATUM), Newark, California or Genewiz, South Plainfield, NJ. All proteins were handled at 4 °C unless otherwise stated. Protein concentrations were determined according to the method of Bradford,[10] using a Tecan Infinite M200 Microplate Reader with bovine serum albumin as the standard. Protein purity and size were estimated using SDS-PAGE and visualized using Coomassie Blue stain and analyzed with a Bio-Rad ChemiDoc MP System. Protein accurate molecular weight was determined on an Agilent 6530 Accurate-Mass Q-TOF LC/MS. Kinetic assays of KR-catalyzed reactions were carried out using the Tecan Microplate Reader. Chiral GC-MS analysis was performed on an Agilent 5977A Series GC-MSD instrument, 70 eV EI in positive ion mode with a Varian CP-Chirasil-DEX CB capillary column (25 m × 0.25 mm) or a Hewlett-Packard Series II GC-MSD instrument, 70 eV EI in positive ion mode, using a Varian CP-Chirasil-DEX CB capillary column (25 m × 0.32 mm).

Figure S1. BonKR2 amino acid sequence. On the basis of the previously illustrated domain boundaries of Bon Module 2 from *Burkholderia gladioli* pv. *cocovenenans*,[4] the synthetic gene encoding BonKR2 domain was subcloned into the corresponding *NdeI* and *EcoRI*-digested pET-28a vector and the recombinant protein was expressed with an *N*-terminal His_6-tag in *E.coli* BL21(DE3). Protein expression and purification procedures are described below.

PLGAPLAAGPLAPAAAPRQTAPAASIATASLASAAVPRRQAGELMLAPIWEAVEETPSQAVA
GLPRTVWLGAETASLDRPAWVARLQAEGRLEQLVWQVPAGVARVALVGLRLIQALLELGYGA
QTLKLTVVTRQAQAVSPTETADPEQAGVHGLVGSLAKSYPKWQVRLLDLPRDGELASLEWQR
VPADPRGDARAWRDGRWYRQRLAPCSLPAVEASRFRHGGVYVVLGGAGSIGAAFSEHLIRRY
RAQLVWLGRRPEDEAIRTQLSRLAALGPAPLYLSADATDRQALERARATILARFGTVHGIVH
SAVVLGEAPLAALDEAGFAEVLATKAITCEQMDAVFGGDALDFQLFFSSAQSFTKTPRLSHY
SAGCCHTDAYAHNLRNRPYAVRVMHWGYWGLAGDGAARFDQLMAQSGFAPIDPARGMEALEQ
LLAGPLGQLAFLSTTHGEGARAALGVSERETVQGLPSTPAVTLPERAEAAAPAGA

(N terminal *NdeI* site)
CATATGCCGCTGGGTGCACCGTTAGCCGCCGGTCCGTTAGCACCTGCAGCAGCACCGCGTCAG
ACAGCACCGGCAGCAAGCATTGCAACCGCAAGTCTGGCAAGCGCAGCCGTTCCTCGCCGTCA
AGCCGGTGAACTGATGCTGGCCCCGATCTGGGAAGCAGTTGAAGAAACCCCGAGCCAAGCAG
TGGCCGGTTTACCGCGCACCGTTTGGCTGGGCGCCGAAACCGCAAGCTTAGATCGCCCGGCA
TGGGTGGCACGTTTACAGGCAGAGGGCCGTCTGGAACAACTGGTGTGGCAGGTGCCGGCCGG
TGTTGCACGTGTTGCATTAGTGGGCCTGCGCCTGATTCAGGCACTGCTGGAGCTGGGCTATG
GCGCACAGACCCTGAAGCTGACCGTGGTTACCCGCCAGGCACAGGCCGTTAGCCCTACAGAG
ACCGCCGATCCGGAACAAGCAGGCGTGCATGGCCTGGTTGGTAGTCTGGCAAAGAGCTACCC
GAAATGGCAGGTGCGCCTGCTGGATCTGCCGCGTGATGGCGAACTGGCAAGTCTGGAATGGC
AACGCGTTCCTGCAGACCCGCGTGGTGATGCCCGCGCCTGGCGTGATGGTCGCTGGTATCGT
CAGCGCCTGGCCCCGTGTAGTTTACCGGCAGTGGAAGCCAGCCGTTTTCGCCATGGTGGCGT
GTATGTGGTTCTGGGTGGTGCCGGCAGCATTGGTGCCGCCTTTAGCGAGCATCTGATCCGTC
GTTATCGTGCACAGCTGGTGTGGCTGGGTCGCCGCCCGGAAGATGAAGCAATTCGCACACAG
CTGAGCCGCCTGGCCGCATTAGGCCCGGCACCGCTGTACCTGAGTGCAGATGCCACCGATCG
CCAAGCATTAGAACGCGCACGTGCAACCATCCTGGCCCGTTTTGGCACAGTGCATGGCATTG
TTCACAGCGCCGTTGTGCTGGGTGAAGCACCGCTGGCCGCACTGGACGAAGCCGGTTTTGCC
GAGGTTCTGGCAACCAAAGCAATCACCTGCGAACAGATGGATGCCGTGTTTGGTGGTGACGC
CCTGGACTTTCAGCTGTTTTTCAGCAGCGCACAGAGTTTCACCAAGACCCCGCGCCTGAGCC
ACTATAGTGCCGGCTGCTGCCATACCGACGCCTATGCACATAACCTGCGCAATCGCCCTTAT
GCAGTTCGCGTGATGCATTGGGGCTATTGGGGTTTAGCCGGCGATGGTGCAGCACGCTTTGA
CCAGCTGATGGCACAGAGCGGTTTTGCCCCGATTGATCCTGCCCGCGGCATGGAAGCACTGG
AACAGCTGCTGGCAGGCCCTCTGGGCCAGCTGGCCTTTCTGAGCACCACCCATGGTGAAGGT
GCACGTGCCGCCCTGGGCGTTAGCGAACGTGAGACCGTTCAGGGCCTGCCGAGTACCCCGGC
AGTGACCTTACCGGAACGTGCCGAAGCAGCAGCACCGGCAGGTGCCTGAATTC (Stop codon and C-terminal *EcoRI* site)

Figure S2. BonDH2 amino acid sequence. The synthetic gene encoding the BonDH2 domain from *Burkholderia gladioli* pv. *cococenanans* was subcloned into the corresponding *NdeI* and *EcoRI*-digested pET-28a vector and the recombinant protein was expressed with an *N*-terminal His_6-tag in *E.coli* BL21 (DE3).

AAPRPAAAATAAARMPADAAGQLHPLVHRNVSTFGRQRYRTVLRGDEFFLRDHLVNGRRVVPGVAQLEWARAAVA
DALADPAMTVRLEQVNWLRPLVAESALEVHVTLVPQQGGRIRFEIHSGSGEHARAHSQGFAVPVPLAGRAQPPRV
DLAAWRARAAQPTEVAARYAHFERVGLRYGPSFRVMDQLRVGADHALAMLRWPAEAPAEGYAWPPSVLDGALQAG
LGLVARDGGLELPFAMQAIEQWAPLPKNGISIVRRAADDSAAVRKLDVEIVDVDGRVAMRVSGFSTRPLGAPLAA
GPLAPAAAPRQT

(N terminal *NdeI* site)
CATATGGCCGCCCCGCGTCCGGCAGCAGCAGCAACAGCCGCAGCACGCATGCCGGCAGATGCAGCAGGTCAGCTG
CATCCGCTGGTTCATCGCAATGTGAGTACCTTTGGCCGTCAGCGCTATCGCACCGTGCTGCGTGGCGATGAGTTC
TTTCTGCGCGACCATCTGGTGAATGGTCGTCGCGTGGTGCCTGGTGTGGCACAGCTGGAATGGGCCCGTGCAGCA
GTTGCAGATGCCCTGGCCGATCCGGCAATGACCGTGCGTCTGGAGCAGGTGAACTGGCTGCGTCCGCTGGTGGCA
GAAAGCGCACTGGAAGTTCATGTTACCCTGGTTCCGCAGCAGGGCGGTCGCATTCGCTTTGAGATTCATAGCGGC
AGCGGCGAACATGCACGCGCCCATAGCCAGGGTTTTGCAGTTCCGGTTCCGTTAGCCGGTCGTGCACAGCCGCCT
CGTGTTGATCTGGCCGCCTGGCGTGCACGTGCAGCACAGCCTACCGAAGTGGCCGCCCGTTATGCCCATTTCGAA
CGTGTTGGCCTGCGTTATGGTCCGAGTTTTCGCGTGATGGATCAGCTGCGCGTTGGTGCAGATCATGCCCTGGCA
ATGCTGCGCTGGCCTGCAGAAGCACCGGCAGAAGGTTACGCCTGGCCGCCGAGTGTTCTGGACGGTGCACTGCAG
GCCGGTCTGGGTTTAGTTGCCCGCGATGGCGGCCTGGAACTGCCTTTTGCCATGCAGGCAATTGAGCAGTGGGCC
CCGCTGCCGAAAAATGGCATTAGCATTGTGCGCCGTGCAGCAGACGATAGTGCCGCCGTGCGCAAACTGGATGTG
GAAATCGTGGATGTGGATGGCCGCGTGGCCATGCGTGTTAGCGGTTTTAGCACCCGTCCTCTGGGTGCCCCTTTA
GCAGCCGGTCCTTTAGCCCCGGCAGCAGCACCTCGCCAGACCTGAATTC (Stop codon and C-terminal *EcoRI* site)

Domain boundaries and design of recombinant OxaDH5, OxaMT5 and OxaACP5

Figure S3. Oxazolomycin PKS modules 3, 4, and 5 and module 3 domain boundaries.

LOCUS ABS90475 4971 aa linear BCT 30-APR-2008
DEFINITION PKS [*Streptomyces albus*].
ACCESSION ABS90475
VERSION ABS90475.1 GI:155061085
DBSOURCE accession EF552687.1

1 MSSHDATDATDATDVSGNDIAIVGMAGRFPGADSVGEFWELLRSGREGITRFSDEELAAA
(.....Oxazolomycin PKS module 3, 4.....)
3061 ADDDDIAVIGIGGRYPHARDLTEFWENLRRGHDAVTEVPGDRWDADAHFDGDHTAPGSSY
3121 GKWGGFLEDVDAFDSLFFRIPPSQAKLMDPQERLFLEAAWSALENAGYPPSRLPRPRFGG
3181 QGRDVGVFAGVMWGDYAQLAAEESVRGNHHTVLTNRSAIANQVSYFGDFRGPSVVVDTAC
3241 SASLVALHQACESVRRGECRYALAGGVNVSAHPSKYAHLSRMRMLSTDGRCRSFGAGGDG
3301 YVPGEGVGAVLLKRLSEAVADGDHIHAVIKATAVNHGGRTSGYTVPSPQAQQALIEETLD
3361 RAGIDPRTIGYVEAHGTGTALGDPIEHTALQQAFAGVVRDAGAIALGSVKSNIGHLEGAA **OxaKS5**
3421 GIAGLTKAVLQLSHGQLVPSLHSEETNPIIDFATSPFRVQRELADWARPVIETDGVRVEQ
3481 PRRASVSSFGAGGTNAHVVLEEYRAPEGSAATASGGHELLVLSARGPERLRAYAAEMAAH
3541 LRRERPALADTAHTLRVGREALPERLAFPAADIDDAADKFAAFADGASVPSAITGNADGH
3601 TALAEVFTDGAGPEFLRTLAAAGDDTRLARLWVSGAFAEWTALHGRAAGRCRTVPLPGYP
3661 FERPRHWLPVTRTELADVPRPPGEKEARPEPPRAADRTLTLDPADPVVRDHVVGGRAVLP
3721 GVGHLDLVVRALGEDAARVFRDVRWLTPLVVPDTGAEVTLTTRPEAALTTRPEAVGAARP
3781 EDGPALGYRLTASSADASPVHSLGQVVATAPDRPAPLSVDLLKADCPRRVSHEELYEGLR
3841 RRGLPYGPHFRRVAQAWTGDRTALARLHRPEECSDAARGPLDPGTLDAALHPLALLLADE **OxaDH5**
3901 GASGRPLLPFAADRVEIHAPLPDEGWSHVRDLGSRRFDVTVTDAGGRVCVRVTGLALREA
3961 KPEPSIDYRPRWAVAPPAVADAVAPRAVLTVTGEEGAALADALREAHPDAEHVRLSIGEG
4021 GLDERATAELLDRVPHIDLVYFLDVGGPHGPAADRRARRAAQDRGTVALYRLVRGLDRAG
4081 LLDGPLALKVLTTDALPLGDDDAVRPEAAGPIGFCEVAAKEFPRLSAACLDVRREELGDG
4141 VRALVREPVRAKVRPVSLRGGVRRIRRLEAVAPAAAPTRFRERGVYLVIGGLGVLGRDTA
4201 RYLARTYRARLVLVGRGAVDERRRADLAAIEELGAEVSYVPCDAGDPVALRQVIDETKDR **OxaKR5**
4261 FGALHGVIHSAMVLVDKPIRRLAEAELRTALDAKADTVWSMFRALRGERPDFVLLYSSAV
4321 TFEGNHGQAGYAAGCHVADAWALAGARTAPYPVRTVNWGYWHAAGDTHRESVLSRFAAAG
4381 IRPIGAEEGMAAVERVLSGTLPQALVVKADQRILAGLGVDTDTVLRAQPELPASSAPLAP
4441 GPGSTEPYTRSGGAAAETETFARRLLVGALRSMGVLRGPGERYTRDGLRARLGVVEAQER
4501 LLGLLVDVLLRGGHLRAEGPELVTTDLVVDPEVLRCVERPEEAAAGLTARHPDAGAVTAL
4561 LLRCVEALPEVLTGRRGHLDVLFPGGSFDLVEAVYAGDPVTDRCNEQVAGAVLRYVTERL
4621 RSRPGDRVRVLEVGAGTGGTSARVLRALATAGLGDHVEYLYTDVSEGFVRHGRKRFGAGH
4681 PFADFRALDIERVPEEQGFESGGYDLVLGSNVFHATGRIDRTLAHTKRLLGTNGVLVLNE **OxaMT5**
4741 GVELRDQMSLIFGLATGWWLFEDAEYRLPHSPLLSTTAWRDVLAHNGFRGVTEAPRPDGG
4801 THQCVLVAESDGFVPVTAAAPGPPVPAASSAPAAVSTVPAGPDPVRDAERRVKAVFARVL
4861 EMEEDLLDARATFENYGVDSLVVLSLTKELEQEYGPLPSTLLFEHITIERLARHLAATGA **OxaACP5**
4921 AGTAAGTAAGPARAAEPEAGIEQLVDSLSDTEVDSLLRQLGSVLQKQEEQR

Figure S4. OxaKR5 amino acid sequence. The synthetic gene encoding OxaKR5 domain was subcloned in the corresponding *NdeI* and *EcoRI*-digested pET-28a vector and the recombinant protein was expressed with an *N*-terminal His_6-tag in *E.coli* BL21(DE3). Protein expression and purification procedures are described below.

IDYRPRWAVAPPAVADAVAPRAVLTVTGEEGAALADALREAHPDAEHVRLSIGEGGLDERATAELLDRVPHIDLV
YFLDVGGPHGPAADRRARRAAQDRGTVALYRLVRGLDRAGLLDGPLALKVLTTDALPLGDDDAVRPEAAGPIGFC
EVAAKEFPRLSAACLDVRREELGDGVRALVREPVRAKVRPVSLRGGVRRIRRLEAVAPAAAPTRFRERGVYLVIG
GLGVLGRDTARYLARTYRARLVLVGRGAVDERRRADLAAIEELGAEVSYVPCDAGDPVALRQVIDETKDRFGALH
GVIHSAMVLVDKPIRRLAEAELRTALDAKADTVWSMFRALRGERPDFVLLYSSAVTFEGNHGQAGYAAGCHVADA
WALAGARTAPYPVRTVNWGYWHAAGDTHRESVLSRFAAAGIRPIGAEEGMAAVERVLSGTLPQALVVKADQRILA
GLGVDTDTVLRAQPELPASSAPLAPG

(N terminal *NdeI* site)

CATATGATTGATTATAGACCACGTTGGGCCGTCGCACCGCCAGCCGTTGCAGATGCTGTCGCACCTCGTGCTGTT
CTGACCGTCACGGGTGAAGAGGGTGCCGCGTTGGCGGACGCCCTGCGCGAGGCACATCCGGATGCTGAACATGTT
CGCCTGTCTATTGGCGAGGGTGGCCTGGATGAGCGTGCGACCGCGGAACTGCTGGACCGTGTTCCGCATATTGAC
CTGGTGTACTTTCTGGATGTGGGTGGCCCGCACGGTCCGGCGGCGGATCGTCGTGCGCGCCGTGCAGCACAGGAT
CGTGGTACCGTTGCCCTGTACCGCCTGGTTCGTGGTCTGGACCGCGCGGGTCTGCTGGACGGTCCGCTGGCGTTG
AAAGTCCTGACGACTGATGCTCTGCCGCTGGGCGATGACGATGCGGTGCGTCCAGAGGCCGCGGGTCCGATCGGT
TTCTGCGAAGTGGCAGCGAAAGAATTTCCGCGCCTGAGCGCAGCCTGTCTGGATGTGCGTCGTGAAGAACTGGGT
GATGGTGTGCGCGCGCTGGTGCGTGAACCGGTTCGTGCGAAAGTTCGTCCGGTGAGCCTGCGTGGCGGCGTTCGT
CGTATTCGTCGTTTGGAAGCGGTGGCGCCTGCAGCGGCTCCAACCCGCTTTCGCGAGCGTGGCGTCTATCTGGTC
ATTGGTGGCCTGGGCGTGCTGGGTCGTGACACCGCGCGTTACCTGGCGCGCACCTATCGCGCGCGCCTGGTCCTG
GTCGGTCGCGGTGCTGTTGACGAGCGCCGCCGTGCGGACCTGGCGGCCATCGAAGAACTGGGCGCTGAGGTGAGC
TACGTCCCGTGCGATGCTGGCGATCCGGTCGCCTTGCGTCAGGTTATCGACGAAACCAAAGACAGATTTGGTGCC
TTACACGGTGTTATTCATTCGGCGATGGTCCTGGTGGATAAGCCGATCCGTCGCTTGGCAGAGGCAGAGCTGCGT
ACCGCTCTGGACGCGAAAGCAGACACGGTGTGGAGCATGTTCCGTGCGCTGCGTGGTGAGCGCCCGGATTTCGTG
CTGTTGTACAGCAGCGCAGTGACCTTCGAGGGTAATCACGGTCAGGCTGGTTACGCCGCGGGTTGCCATGTTGCC
GATGCGTGGGCATTAGCGGGCGCCCGTACCGCACCGTATCCGGTCCGTACTGTCAACTGGGGCTACTGGCACGCT
GCCGGCGATACGCACCGTGAGTCTGTTCTGTCCCGTTTCGCGGCCGCGGGCATCCGTCCGATCGGTGCGGAAGAG
GGCATGGCAGCGGTGGAGCGTGTGCTGAGCGGCACCCTGCCGCAAGCGCTGGTTGTTAAAGCAGACCAACGCATT
CTGGCTGGTTTGGGTGTTGATACGGATACCGTATTGCGTGCGCAGCCTGAACTTCCGGCGAGCAGCGCACCGCTG
GCTCCGGGTTGAATTC (Stop codon and C-terminal *EcoRI* site)

OxaDH5 amino acid sequence. The synthetic gene encoding OxaDH5 domain was subcloned in the corresponding *NdeI* and *EcoRI* digested pET-28a vector and the recombinant protein was expressed with N-terminal His6-tag in *E.coli* BL21 (DE3).

LPGYPFERPRHWLPVTRTELADVPRPPGEKEARPEPPRAADRTLTLDPADPVVRDHVVGGRAVLPGVGHLDLVVR
ALGEDAARVFRDVRWLTPLVVPDTGAEVTLTTRPEAALTTRPEAVGAARPEDGPALGYRLTASSADASPVHSLGQ
VVATAPDRPAPLSVDLLKADCPRRVSHEELYEGLRRRGLPYGPHFRRVAQAWTGDRTALARLHRPEECSDAARGP
LDPGTLDAALHPLALLLADEGASGRPLLPFAADRVEIHAPLPDEGWSHVRDLGSRRFDVTVTDAGGRVCVRVTGL
ALREAKPEPSID

(N terminal *NdeI* site)
CATATGCTGCCGGGCTATCCGTTTGAACGTCCGCGCCATTGGCTGCCTGTGACCCGTACCGAACTGGCCGATGTT
CCGCGTCCGCCGGGTGAGAAAGAAGCCCGTCCTGAACCTCCGCGTGCCGCAGATCGTACCCTGACACTGGATCCG
GCAGATCCTGTTGTTCGCGATCATGTTGTGGGTGGTCGTGCCGTTCTGCCGGGTGTGGGTCATCTGGATCTGGTG
GTTCGCGCACTGGGTGAAGATGCAGCCCGCGTGTTTCGCGATGTGCGTTGGCTGACACCGCTGGTTGTTCCGGAT
ACCGGCGCAGAAGTGACCCTGACAACACGTCCGGAAGCAGCACTGACAACCCGCCCGGAAGCCGTTGGTGCCGCA
CGTCCTGAAGATGGCCCGGCACTGGGTTATCGCCTGACAGCAAGCAGCGCCGATGCAAGCCCGGTTCATAGCCTG
GGTCAGGTGGTTGCAACAGCCCCGGATCGTCCGGCACCGCTGAGCGTTGACCTGCTGAAAGCCGATTGCCCTCGC
CGCGTTAGCCATGAAGAACTGTATGAGGGCCTGCGTCGTCGCGGTCTGCCGTATGGTCCTCATTTCCGTCGCGTT
GCACAGGCATGGACCGGTGATCGTACCGCCCTGGCACGTCTGCATCGTCCGGAAGAATGCAGCGATGCAGCACGT
GGTCCGCTGGATCCTGGCACACTGGATGCAGCCCTGCATCCGCTGGCACTGCTGCTGGCCGATGAAGGCGCAAGC
GGTCGTCCGCTGCTGCCTTTTGCAGCCGATCGCGTTGAAATCCATGCACCGCTGCCGGATGAAGGCTGGAGCCAT
GTGCGCGATCTGGGTAGCCGCCGTTTTGATGTGACCGTGACCGATGCAGGTGGTCGCGTGTGCGTTCGTGTTACC
GGTCTGGCCCTGCGTGAAGCCAAACCGGAACCGAGCATCGATTGAGAATTC (Stop codon and C-terminal *EcoRI* site)

General procedures for recombinant protein expression and purification. The synthetic BonKR2, BonDH2, OxaKR5 and OxaDH5 genes, sub-cloned into the pET28a vector, were each transformed into *E. coli* BL21(DE3), and the resultant recombinants were cultured under standard conditions. A single recombinant clone of each transformant was inoculated into 10 ml of LB media containing 50 mg/L kanamycin and grown at 37 °C overnight. The overnight culture was then inoculated into 500 ml of Super Broth containing 50 mg/L kanamycin in a 2.5-L flask and grown until an OD_{600} of 0.4-0.8. The culture was cooled to 18 °C for 30 min, after which protein expression was induced by addition of 0.2 mM IPTG. The culture was continuously shaken for an additional 40-48 h at 18 °C. The cells were harvested by centrifugation at 4,200*g* for 20 min and the cell pellet was dissolved in 35 ml lysis buffer (500 mM NaCl, 50 mM phosphate, 50 mM imidazole, 1 mg/ml of lysozyme, pH 7.8) and stored at -80 °C. The cells in lysis buffer from the -80 °C freezer were thawed at room temperature, followed by sonication. The cell supernatant and the pellet were separated by centrifugation at 23,000*g* for 30 min and the supernatant was filtered using an 0.8 μm filter and then loaded onto a pre-charged 5-ml HisTrap FF column (GE Healthcare) pre-equilibrated with lysis buffer. The column was washed with 25 mL lysis buffer and then 25 mL washing buffer (50 mM sodium phosphate, 500 mM NaCl, 60 mM imidazole, pH 7.6). Proteins were eluted from the Ni-column by elution buffer (150 mM NaCl, 50 mM phosphate, 150 mM imidazole, pH 7.5). The eluted fractions were collected and concentrated with an Amicon filter MWCO 30 KDa. The buffer was exchanged with exchange buffer (50 mM sodium phosphate, 10% glycerol, 100 mM NaCl, pH 7.2), concentrated, and stored at −80 °C until use. Protein purity was assessed as >90% by 12% acrylamide SDS-PAGE, and the His_6-tagged fusion proteins were utilized without further modification. The molecular weight (MW) of each protein was verified on an Agilent Technologies QTOF LC-MS and matched the predicted values.

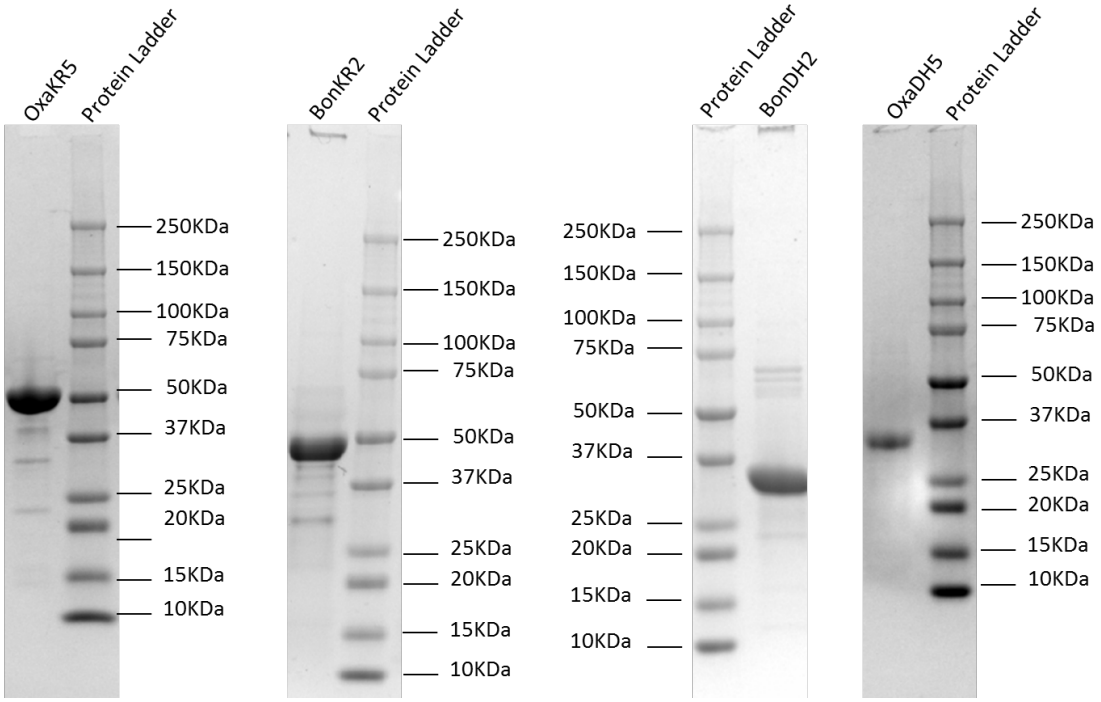


Figure S5. SDS PAGE of recombinant OxaKR5, OxaDH5, BonKR2 and BonDH2.

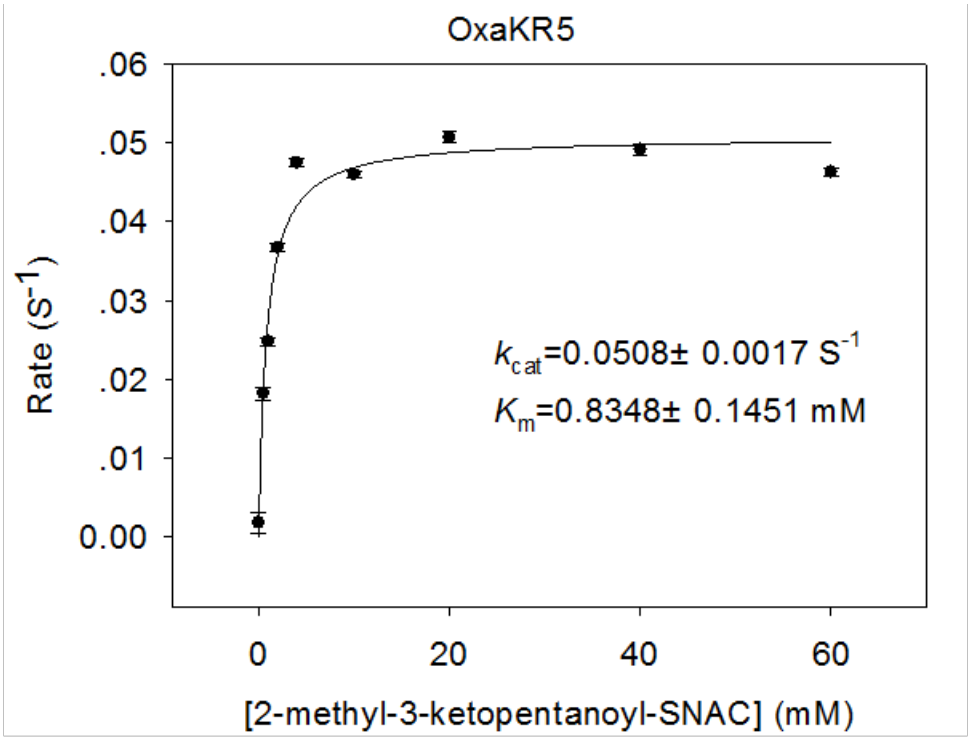
Table S1. Predicted MW and observed M_D of OxaKR5, OxaDH5, BonKR2 and BonDH2.

Protein	MW (cal, Da)	LC-QTOF (M_D, Da)
OxaKR5	53015.54	53017.97
OxaDH5	35807.51	35808.31
BonKR2	54025.59	54027.45
BonDH2	35565.68	35565.72

Kinetic assay of reductase activity of recombinant KR domains with (±)-2-methyl-3-ketopentanoyl-SNAC (4) and (±)-2-methyl-3-ketobutanoyl-SNAC (3). The continuous NADPH-linked assay, used for determination of the steady-state kinetic parameters, was carried out using a microplate plate reader and Falcon polystyrene 96-well plates to monitor NADPH consumption at 340 nm.[11] The reductase activity of each KR domain was assayed using variable concentrations from 0 to 60 mM of (±)-2-methyl-3-ketopentanoyl-SNAC (**4**) or from 0 to 20 mM of (±)-2-methyl-3-ketobutanoyl-SNAC (**4**) as the reference substrate. KR proteins were diluted in assay buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.2; NaCl concentration is very important to keep OxaKR5 in a soluble state) to give a 50 μM stock solution. The (±)-2-methyl-3-ketopentanoyl-SNAC (**4**) and (±)-2-methyl-3-ketobutanoyl-SNAC (**3**) thioesters were diluted with DMSO:H_2O (1:1) solution so as to give 9 different substrate concentrations of between 0 and 60 mM or 0-20 mM in the final mixture (the total amount of DMSO was kept constant at 5% v/v). Each incubation was carried out with 5 μM OxaKR5 or BonKR2, and 2 mM NADPH. The reaction was initiated by addition of substrate. The solutions were quickly mixed by pipetting up and down 3 times. The course of the reaction was followed by monitoring the change in absorbance at 340 nm every 1 min for 30 min. Each substrate concentration was assayed in duplicate. The steady-state kinetic parameters were calculated by fitting the observed rate and substrate concentration data to the Michaelis-Menten equation by nonlinear least squares regression using the SigmaPlot 12.5 program. The reported errors are the statistical deviations calculated by the nonlinear regression analysis.

Table S2. Steady-state kinetic parameters for reduction of (±)-2-methyl-3-ketopentanoyl-SNAC (**4**) and (±)-2-methyl-3-ketobutanoyl-SNAC (**3**) by OxaKR5 and BonKR2. See Figure S6 and S7 for plots of v vs [S] for each KR domain.

KR	Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
OxaKR5	(±)-2-methyl-3-ketopentanoyl-SNAC	0.051±0.002	0.84±0.15	0.061
	(±)-2-methyl-3-ketobutanoyl-SNAC	0.053±0.005	1.6±0.5	0.033
BonKR2	(±)-2-methyl-3-ketopentanoyl-SNAC	0.060±0.004	2.9±0.9	0.021
	(±)-2-methyl-3-ketobutanoyl-SNAC	0.066±0.007	0.82±0.4	0.08



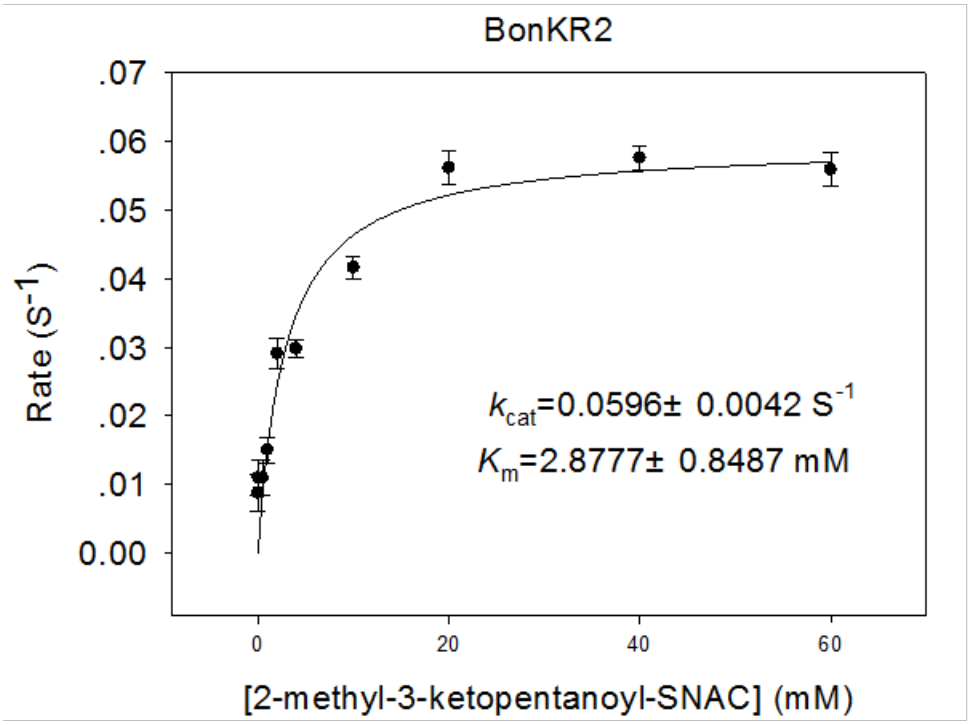


Figure S6. Assay of ketoreductase activity with (±)-2-methyl-3-ketopentanoyl-SNAC (**4**). See Table S2 for comparison of steady-state kinetic parameters.

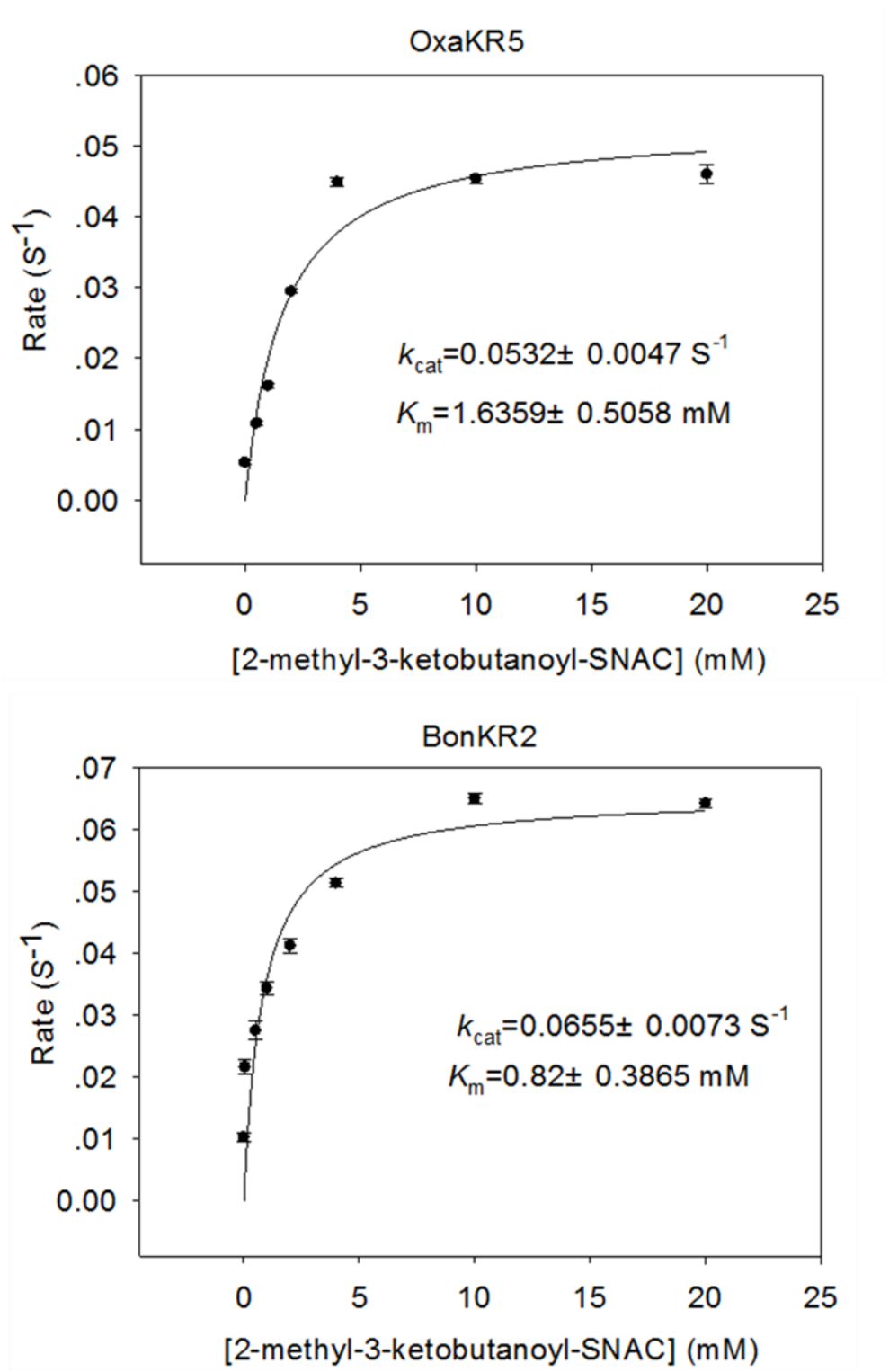


Figure S7. Assay of ketoreductase activity with (±)-2-methyl-3-ketobutanoyl-SNAC (**3**). See Table S2 for comparison of steady-state kinetic parameters.

Incubation of BonKR2 and OxaKR5 with NADPH and acyl-SNAC substrates 3 and 4. The protocol for reduction of (±)-2-methyl-3-ketopentanoyl-SNAC (**4**) was based on the previously described procedure.[11] In a typical incubation, in a total volume of 500 μL containing 1 mM (±)-2-methyl-3-ketopentanoyl-SNAC (**4**) or (±)-2-methyl-3-ketobutanoyl-SNAC (**3**), 2 mM NADPH, 50 mM NaH_2PO_4 buffer (100 mM NaCl, pH 7.2) containing tris-2-carboxyethyl-phosphine (TCEP, 2.5 mM) and 50 μM BonKR2 or OxaKR5. (The incubation mixture also contained ～1% glycerol from protein solutions and DMSO from SNAC substrate solutions). The enzyme mixtures were incubated at room temperature for 2 h, and the reaction was then quenched by addition of 150 μL of 0.5 M NaOH solution and heated to 65 °C for 20 min. The basic mixture was treated with 200 μL of 1 M HCl solution. After centrifugation at 13,000*g* for 5 min to remove precipitated protein, the supernatant was extracted with 4 × 800 μL of ethyl acetate. After evaporation of the solvent, the concentrated organic extract was derivatized with TMS-CHN_2 and analyzed by GC-MS, with XIC monitoring of the *m*/z 88 base peak, as described below.

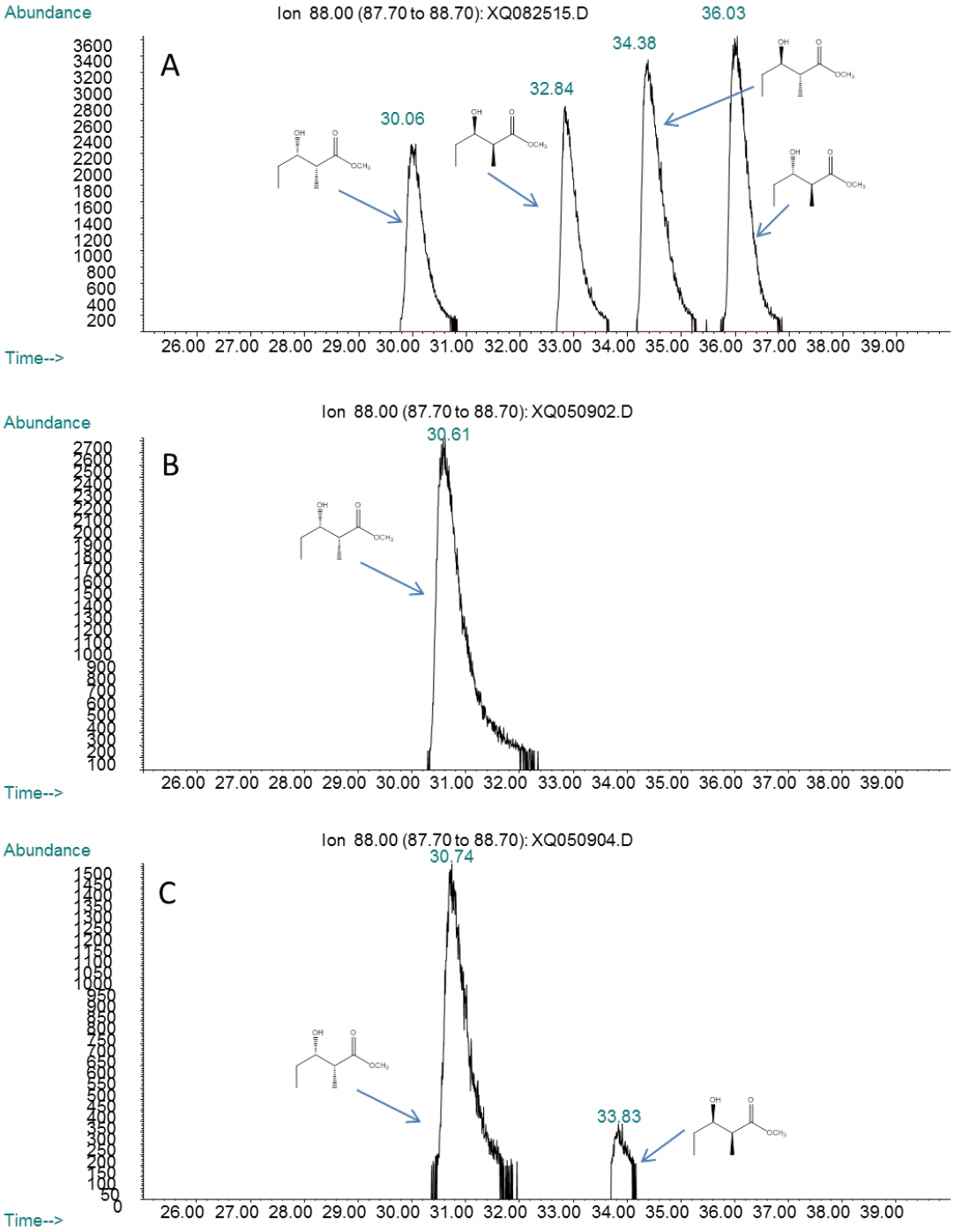


Figure S8. GC-MS analysis [XIC *m*/z 88 (base peak)] of methyl 2-methyl-3-hydroxypentanoates produced by BonKR2-catalyzed reduction of (±)-2-methyl-3-ketopentanoyl-SNAC (**4**). A. Reference Standard. B. BonKR2. C. B plus added methyl (2*S*, 3*R*)-2-methyl-3-hydroxypentanoate.

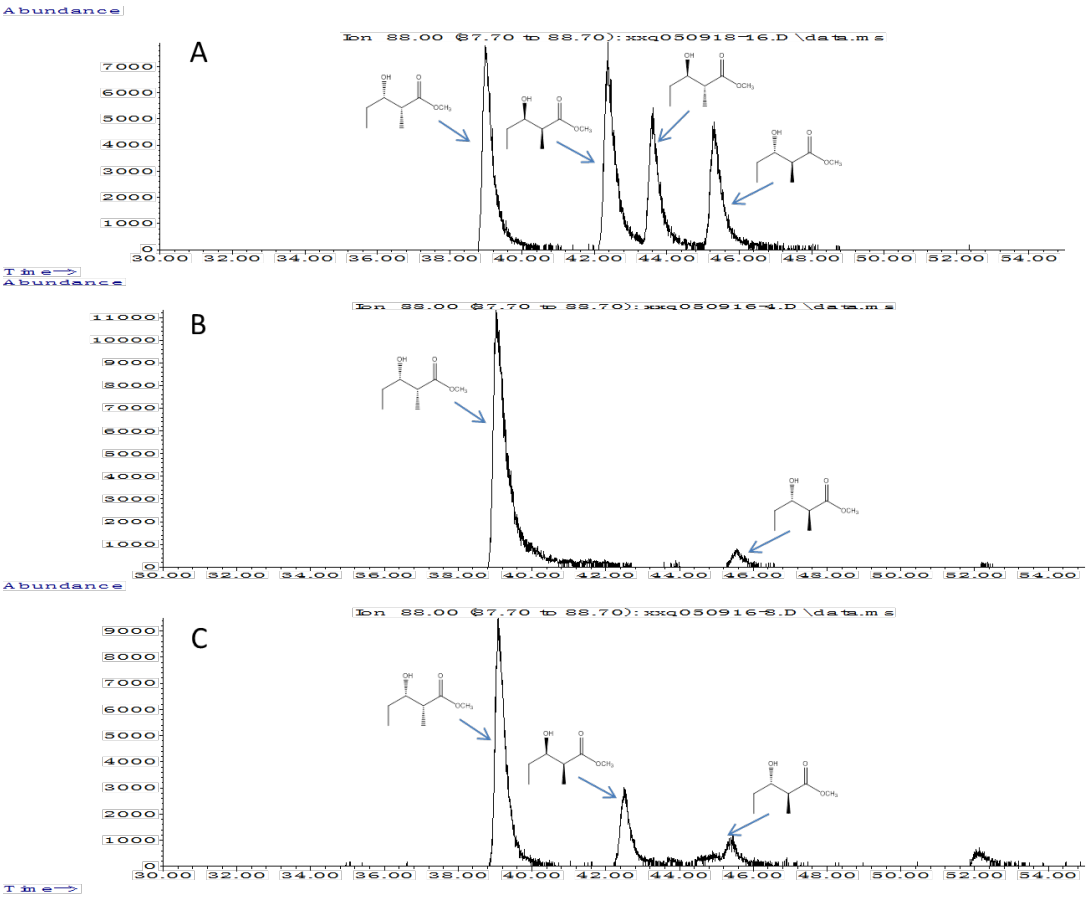


Figure S9. GC-MS analysis [XIC *m*/z 88 (base peak)] of methyl 2-methyl-3-hydroxypentanoates produced by OxaKR5-catalyzed reduction of (±)-2-methyl-3-ketopentanoyl-SNAC (**4**). A. Reference Standard. B. OxaKR5. C. B plus added methyl (2*S*, 3*R*)-2-methyl-3-hydroxypentanoate.

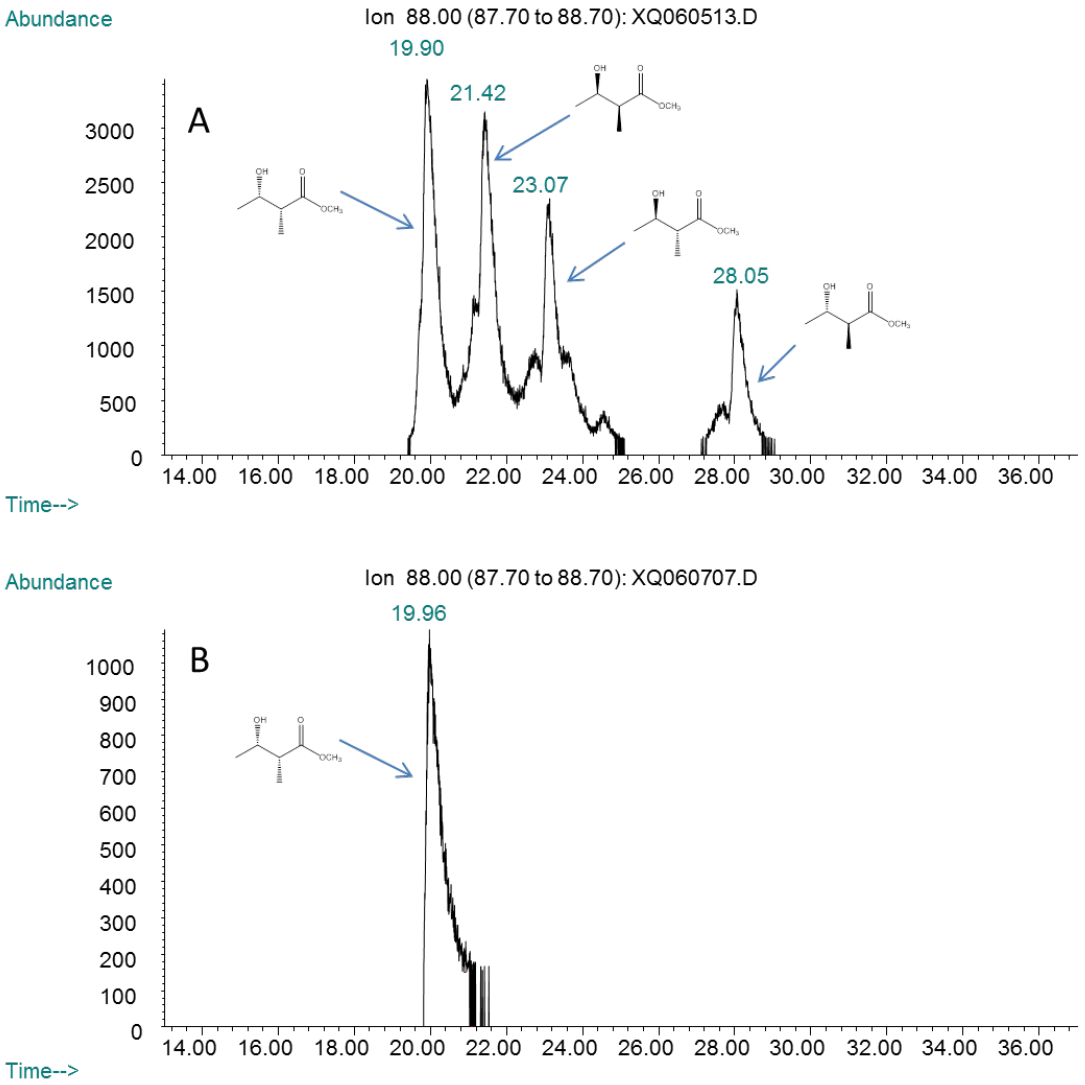


Figure S10. GC-MS analysis [XIC *m*/z 88 (base peak)] of methyl 2-methyl-3-hydroxybutyrates produced by BonKR2-catalyzed reduction of (±)-2-methyl-3-ketobutanoyl-SNAC (**3**). A. Reference Standard. B. BonKR2.

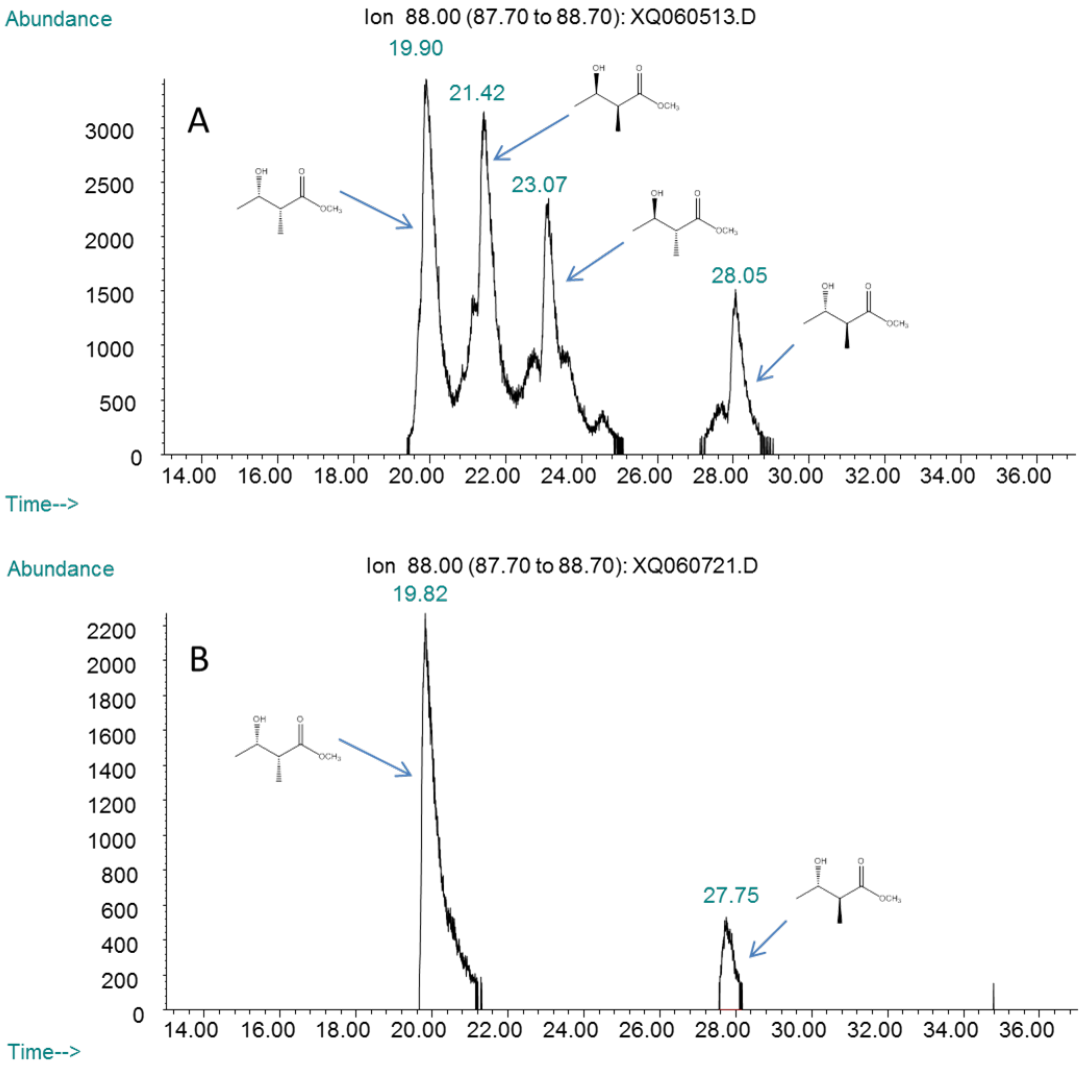


Figure S11. GC-MS analysis [XIC *m*/z 88 (base peak)] of methyl 2-methyl-3-hydroxybutyrates produced by OxaKR5-catalyzed reduction of (±)-2-methyl-3-ketobutanoyl-SNAC (**3**). A. Reference Standard. B. OxaKR5.

Incubation of BonKR2 and OxaKR5 with chemoenzymatically generated (2*R*)-2-methyl-3-ketopentanoyl-SEryACP6.

The chemoenzymatic preparation of (2*R*)-2-methyl-3-ketopentanoyl-SEryACP6 and its use for the determination of the stereochemistry KR reduction has been previously described.[1, 2] In a typical assay, 5 mM propionyl-SNAC was pre-incubated with 40 μM Ery[KS6][AT6] in a total volume of 500 μL of phosphate buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.2) containing tris-2-carboxyethyl-phosphine (TCEP, 2.5 mM). After 30 min, 200 μM of *holo*-EryACP6, 300 μM methylmalonyl-CoA, 2 mM NADPH and 300 μM BonKR2 or OxaKR5 were added. The enzyme mixture was incubated at room temperature for an additional hour. The reaction was quenched by addition of 150 μL of 0.5 M NaOH, the mixture incubated at 65 °C for 20 min. The basic reaction mixture was acidified with 200 μL of 1 M HCl and then extracted with ethyl acetate (600 μL). The concentrated organic extract was derivatized with TMS-CHN_2 and the resulting methyl ester analyzed by GC-MS, as described below.

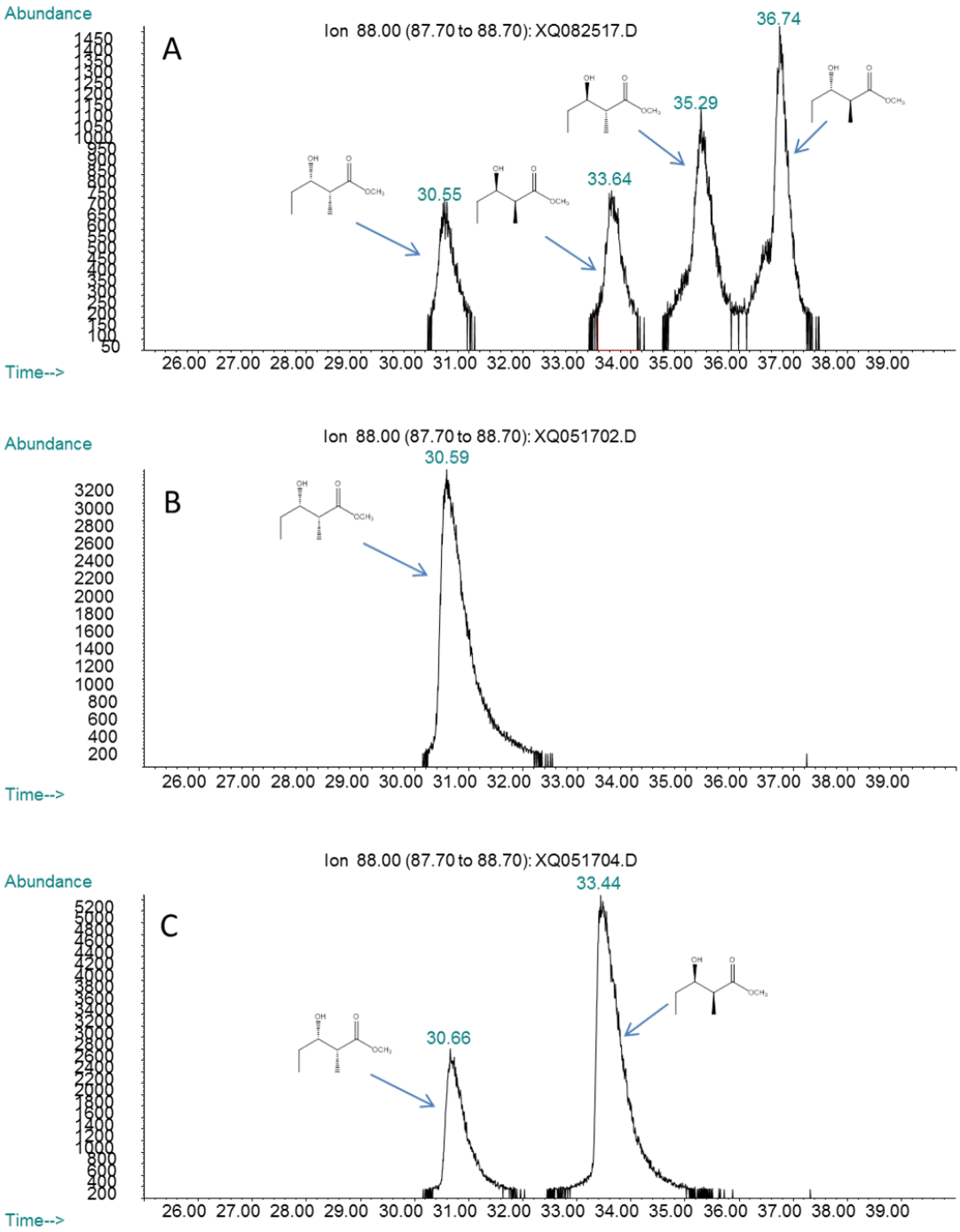


Figure S12. GC-MS analysis [XIC *m*/z 88 (base peak)] of methyl 2-methyl-3-hydroxypentanoate produced by BonKR2-catalyzed reduction of (2*R*)-2-methyl-3-ketopentanoyl-EryACP6 produced by incubation of Ery[KS6][AT6], propionyl-SNAC, methylmalonyl-CoA and *holo*-EryACP6. A. Reference Standard. B. BonKR2. C. B (**8-Me**) plus added methyl (2*S*, 3*R*)-2-methyl-3-hydroxypentanoate.

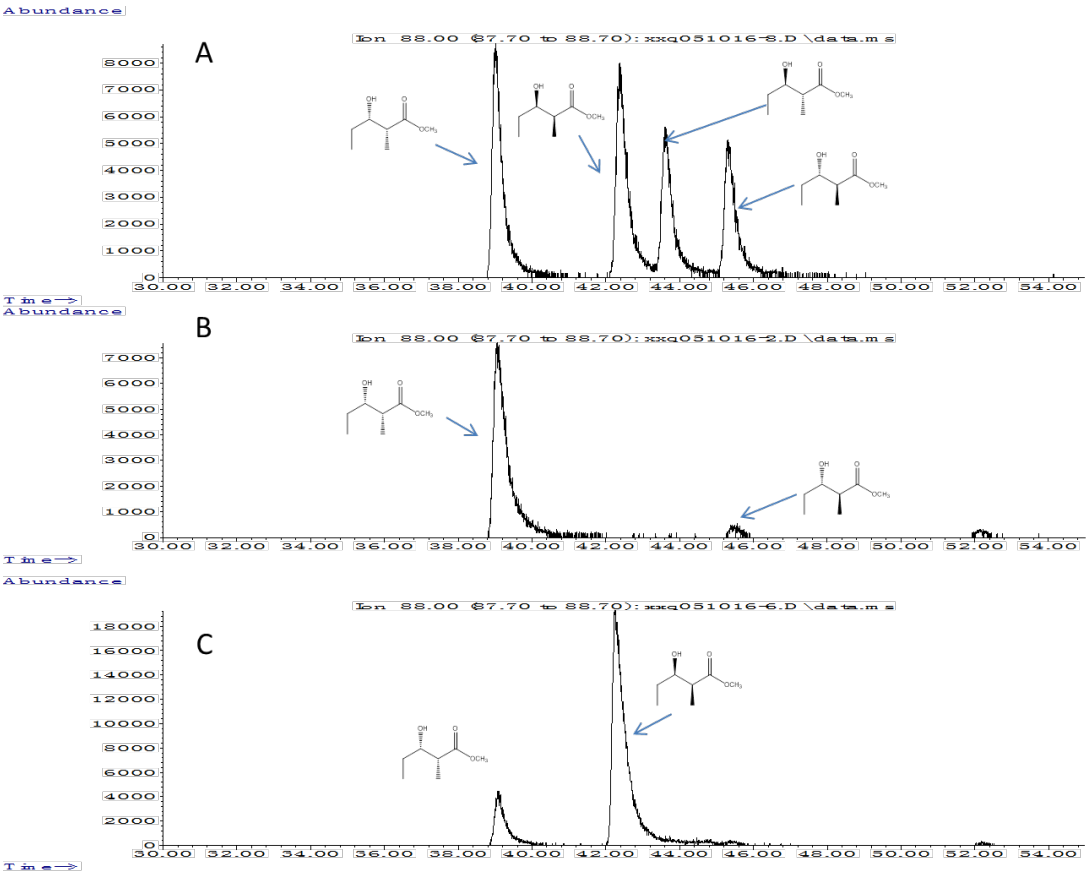


Figure S13. GC-MS analysis [XIC *m*/z 88 (base peak)] of methyl 2-methyl-3-hydroxypentanoates produced by OxaKR5-catalyzed reduction of (2*R*)-2-methyl-3-ketopentanoyl-EryACP6 produced by incubation of Ery[KS6][AT6], propionyl-SNAC, methylmalonyl-CoA and *holo*-EryACP6. A. Reference Standard. B. OxaKR5. C. B (**8-Me**) plus added methyl (2*S*, 3*R*)-2-methyl-3-hydroxypentanoate.

Incubation of recombinant BonKR2 and OxaKR5 with reconstituted BonKS2, BonACP2, and BonMT2. The (2*R*)-2-methyl-3-ketopentanoyl-BonACP2 (**13**) or (2*R*)-2-methyl-3-ketobutanoyl-BonACP2 (**12**) were generated as previously described.[4] In a typical assay, 5 mM propionyl-SNAC or acetyl-SNAC was pre-incubated with 200 μM BonKS2 in a total volume of 200 μL of phosphate buffer (50 mM sodium phosphate, 100 mM NaCl, 2.5 mM TCEP, pH 7.2). In another tube, 200 μL of phosphate buffer (50 mM sodium phosphate, 100 mM NaCl, 2.5 mM TCEP, pH 7.2) containing 500 μM *apo*-BonACP2, 10 μM Sfp, 10 mM $MgCl_2$ and 600 μM malonyl-CoA were incubated at room temperature for 30 min. The solution mixture containing fresh chemoenzymatically-generated malonyl-BonACP2 was transferred into the tube with the preincubated mixture of propionyl-SNAC or acetyl-SNAC and BonKS2 and the solution was incubated at room temperature for 2 h. To this solution was added (final concentration) 50 μM BonMT2, 15 mM SAM, and 50 μM SAH nucleosidase. After 15 min, 100 μL solutions containing 6 mM NADPH and 600 μM BonKR2 or OxaKR5 in assay buffer (50 mM sodium phosphate, 100 mM NaCl, 2.5 mM TCEP, pH 7.2) were added. After 1 h, the enzyme reaction mixture was quenched by mixing with 100 μL of 0.5 M NaOH. The resulting mixture was incubated at 65 °C for 30 min. After acidification with 150 μL of 1 M HCl and extraction with ethyl acetate (4×600 μL), the concentrated organic extract was methylated by treatment with TMS-CHN_2 and analyzed by chiral GC-MS, as described below.

Chiral GC-MS analysis of BonKR2 and OxaKR5 reduction products. The concentrated organic extracts of the above-described enzymatic reductions with BonKR2 or OxaKR5 were dissolved in 200 μL of methanol and then treated with 5 μL of TMS-diazomethane (2 M in hexane) for 10 min at room temperature. The derived reduced 2-methyl-3-hydroxybutyryl and 2-methyl-3-hydroxypentanoyl methyl esters were directly analyzed by chiral GC-MS. For analysis of stereochemistry of 2-methyl-3-hydroxypentanoic acid methyl esters generated by OxaKR5, GC-MS spectra were recorded on an Agilent 5977A Series GC-MSD instrument, 70 eV EI in positive ion mode, using a Varian CP-Chirasil-DEX CB capillary column (25 m × 0.25 mm, Agilent Technologies) and a temperature program of (1) initial temp 60 °C for 1 min, (2) increase at rate 0.3 °C/min up to 75 °C, (3) 5 °C/min up to 90 °C, and then 20 °C/min to final temp 200 °C. For analysis of the stereochemistry of 2-methyl-3-hydroxypentanoic acid methyl esters generated by BonKR2, GC-MS spectra were recorded on a GC-MS Hewlett-Packard Series II GC-MSD instrument, 70 eV EI in positive ion mode, using a Varian CP-Chirasil-DEX CB capillary column (25 m × 0.32 mm, Agilent Technologies) and a temperature program of (1) initial temp 60 °C for 1 min, (2) increase at rate 0.3 °C /min up to 70 °C, (3) 5 °C/min up to 90 °C, and then 20 °C/min to final temp 200 °C. The extracted ion current (XIC) of the chromatogram was analyzed set on the base peak at *m/z* 88 of methyl 2-methyl-3-hydroxypentanoate. The eluent peaks were directly compared by ret. time and MS with authentic standards of all four diastereomers, as previously described,[8] and confirmed by co-injection of authentic methyl (2*S*,3*R*)-2-methyl-3-hydroxypentanoate (**8-Me**).

For the analysis of methyl 2-methyl-3-hydroxybutanoate generated by incubations with BonKR2 or OxaKR5, GC-MS spectra were recorded on the Hewlett-Packard Series II GC-MSD instrument, 70 eV EI in positive ion mode using a Varian CP-Chirasil-DEX CB capillary column (25 m × 0.32 mm, Agilent Technologies) and a temperature program of (1) initial temp 60 °C for 1 min; (2) increase at rate 0.3 °C/min up to 70 °C, hold for 1 min at 70 °C; (3) 5 °C/min up to 90 °C, hold for 1 min at 90 °C and then 20 °C/min to final temp 200 °C. The extracted ion current (XIC) of the chromatogram was analyzed set on the base peak at *m/z* 88 of methyl 2-methyl-3-hydroxybutyrate.

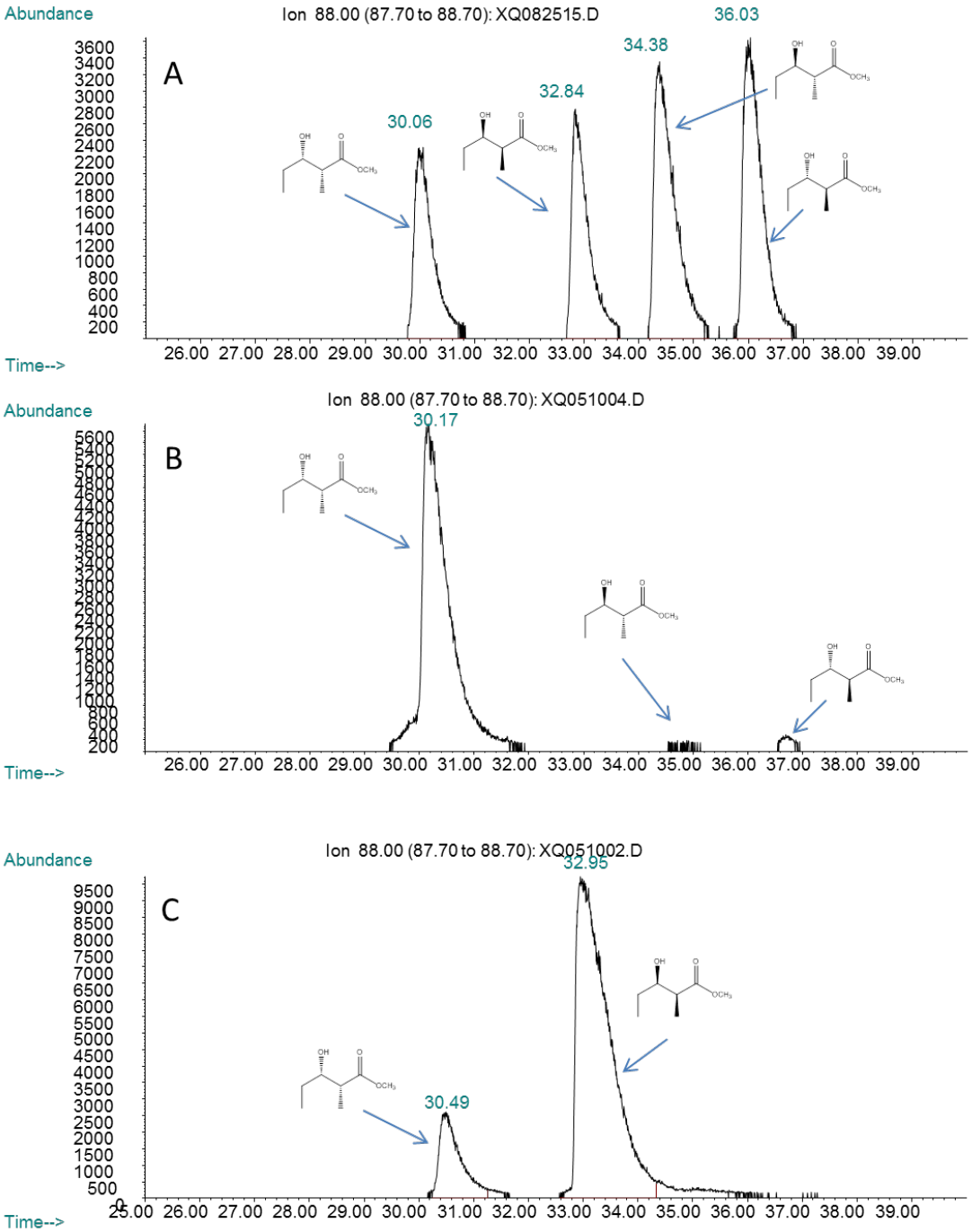


Figure S14. GC-MS analysis [XIC *m*/z 88 (base peak)] of methyl 2-methyl-3-hydroxypentanoates produced by BonKR2-catalyzed reduction of (2*R*)-2-methyl-3-ketopentanoyl-BonACP2 (**13**) generated by incubation of propionyl-SNAC, BonKS2, BonACP2, BonMT2, malonyl-CoA, SAH nucleosidase and SAM. A. Reference Standard. B. BonKR2. C. B (**8-Me**) plus added methyl (2*R*,3*S*)-2-methyl-3-hydroxypentanoate.

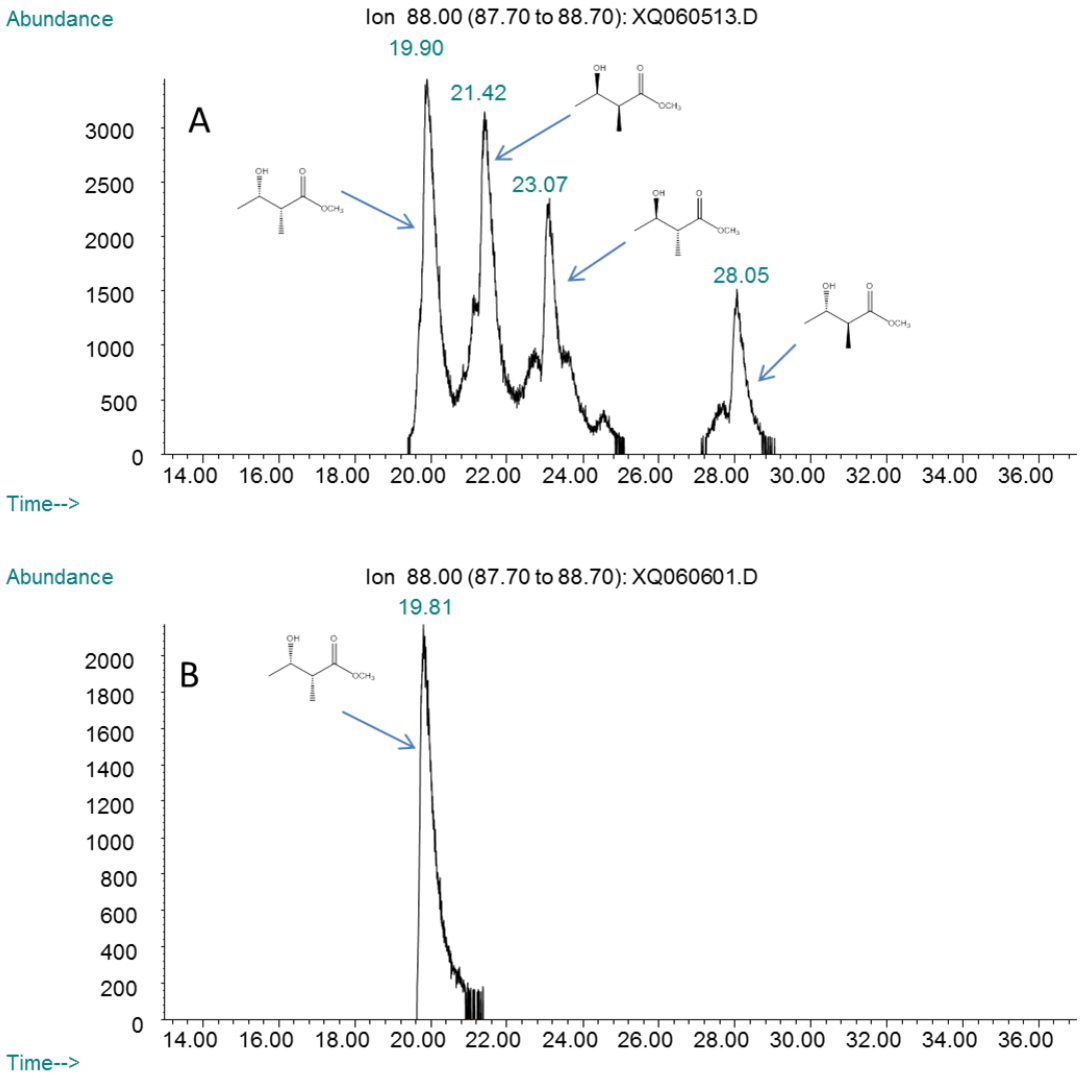


Figure S15. GC-MS analysis [XIC *m*/z 88 (base peak)] of methyl 2-methyl-3-hydroxybutyrates produced by BonKR2-catalyzed reductions of (2*R*)-2-methyl-3-ketobutyryl-BonACP2 (**12**) generated by incubation of acetyl-SNAC, BonKS2, BonACP2, BonMT2, malonyl-CoA, SAH nucleosidase and SAM. A. Reference Standard. B. BonKR2.

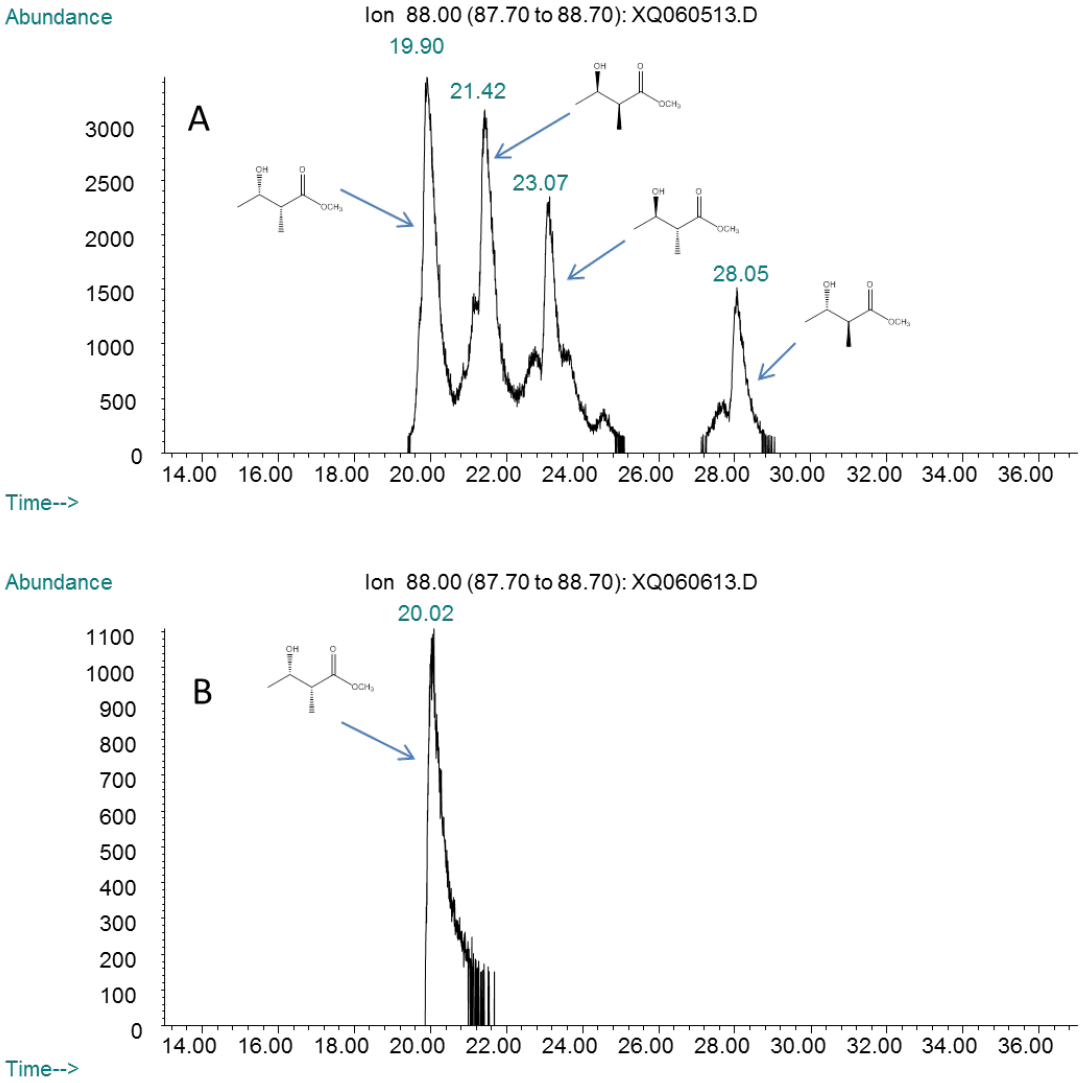


Figure S16. GC-MS analysis [XIC *m*/z 88 (base peak)] of methyl 2-methyl-3-hydroxybutyrates produced by OxaKR5-catalyzed reduction of (2*R*)-2-methyl-3-ketobutyryl-BonACP2 (**12**) generated by incubation of acetyl-SNAC, BonKS2, BonACP2, BonMT2, malonyl-CoA, SAH nucleosidase and SAM. A. Reference Standard. B. OxaKR5.

Table S3. Stereospecificity of the KR-catalyzed reduction of (±)-2-methyl-3-ketopentanoyl-SNAC (**4**) or (2*R*)-2-methyl-3-ketopentanoyl-EryACP6, generated by incubation of propionyl-SNAC, Ery[KS6][AT6], *holo*-EryACP6 and methylmalonyl-CoA, or of (2*R*)-2-methyl-3-ketopentanoyl-BonACP2 (**13**), generated by incubation of propionyl-SNAC, BonKS2, *apo*-BonACP2, Sfp, malonyl-CoA, BonMT2 and SAM.

protein	**Methyl 2-methyl-3-hydroxypentanoate (C5)**			
	(2*R*,3*S*)	**(2*S*,3*R*)**	**(2*R*,3*R*)**	**(2*S*,3*S*)**
	(%)	(%)	(%)	(%)
Incubation with (±)-2-methyl-3-ketopentanoyl-SNAC (**4**)				
OxaKR5	>99	0	0	<1
BonKR2	100	0	0	0
Propionyl-SNAC + Ery[KS6][AT6] + EryACP6 + MMCoA				
OxaKR5	>99	0	0	<1
BonKR2	100	0	0	0
Propionyl-SNAC + BonKS2 + *apo*-BonACP2 + Sfp + MCoA+BonMT2+SAM				
BonKR2	>98	0	<1	<1

Table S4. Stereospecificity of the KR-catalyzed reduction of (±)-2-methyl-3-ketobutyryl-SNAC (**3**) or of (2*R*)-2-methyl-3-ketobutyryl-BonACP2, generated by incubation of acetyl-SNAC, BonKS2, *apo*-BonACP2, Sfp, malonyl-CoA, BonMT2 and SAM.

protein	**Methyl 2-methyl-3-hydroxypentanoate (C4)**			
	(2*R*,3*S*)	**(2*S*,3*R*)**	**(2*R*,3*R*)**	**(2*S*,3*S*)**
	(%)	(%)	(%)	(%)
Incubation with 2-methyl-3-ketobutyryl-SNAC (**3**)				
OxaKR5	90	0	0	10
BonKR2	100	0	0	0
Acetyl-SNAC + BonKS2 + *apo*-BonACP2 + Sfp+MCoA+BonMT2+SAM				
OxaKR5	100	0	0	0
BonKR2	100	0	0	0

Incubation of BonDH2 and OxaDH5 with chemoenzymatically generated (2*R*,3*S*)-2-methyl-3-hydroxybutyryl-BonACP2. Chemoenzymatically generated (2*R*,3*S*)-2-methyl-3-hydroxybutyryl-BonACP2 was incubated with 50 μM BonDH2, OxaDH5, or NigDH1 (as negative control) in assay buffer (50 mM sodium phosphate, 100 mM NaCl, 2.5 mM TCEP, pH 7.2). After 30 min, the diketide product was hydrolytically released from BonACP2 by treatment with PICS TE (50 μM) for 10 min at room temperature. The reaction mixture was acidified to pH <3 by addition of 1 M HCl. The C4 acids were extracted with 3 × 800 μL ethyl acetate and the solvent was removed by rotary evaporation. The concentrated organic extract was taken up in 200 μL of dichloromethane and directly analyzed by GC-MS. GC-MS spectra were recorded on GC-MS Hewlett-Packard Series II GC-MSD instrument, 70 eV EI in positive ion mode, using a Varian CP-Chirasil-DEX CB capillary column (25 m × 0.32 mm, Agilent Technologies) and a temperature program of (1) initial temp 80 °C for 1 min; (2) increase at rate 0.5 °C /min up to 90 °C, hold at 90 °C for 5 min; (3) 20 °C /min up to 200 °C and then 20 °C /min to final temp 210 °C for 1 min. The extracted ion current (XIC) of the chromatogram was analyzed set on the base peak at *m/z* 100 of the (*Z*)- and (*E*)-2-methyl-2-butenoic acid, with direct comparison of both ret. time and MS to authentic standards of each geometric isomer of 2-methyl-2-butenoic acid. There was no detectable dehydrated product from the control incubation with NigDH1.

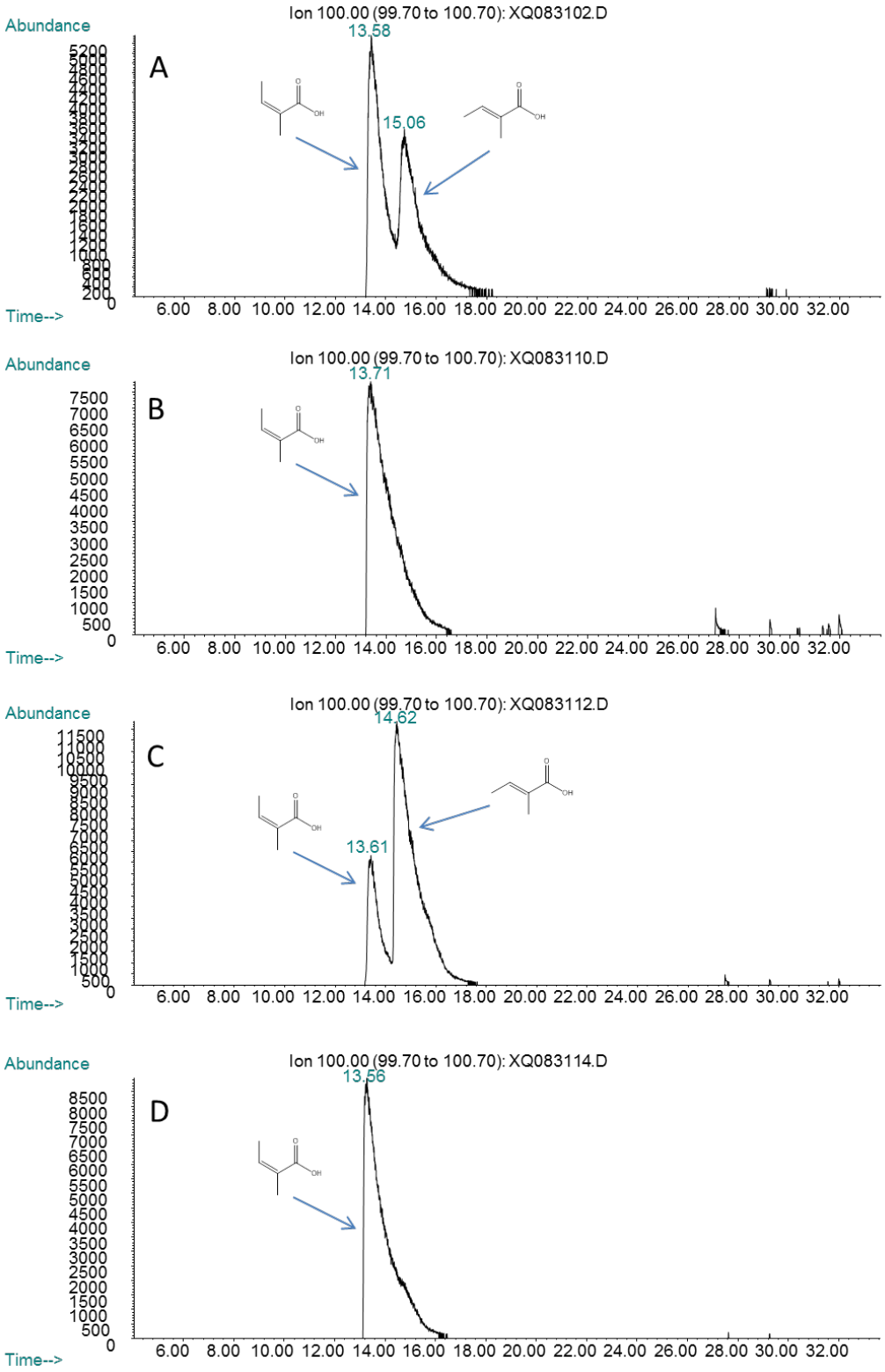


Figure S17. GC-MS analysis [XIC *m*/z 100 (base peak)] of 2-methyl-2-butenoic acid ((*Z*)-**17**) produced by BonDH2-catalyzed dehydration of (2*R*,3*S*)-2-methyl-3-hydroxybutyryl-BonACP2 (**14**), generated by incubation of acetyl-SNAC, BonKS2, *apo*-BonACP2, Sfp, BonMT2, malonyl-CoA, SAH nucleosidase, SAM, BonKR2 and NADPH, followed by PICS TE-catalyzed hydrolysis. A. Reference Standard. B. BonDH2. C. B + (*E*)-2-methyl-2-butenoic acid. D. B + (*Z*)-2-methyl-2-butenoic acid (**17**).

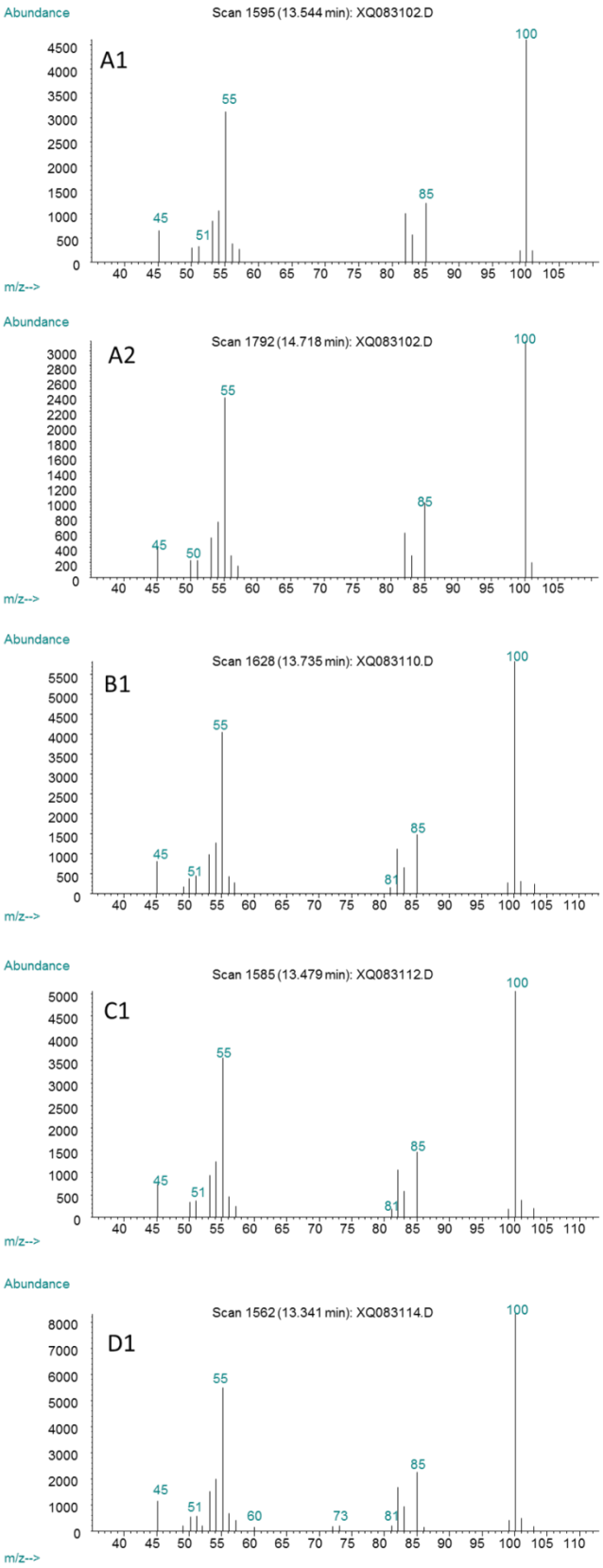


Figure S17 (cont). A1 and A2. MS of *(Z)*- and (*E*)-2-methyl-2-butenoic acid from A; B1. MS of (*Z*)-2-methyl-2-butenoic acid from B; C1. MS of (*Z*)-2-methyl-2-butenoic acid from C; D1. MS of (*Z*)-2-methyl-2-butenoic acid (**17**) from D.

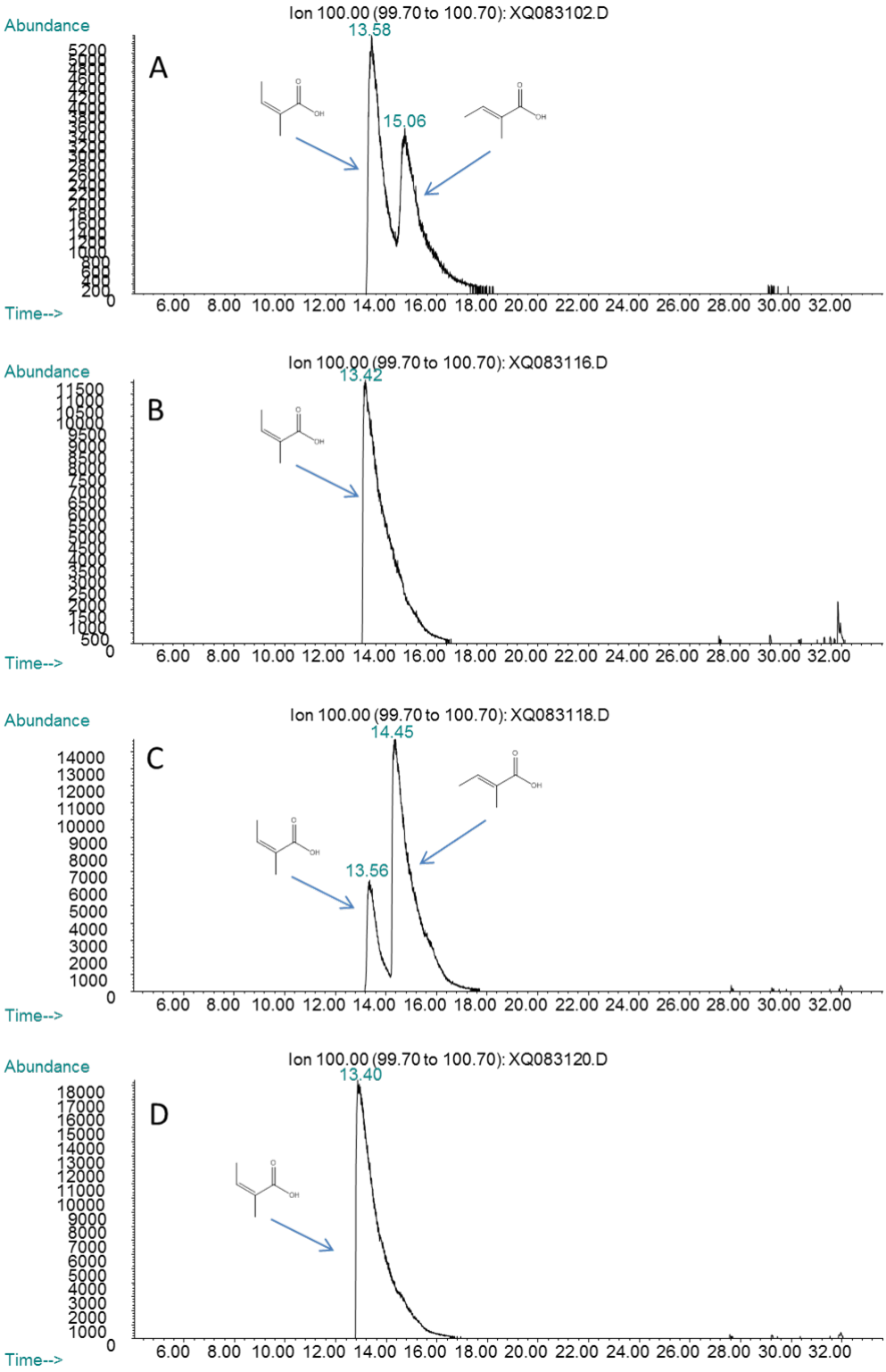


Figure S18. GC-MS analysis [XIC *m*/z 100 (base peak)] of 2-methyl-2-butenoic acid (*Z*)-**17** produced by OxaDH5-catalyzed dehydration of (2*R*,3*S*)-2-methyl-3-hydroxybutyryl-BonACP2 (**14**), generated by incubation of acetyl-SNAC, BonKS2, *apo*-BonACP2, Sfp, BonMT2, malonyl-CoA, SAH nucleosidase, SAM, BonKR2 and NADPH, followed by PICS TE-catalyzed hydrolysis. A. Reference Standard. B. OxaDH5. C. B + (*E*)-2-methyl-2-butenoic acid. D. B + (*Z*)-2-methyl-2-butenoic acid (**17**).

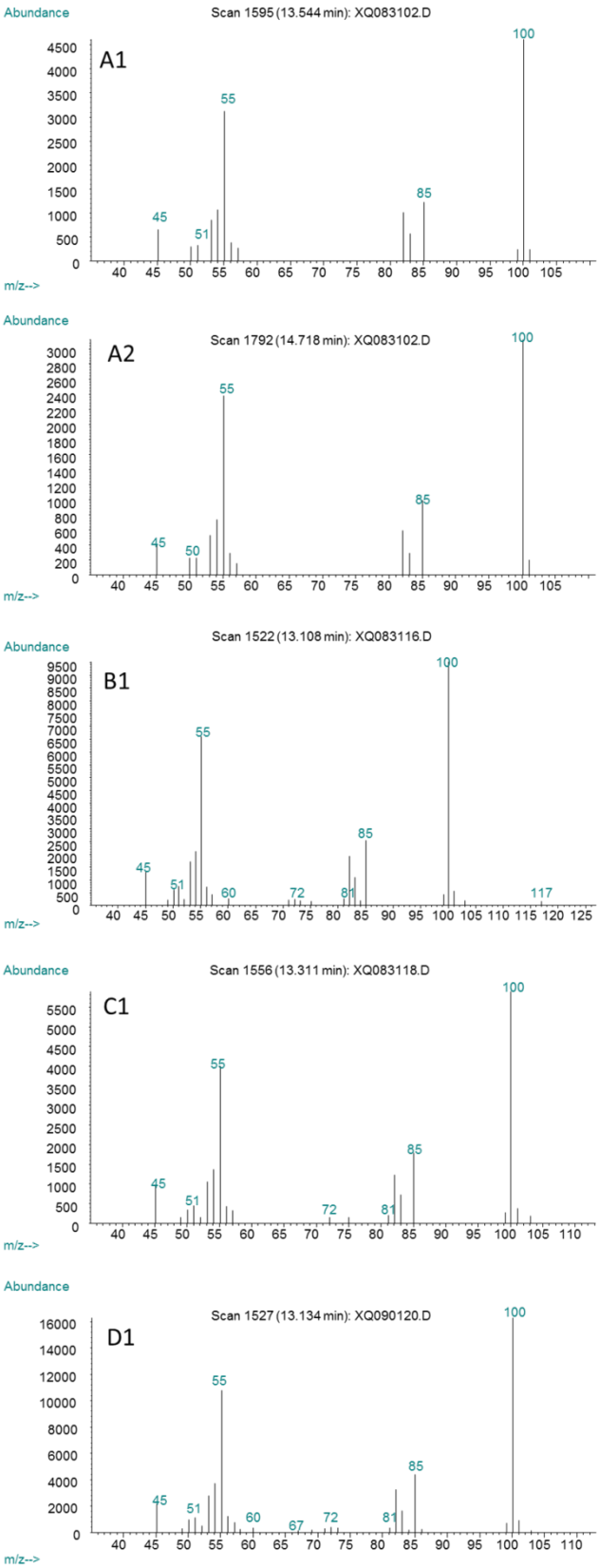


Figure S18 (cont). A1 and A2. MS of 2-methyl-2-butenoic acid *(Z)*- and (*E*)-2-methyl-2-butenoic acid from A; B1. MS of (*Z*)-2-methyl-2-butenoic acid from B; C1. MS of (*Z*)-2-methyl-2-butenoic acid from C; D1. MS of (*Z*)-2-methyl-2-butenoic acid from D.

DH-Catalyzed hydration of (*Z*)-2-methyl-2-butenoyl-BonACP2 ((*Z*)-16).

(*Z*)-2-Methyl-2-butenoyl-CoA was synthesized as previously described.[12] To a stirred solution of 0.2 mmol of (*Z*)-2-methyl-2-butenoic acid in 1 mL of anhydrous dichloromethyl, triethylamine (70 μL, 47 mg, 4.0 equiv) was added and the solution was stirred for 10 min under N_2 at 0 °C. Ethylchloroformate (50 μL, 45.8 mg, 3.0 equiv) was added and the mixture allowed to react for 2 h under N_2 at 0 °C. The solvent was removed by rotary evaporation, the residue was dissolved in 2 mL of THF, and the insoluble salts were removed by centrifugation. The THF solution was then added dropwise to a separate round-bottom flask containing 20 mg of CoASH in 1 mL of $NaHCO_3$ buffer (pH 8.0). The reaction mixture was stirred 1−3 h at room temperature with monitoring by LC−MS. The organic solvent was removed by rotary evaporation and the aqueous phase was extracted with ether to remove organic byproducts. The aqueous crude acyl-CoA mixture was purified by HPLC using a Phenomenex Gemini semipreparative C18 column, 150×10 mm, equilibrated with 5% CH_3CN/H_2O. The sample was eluted with a linear gradient from 5% to 95% of CH_3CN/H_2O. HPLC peaks were collected and lyophilized. Each fraction was analyzed by HPLC−ESI(+)−MS using an Agilent Zorbax C18 column (2.1×50 mm, 3.5 μm) and a linear gradient from 5% to 95% CH_3CN/H_2O.

(*Z*)-2-Methyl-2-butenoyl-BonACP2 (**16**) was chemoenzymatically prepared by incubation of (Z)-2-methyl-2-butenoyl-CoA, *apo*-BonACP2 and Sfp. In a typical procedure, 500 μM (*Z*)-2-methyl-2-butenoyl-CoA, 250 μM *apo*-BonACP2, 20 μM Sfp, 10 mM $MgCl_2$ and 1 mM TCEP were incubated in 50 mM phosphate buffer (pH 7.2), in a total volume of 500 μL. After 30 min, 50 μL of enzyme reaction mixture was diluted by 450 μL H_2O and passed through a Millipore 10 kDa MWCO 500 μL filter and centrifuged at 14,000 *g* to concentrate the acyl-BonACP2 (**16**), which was desalted using a 10 kDa MWCO ultra-filter and C18 ZipTip (Millipore) pipette tips. The resulting sample was diluted to a total volume of 200−300 μL and analyzed by LC-ESI-MS.

Then 50 μM BonDH2 or OxaDH5 or NigDH1 (as negative control) in assay buffer (50 mM sodium phosphate, 100 mM NaCl, 2.5 mM TCEP, pH 7.2) was added to (*Z*)-2-methyl-2-butenoyl-BonACP2 (**16**) solution. After 30 min, the 400 μL of enzyme mixture was immediately quenched by mixing with 100 μL of 0.5 M NaOH and the resulting mixture was incubated at 65 °C for 30 min. After acidification with 150 μL of 1 M HCl and extraction with ethyl acetate (4×600 μL), the concentrated organic extract was methylated by treatment with TMS-CHN_2 and analyzed by chiral GC-MS.

Alternatively, the 50 μL of enzyme mixture was diluted by 450 μL H_2O, passed through a Millipore 30 kDa MWCO 500-μL filter and centrifuged at 14,000*g* to remove DH proteins. The ultrafiltrate containing acyl-BonACP2 and a small amount of Sfp was concentrated and desalted using a 10 kDa MWCO ultrafilter and C18 ZipTip (Millipore) pipette tips and the resulting sample was diluted to a total volume of 200−300 μL and analyzed by LC-ESI-MS.

Table S5. Predicted MW and observed ESI-MS M_D (daltons) of acyl-BonACP2 derivatives.

Acyl-ACP	**MW (cal, Da)**	**LC-ESI-MS (M_D, Da)**
(*Z*)-2-methyl-2-butenoyl-BonACP2 (**16**)	15527	15527
2-methyl-3-hydroxypentanoyl-BonACP2 (**14**)	15545	15545

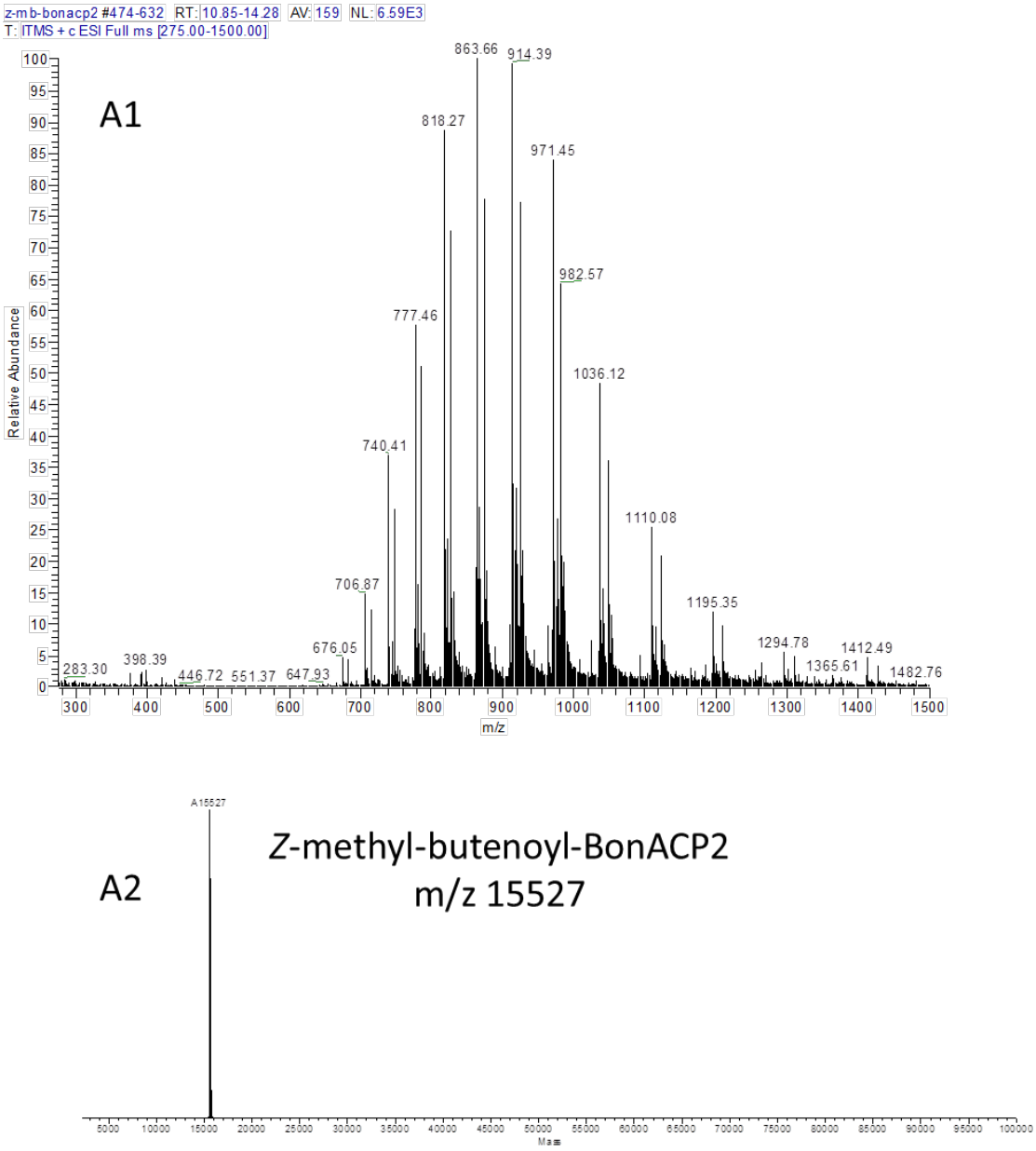


Figure S19. LC-MS analysis of acylated BonACP2 derivatives. A. (*Z*)-2-methyl-2-butenoyl-BonACP2 (**16**) from incubation of (*Z*)-2-methyl-2-butenoyl-CoA, Sfp and *apo*-BonACP2; B. 2-Methyl-3-hydroxybutyryl-BonACP2 (**14**) from incubation of A and BonDH2; C. 2-Methyl-3-hydroxybutyryl-BonACP2 (**14**) from incubation of A and OxaDH5.

Each ESI-MS also showed peaks corresponding to 20-35% of the corresponding *N*-gluconyl-ACP derivative (M+178), typical of His_6-tag proteins expressed in *E. coli*.[13]

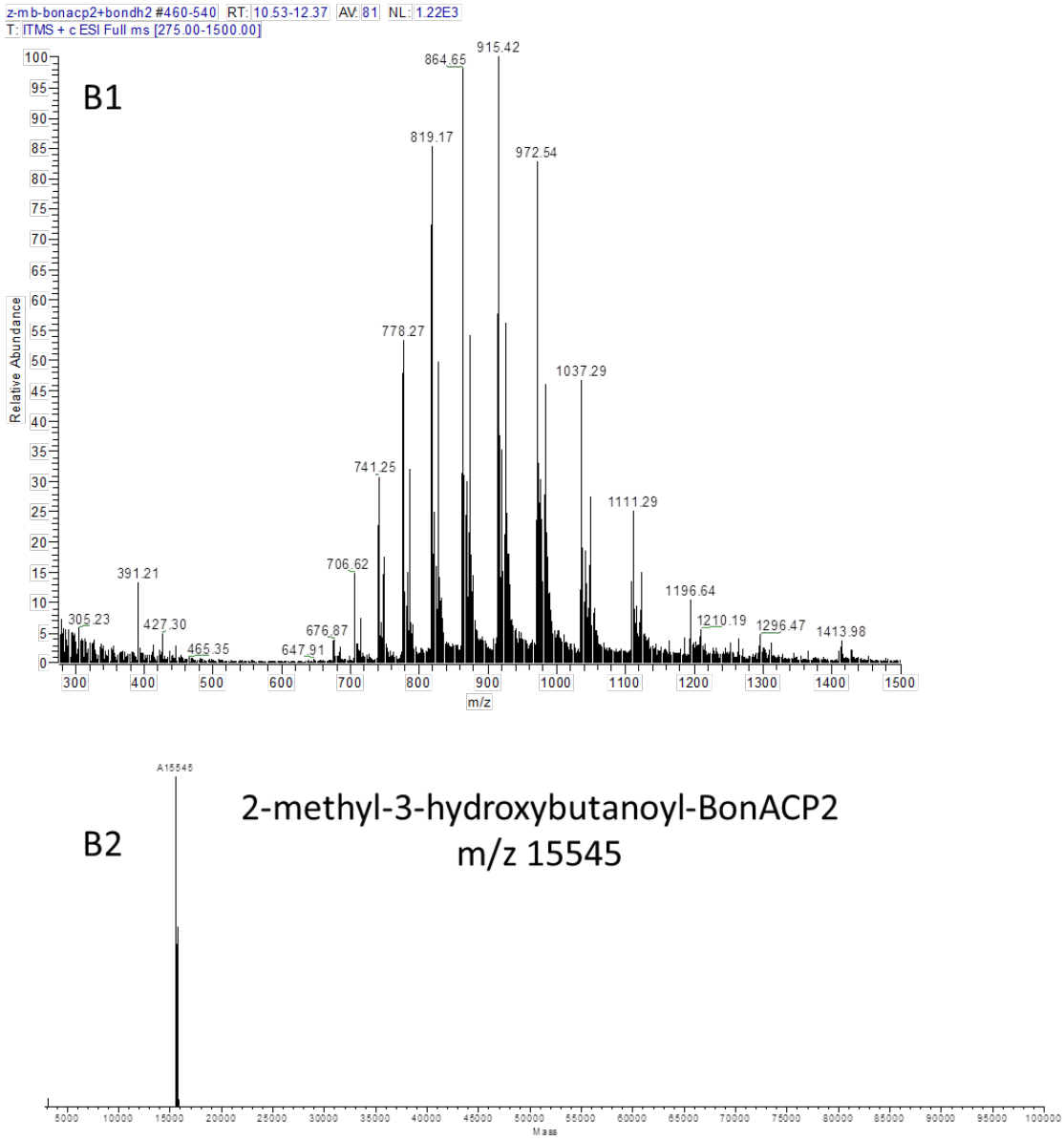


Figure 19 (cont). B. 2-Methyl-3-hydroxybutyryl-BonACP2 (**14**) from incubation of A and BonDH2.

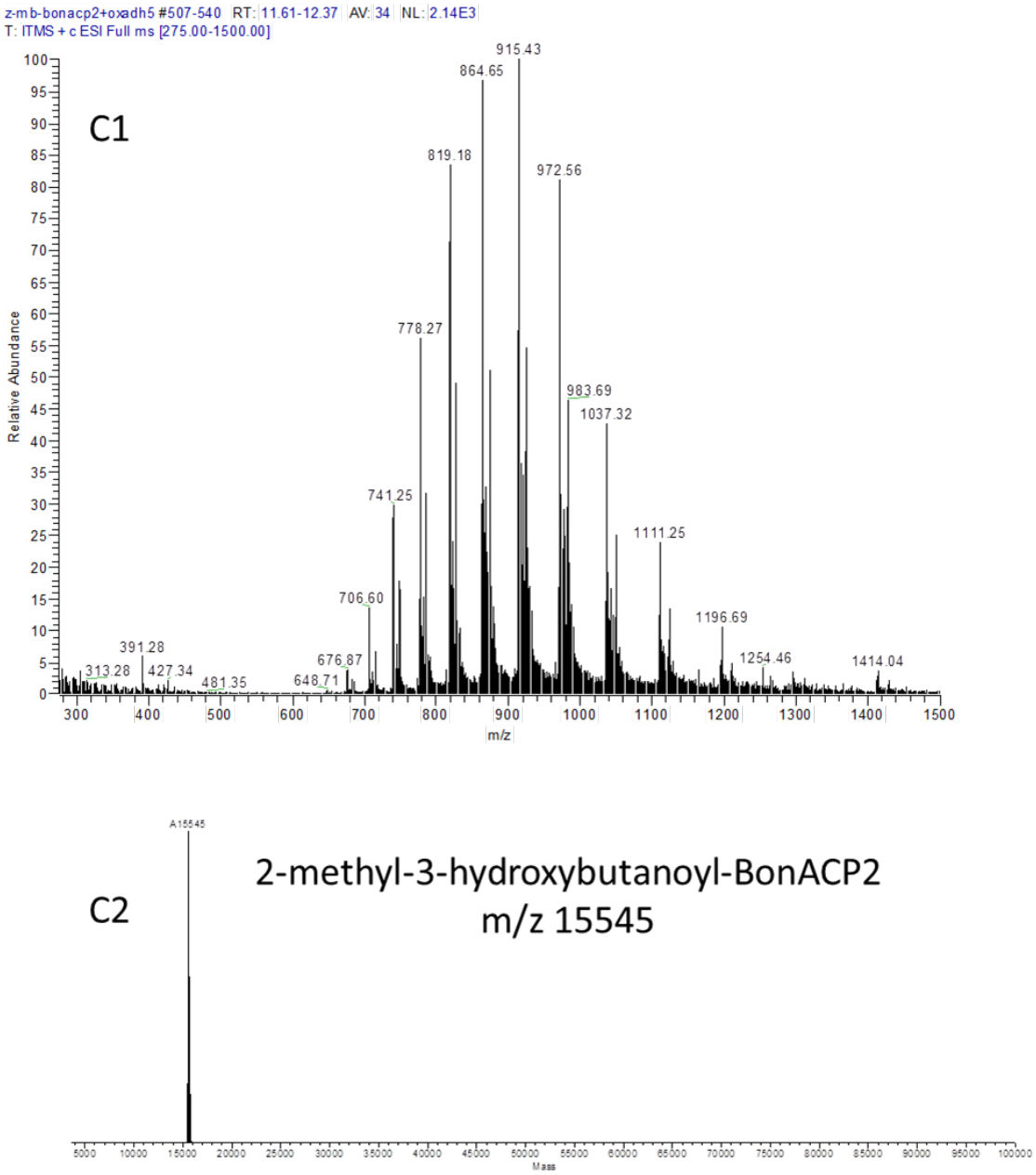


Figure 19 (cont) C. 2-Methyl-3-hydroxybutyryl-BonACP2 (**14**) from incubation of A and OxaDH5.

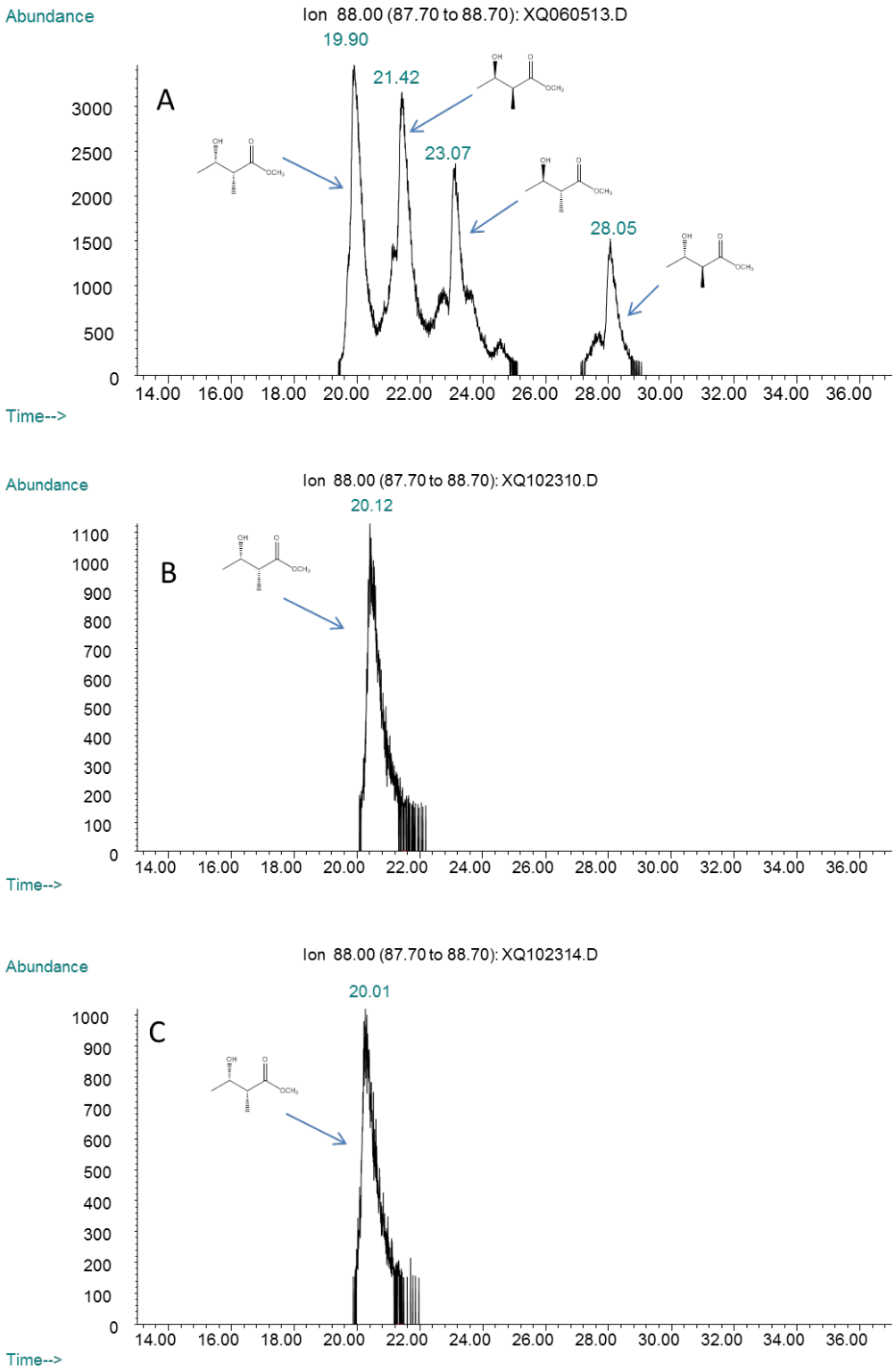


Figure S20. GC-MS analysis [XIC *m*/z 88 (base peak)] of methyl 2-methyl-3-hydroxybutyrates resulting from DH-catalyzed hydration of (*Z*)-2-methyl-2-butenoyl-BonACP2 (**16**). A: Standard. B: **7-Me** derived from (2*R*,3*S*)-**14** produced by incubation of (*Z*)-2-methyl-2-butenoyl-BonACP2 (**16**) with BonDH2; C. **7-Me** derived from (2*R*,3*S*)-**14** produced by incubation of (*Z*)-2-methyl-2-butenoyl-BonACP2 (**16**) with OxaDH5.

Figure S21. Mega3.0 (http://www.megasoftware.net) sequence alignment of PKS DH domains. PKS source: Bae, Bacillaene; Bon, Bongkrekic acid; Dif, Difficidin; Ery, erythromycin; Nan, Nanchangmycin; Oxa, Oxazolomycin; Pic, picromycin; Rif, Rifamycin.

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