

Supporting Information

Enzyme-directed Mutasynthesis: A Combined Experimental and Theoretical Approach to Substrate Recognition of a Polyketide Synthase

Uschi Sundermann^{†,§}, Kenny Bravo-Rodriguez^φ, Stephan Klopries[†], Susanna Kushnir[†], Hansel Gomez^{φ,#}, Elsa Sanchez-Garcia^{φ*}, Frank Schulz^{†,§*}

[†]Fakultät für Chemie, Chemische Biologie, Technische Universität Dortmund, Otto-Hahn-Str. 6, 44221 Dortmund, Germany

[§]Max-Planck-Institut für molekulare Physiologie, Abteilung für Chemische Biologie, Otto-Hahn-Str. 11, 44227 Dortmund, Germany

^φMax-Planck-Institut für Kohlenforschung, Kaiser-Wilhelm-Platz 1, 45470 Mülheim an der Ruhr, Germany

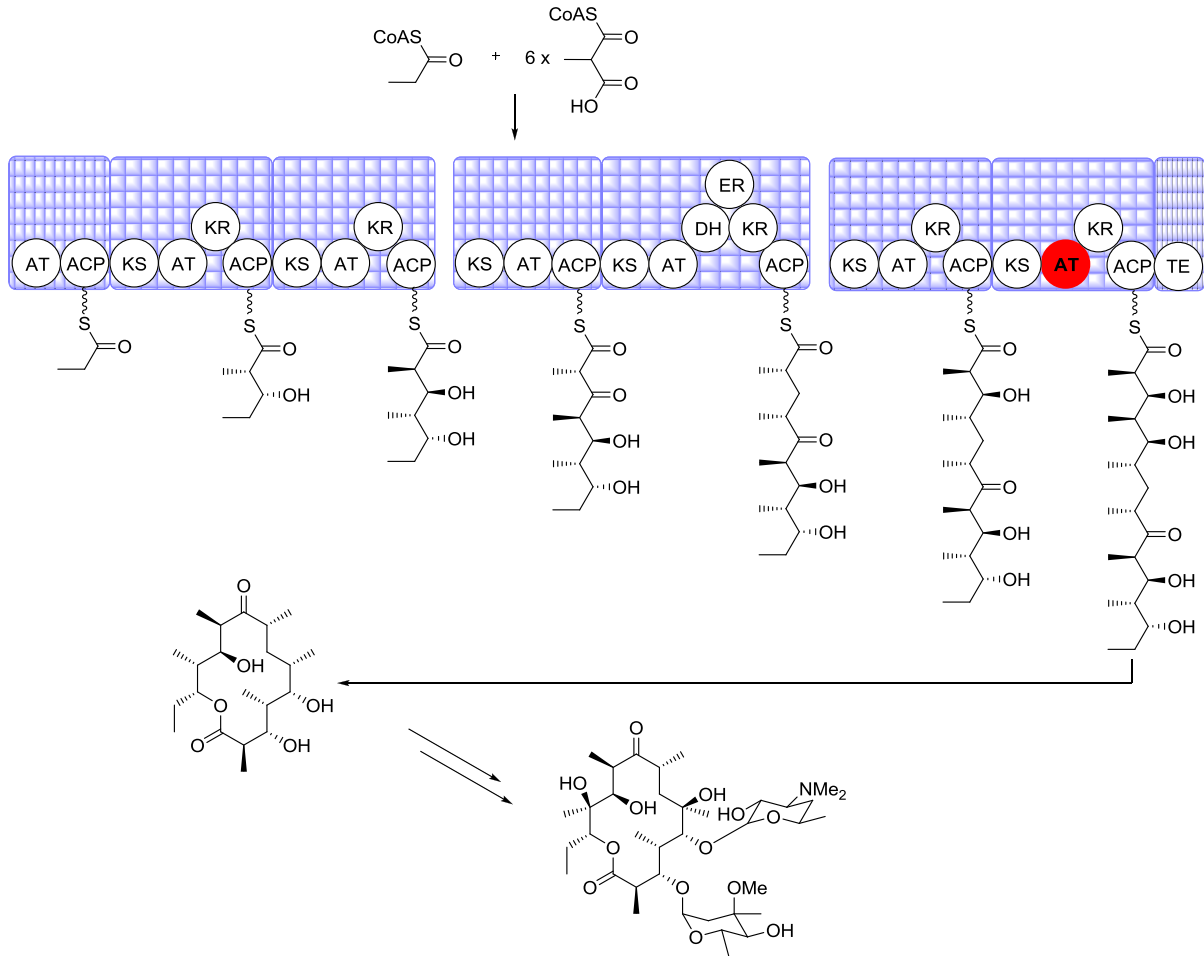
[#]Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès (Bellaterra), Spain

*corresponding authors

Supporting Information

- I. Supporting methods: Mutagenesis and microbiological techniques (pages 3-14)
- II. Supporting methods for fermentation experiments using *Saccharopolyspora erythraea* and characterization of fermentation products (pages 15-22)
- III. Supporting Computational methods (pages 23-24)
- IV. Supporting results: Analysis of the wild-type system (pages 24-29)
- V. Supporting results: Analysis of AT6-variants (pages 30-32)
- VI. Supporting methods: Synthesis of malonic acid derivatives for feeding studies (pages 33-42)
- VII. Supporting references (page 43-44)

Supplementary Figure 1: Overview of the biosynthetic pathway towards erythromycin A. In cascade of Claisen condensations, the polyketide is assembled from propionyl-CoA as starter unit and methylmalonyl-CoA as extender units. The resulting β -keto thioester is in optional steps reduced by further catalytic domains. The primary product 6-Desoxyerythronolide B is cyclized and cleaved from the PKS multienzyme complex before being furnished with hydroxylations and glycosylations to yield erythromycin A. The Acyltransferase domain in module 6 which was investigated in this study is highlighted in red.



I. Mutagenesis and Microbiological Methods

1. Acyltransferase Sequence Alignments

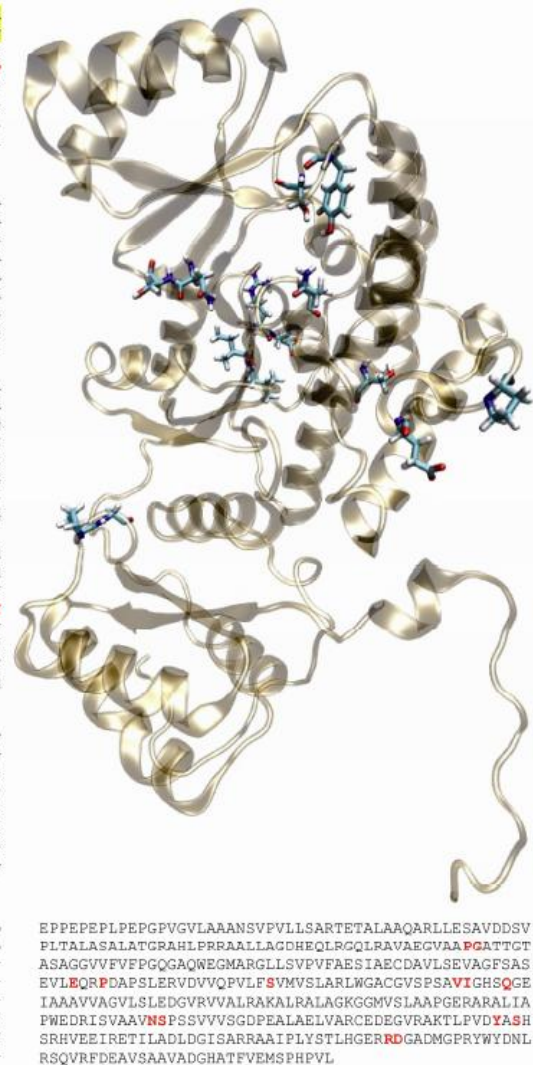
Domain boundaries were identified using the SBSPKS – server (NRPS – PKS Analysis) and extended by approximately 100 AA at the N-terminus and 50 AA at the C-terminus for theoretical analysis.¹ Candidates for critical residues were identified using protein sequence alignments employing AlignX from the VectorNTI Advance 11.0 program package and the T-COFFEE server.²

Supplementary Figure 2: Identification of mutagenesis sites. A CLUSTAL W (1.83) multiple sequence alignment of AT domains from the Epothilon PKS (EpoCAT), the erythromycin PKS (EryAT), the Niddamycin PKS (NidAT), and the Rapamycin PKS (RapaAT) with different substrate specificities reveals a number of potentially influential positions in DEBS AT6, guided by the differences between EpoAT2 and EpoAT3 (highlighted in yellow). AT specificities are: EpoAT2: Malonyl-, EpoAT3: Malonyl- & methylmalonyl-, EryAT: both methylmalonyl-, NidAT4: Methylmalonyl-, NidAT5: Methyl- & ethylmalonyl-, RapaAT2: Methylmalonyl-, RapaAT4: Malonyl-CoA. Genbank numbers: EpoAT: AAF26921.1; Ery AT6: CAM00065.1 Region 2020-2291; Ery AT3: CAM00064.1 Region 562-832; NidAT4: AAC46026.1 Region 560-581; NidAT5: AAC46026.1 Region 2010-2306; RapaAT2: CAA60459.1 Region 2137-2418; RapaAT4: CAA60459.1 Region 5758-6022. Inspection of I-TASSER homology models of DEBS AT6 and the EpoAT2/EpoAT3 domains indicated further candidate residues, required for example by gap regions in the alignments. All candidate sites are highlighted in the model of DEBS AT6 and its sequence shown below. These go beyond the domain boundaries as predicted by the SBSPKS-server. DEBS AT6 sites are highlighted in red.

```

EpoAT2      ---GQGAQTPGMGRGLCAAWPAFREAFDRCVALFDRELDRLPLREVMWARA
EpoAT3      ---GQGAQTPGMGRGLCAAWPAFREAFDRCVALFDRELDRLPLREVMWARA
EryAT3      LFPGQGSQWAGMGAE LLS SPVFAKTRACDESMADPQDWKVS DVL RQAP
EryAT6      -FPGQGAQWEGMARGLLS-VPVFAESIAECDAVLSEVAGFSASEVLEQR
NidAT3      VFPGQGAQWAGMGAE LLDSSPVFAELARCEAALEPFVDWLSLTDVLRGAP
NidAT5      VFPGQGSQWAGMAEGLLERSGAFRSAADSCDAALRPYLWGSVLSVLRGEP
RapaAT2     VFPGQGSQWGLMGSA LRDS SI VFAERMAECAPALREPFVDWDLFTVLD---
RapaAT4     VFPGQSSQRAGMGEELAAAFPVFARIHQQVW-----DLLD-----
          *** * * * *
EpoAT2      GSAESLLLDQTAFTQPALFAVEYALTALWRSWGVPELVGHSTIGELVAA
EpoAT3      GSAESLLLDQTAFTQPALFTEYALTALWRSWGVPELVAGHSAGELVAA
EryAT3      GA---PGLDRVDVVQPVLFAVMVSLAELWRSYGVPEAAVVGHSQGEIAAA
EryAT6      DA---PSLERVDVVQPVLFSVMVSLARLWGCAGCVSPSAVIGHSQGEIAAA
NidAT3      GA---PGLDRVDVVQPVTFAVVVALAAMWRWLGVEPAAVVGHSQGEIAAA
NidAT5      DA---PSLDRVDVVQPVLFTMMVSLAAVWRALGVEPAAVVGHSQGEIAAA
RapaAT2     DP---AVVDVVDVVQPASWRMMSLAAVVQAAGVPRDAVIGHSQGEIAAA
RapaAT4     -VP-DLEVNETGYAQPALFALQVALFGLLESWGVPRDAVVGHSVGLAAG
          ::.. .** : : * : * * : * * * : *
EpoAT2      CVAGVFSLEDGVRVLAARGRLM-QGLSAGGAMVSLGAPAEVAAVAFPH
EpoAT3      CVAGVFSLEDGVRVLAARGRLM-QGLSAGGAMVSLGAPAEVAAVAFPH
EryAT3      HVAGALTLEDAAKLVVGRSRLM-RSLSGEGMRAVALGEEAARERLRFWQ
EryAT6      VVAGVLSLEDGVRVVALRAKAL-RALAGKGMVSLAAPGERARALIAFWE
NidAT3      HVAGVLSLEDAARVVALRSQLIARELAGRGSMAVALAAADVESRLAGAE
NidAT5      HVAGALSLEDDARIVALRSRAW-LGLAGKGMVAVPMPAEELRPLVTVG
RapaAT2     CVAGAVSMRDAARIVTLRSEATARGLAGRGAMASVALPAQDVELV----
RapaAT4     YVSGLWSLEDACTLVSARARLM-QALPAGGVVAVPVSEDEARAVL---G
EpoAT2      ----ASVSIAAVNGPEQVVIAGVEQAVQAIAGFAARGARTKRLH-VSH
EpoAT3      ----ASVSIAAVNGPEQVVIAGVEQAVQAIAGFAARGARTKRLH-VSH
EryAT3      ----DRLSVAAVNGPRSVVVSSEFGALRAFSEDCAAEGIRVRDID-VDY
EryAT6      ----DRISVAAVNSPSSVVVSGDPEALAEVARCEDEGVRAKTLP-VDY
NidAT3      ACCCVRDVEIAAVNCPETTIVCCAPCAVDSLLCVLQCECVRRRID-VDY
NidAT5      ----DRLAFAAVNSPGSCAVAGDPEALAEVALLTGEVHARPIPGVDT
RapaAT2     ----DGAWIAAHNGPASTVIAGTPEAVDHVLTAEHARGVRRIT-VDY
RapaAT4     ----EGVEIAAVNGPSSVVLSSGDEAAVLQAAE---GLG-KWTRIA-TSH
          : ** . * . : . * * : * : : .
EpoAT2      AFHSPLMPEMLEEFGVRAASVTYRRPSVSLVSNLSGKVVYDELSAPGYWV
EpoAT3      AFHSPLMPEMLEEFGVRAASVTYRRPSVSLVSNLSGKVVYDELSAPGYWV
EryAT3      ASHSPQIERVREELLETGDIAPRPARVTFHSTVESRSMDGTELDARYWY
EryAT6      ASHSRHVEEIRETILADLDGISARRAAIPLYSTLHGERDGDAMGPRYWY
NidAT3      ASHSRHVEGIRDELAAVLAGLRPRAGRVPFYSTVEAEPLDGTALDAGYWY
NidAT5      AGHSPQVDALRAHILLEVLAFVAPRPADIPFYSTVTGGLDGTELDARYWY
RapaAT2     ASHTPHVELIRDELDDITSDSSSQAPVVPWLVSTVDGWSVDS-PLDVEYWY
RapaAT4     AFHSARMEPMLEEFRVAEGLTYRTPQVSMMA-----GDQLTTTEYWV
          * * : : : : : : : : : : :
EpoAT2      RHVREAVRFADGVKALHEAGAGTVEVVGPKPTLLGLSPACLPEA--EPTL
EpoAT3      RHVREAVRFADGVKALHEAGACTFVVRGPKPTLIGLIPACIPRA--EPTL
EryAT3      RNLRETVRFADAVTRLAESGYDAFIEVSPH-----
EryAT6      DNLRQVRVFEAVSAAVADGHATFVEMSPHPVL-----
NidAT3      RNLRQVRVFEASALRAMLADGVDAFVECSPHVLTVPVPRQTLLEDAG----
NidAT5      RNMREPVFERATRALIADGHDFLETSHPMLAVALQVTDAGTDAAV----
RapaAT2     RNLREPVGFHPAVGQLQAEQDTVEVVSASPVLLQAMDDV-----VT
RapaAT4     RQVRDTRVRFGEQVASYE---DAVVELGADRSLARLDVGVAMLH-----

```



Supplementary Figure 3: A CLUSTAL W (1.83) multiple sequence alignment of the methylmalonyl-CoA specific AT6 domain of the erythromycin PKS (Ery, from *Saccharopolyspora erythraea*, Genbank: CAM00065.1, region 2020..2291) and AT domains accepting larger malonyl-CoA derivatives using the T-COFFEE server.²

HbmAI: Herbimycin PKS from *Streptomyces hygroscopicus*, Genbank: AAY28225, region 3706..4058, accepts Methoxymalonyl-CoA³

LeuA: Leupyrrin PKS from *Sorangium cellulosum* So ce690, Genbank: ADZ24995, region 1676..1974, accepts 2-carboxyl-5-methylhexanoyl-CoA⁴

Nid: Niddamycin PKS from *Streptomyces caelestis*, Genbank: AAC46026.1, region 2010 – 2306, accepts Ethylmalonyl-CoA⁵

Pks13: from *Mycobacterium avium* subsp. Paratuberculosis, Genbank: AAS02537, region 720..1020, accepts long chain acyl-CoA with a predilection for carboxylated substrates⁶

Sala: Salinosporamide PKS from *Salinispora tropica*, Genbank: ABP73645, region 1071..1365, accepts chlorethylmalonyl-CoA⁷

SamR: Stambomycin PKS from *Streptomyces ambofaciens*, Genbank: CAJ88184, region: 2601..2897, accepts Hexyl/Heptylmalonyl-CoA⁸

```

Ery AT6      -----FPGQGAQWEGMARGLLS-VPVFAESIAECDVLESEVAGFSASEVL
HbmAI AT2    KTVFVFPFGQGAQWAGMGAQLLDTSPVFAIRLHECAKALAPYTDWSLIDVI
LeuA        -----LFGSFGSESVGMGRELYETEPAFREAMDRCADLLAPHLPRRLTDV
Nid AT5      -----VFPGQSQWAGMAEGLLERSGAFRSAADSCDAALRPYLGWSVLSVL
Pks13       -----FGAQHRKMGKNLYLRNEVFAEWIEKVDALIQDERGYSVLELI
Sala        -----SGGGTHWAGMGRALMDWHAGFRASMHECDAVFRELIGWSVIDEL
SamR0477 AT13 -----VLPQGSQWPGMADRLLAESATFRNTLRTCAQALEEHLDWSVEDTTL
                *: . * . * . * : . :

```

```

Ery AT6      EQRPDA-----PSLDRVVDVQPVLFVSMVSLARLWGACGVSPPSAVIGHSQ
HbmAI AT2    TGAPDA-----PSLDRVVDVQPTTFAIMVSLAALWQANGIHPDAVIGHSQ
LeuA        YPARDAAGGAAASLGDLSSYAQPALFALEYCLAELWKSWGITPSPAVVGHS
Nid AT5      RGEFDA-----PSLDRVVDVQPVLFMMVSLAAVWRALGVEPAAVVGHSL
Pks13       LDDSH-----YGI---ETSNVVI FAIQIALGELLRRHHGAKPAAVVGQSL
Sala        SLP AER-----SRLDADIQQPVLFTLQVSLARLWMELGIEPEAFVGHSI
SamR0477 AT13 RGLPGA-----GNMERA EVIQPVLFATMVLAALWREHGVEPEAVVGHSL
                : . : . * : * * * : * : *

```

```

Ery AT6      GEIAAAVAVGLSLEDGVRVVALRAKALR-ALAGKGG-----MVS
HbmAI AT2    GEIAAACVAGHLTLTNAAKIVTLRSQTIAHHLTGHA-----MMS
LeuA        GECVAACVAGVFSLEDAITLVAARGRLME-SLAGEGE-----TFL
Nid AT5      GEIAAAHVAGALSLEDDARSIVALRSRAWL-GLAGKGG-----MVA
Pks13       GEPASAYFSGGLSLADATRVCISRSRLM-----GEGEAMLFGEYIRFMAL
Sala        GEVAAVCVAGGLSVRDAARVTIARSHLIQ-HRAAKAA-----MIA
SamR0477 AT13 GEIAAAHLAGALSLEDAARVVTHRSRLLS-RVVQGA-----VAS
                ** ... : * : : : : * : : : . . .

```

```

Ery AT6      LAAPGERARAL IAPWEDRISVAAVNSPSSVVVSGDPEALAEVVARCEDEG
HbmAI AT2    VLASPTWVQETLAPWHGHLWIAAVNGPASVSVSGDPDALAEFGTTLSKAK
LeuA        VSADEATVRRVIA--SDPVSIGSINGPANIVISGAPAGVKSIVVERLSQEG
Nid AT5      VPMPAEELRPRLV TWGDR LAVAAVNSPGSCAVAGDPEALAEVALLTGEG
Pks13       VEYSADELKT V FADFP-GLEV CVYAAPSQT VIGGPPEQIDAIVARAEAE
Sala        VQAGDEEII PFLAPY GGRVAIAALNSPTSSAVSGPPEEIRALEVALNRAG
SamR0477 AT13 VSLPAQEALARLERWGDALSIAAVNGVSSVSVAGDEAPLDEF LAELETEG
                : : : . . : * : .

```

```

Ery AT6      VRAKTL-PVDYASHSRHVEEIRETILADLDG I SARRAAIPLYSTLHGERR
HbmAI AT2    VYRWQLPGVDFAGHSGHVDTIKQDLHNVLDGITATPGHTAWMSTVDADWA
LeuA        IEVKKLD-VRRAAHSP LMDPML EAFGKVARSI RYARPTIDL VANLTGEVA
Nid AT5      VHARPIPGVDTAGHSQVDALRAHLLLEVLAPVAPRPADIPFYSTVTGGLL
Pks13       RFARKLQ-TKGAGHTSQMDPLLGEFSAELQGIKPMSPVTGIFSTVHGTY
Sala        ISSRAVR-VDRPGHSPGMDPLLSPLREALTNIEPRAFWARFHSTALDGAV
SamR0477 AT13 VRCRKL R- I KGAHSAVVEPLREELAVLAPVVRPRASRIPFYSTVTGGLL
                : . * : : : : : .

```

```

Ery AT6      -----DGADMGPYRWYDNLRSQVRFDEAVSAAV-ADGHATFVEMSPHPVL-
HbmAI AT2    -----NPTHIDPDYWRNLRDTRVFEEATRALL-TQGYRVFIEVSTHPVLT

```

```

LeuA          ----GEEIATPEYWCRQIRETVRMSACLRTLHDALGFEVFLELGPSPALV
Nid AT5      ----DGTELDATYWYRNMREPVEFERATRALI-ADGHDVFLETSPHPMLA
Pks13        IKPGSEPVHDVAYWVKGMRSVYFTHGIRNAV-DSGHTTFLELAPNPVAL
Sala         ----DPVVNADYWAHNLRNQVRFAPTVAAALA-DAGIDTFVEISPHGTLR
SamR0477 AT13 ----DTTELDAEYWYRNMQRQVQFAPATRALL-ADGFGVFVECSPPALA
                **      :*  *  :          *  .:*  ..

```

```

Ery AT6      -----
HbmAI AT2    TA-IQDTTESLPDTPPTITGTLRRDDGGPDRVLTSLAELSAAGIPVHWPT
LeuA         WNGMQCVPKR----SGAWIASLRP-----
Nid AT5      VA-LEQTVTDAG-TDAAVLGTLR-----
Pks13        MQ-IGLTAAAGLHDAQLIPTLAK-----
Sala         GA-IEEITQAQGA-SVVVADSIRR-----
SamR0477 AT13 GA-VQETAEDAGASDPVLLASLR-----

```

```

Ery AT6      -----T
HbmAI AT2    AYAGTTPSQVPLPTYPFQHQHYWL
LeuA         -----G
Nid AT5      -----R
Pks13        -----K
Sala         -----G
SamR0477 AT13 -----R

```

2. Bacterial strains and media

E. coli strain OmniMax (Invitrogen) was used for cloning, and strain ET12567/pUZ8002 was used to drive conjugative transfer of plasmid DNA from *E. coli* to *S. erythraea* and *S. erythraea* Δ AT6hyg^R.

B. subtilis DSM10 was used as test culture for biological testing (zone-of-inhibition).

S. erythraea NRRL-B-24071, *S. erythraea* Δ AT6hyg^R and *S. erythraea* AT6* were used for fermentation.

Cultivation of *E. coli* strains was performed using standard techniques⁹. *S. erythraea* strains were grown on ABB13¹⁰, SY¹¹ or LB plates and cultivated in tryptone soy broth (TSB)¹² and SM3¹³ as fermentation medium. For selection of *E. coli* transformants and *S. erythraea* transconjugants the following antibiotics were used: Apramycin (50 or 25 μ g/ml respectively), Hygromycin (50 μ g/ml) and Phosphomycin (100 μ g/ml).

3. Construction of pKSSU96 for site-directed mutagenesis

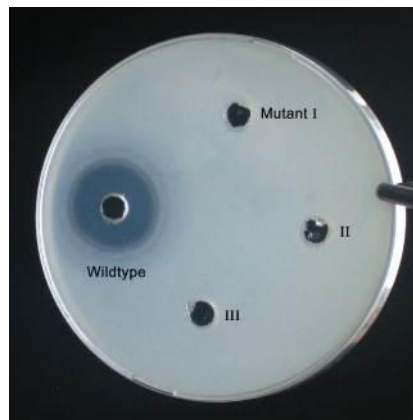
An erythromycin-negative strain for analyzing the impact of mutations was obtained by insertional inactivation via homologous recombination using a temperature-sensitive replicon where the AT6 encoding gene of DEBS3 was replaced by an Δ hyg fragment encoding hygromycin resistance¹⁴. REDIRECT PCR-targeting technology¹⁵ was used for this purpose. The disruption cassette of the AT6 gene was generated by the following strategy: two fragments corresponding to the left and right chromosomal regions flanking AT6+100bp were PCR-amplified from plasmid pIB23 using oligonucleotides 1&2 (fragment A) and 3&4 (fragment B), respectively. The Δ hyg gene was amplified from plasmid pKShyg using oligonucleotides 5&6 (fragment C). PCR was carried out using 2xPhusion PCR Master mix with 50-80 ng of template and betain as an additive in 20-40 μ l reaction volume in a Piko thermocycler (Finnzymes). ScaI endonuclease recognition sites were included into oligonucleotides 7&8 to be used further for mutagenesis of AT6 gene. PCR-product of fragment A was digested by NotI resulting in the A-150bp fragment, PCR-product of fragment B was digested by SalI resulting in the B-100bp fragment. A-150bp, B-100bp and Δ hyg were gel-purified and used in overlap-extension PCR as a template with oligonucleotides 1&3. The generated cassette was introduced into *E. coli* BW25113 (pIJ790) harbouring the plasmid pKSSU89¹⁶ and preinduced for λ Red functions by the addition of arabinose, to obtain a target gene-disrupted version of the plasmid. The resulting recombinant plasmid pKSSU96 (pSET152_lacZ_LIC-Pact-DEBS3-hygScaI-2) was confirmed by restriction analysis and sequencing (oligonucleotides 7&8).

4. Generation of *S. erythraea* Δ AT6hyg^R

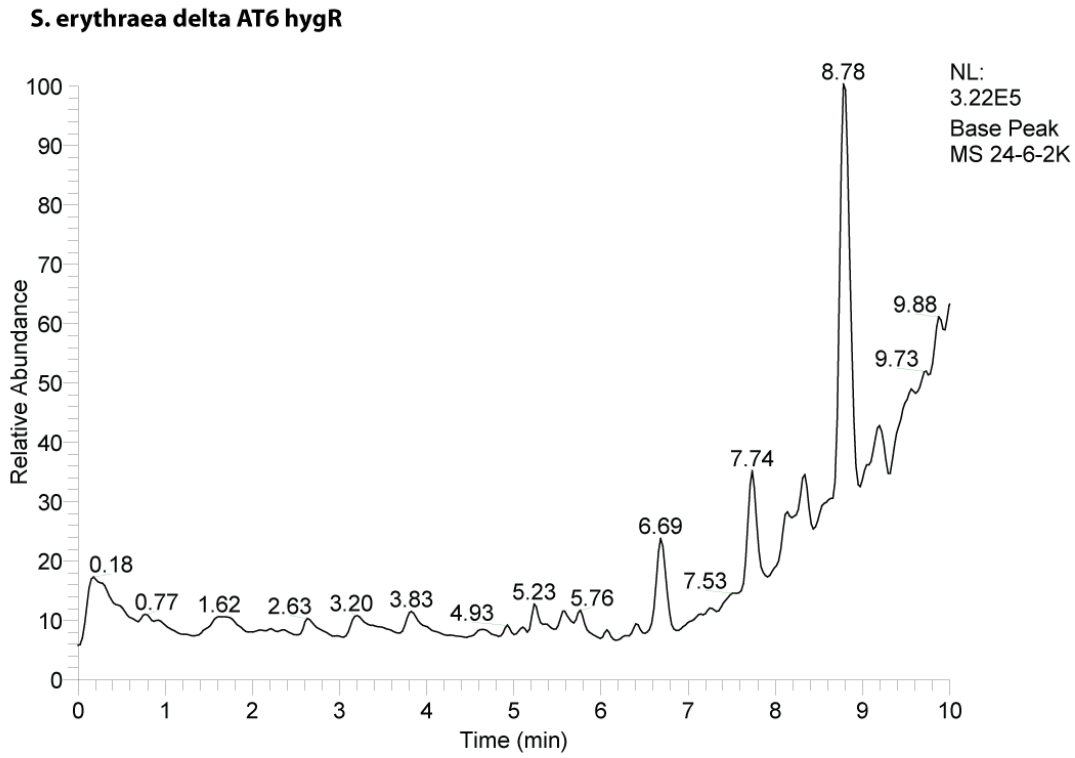
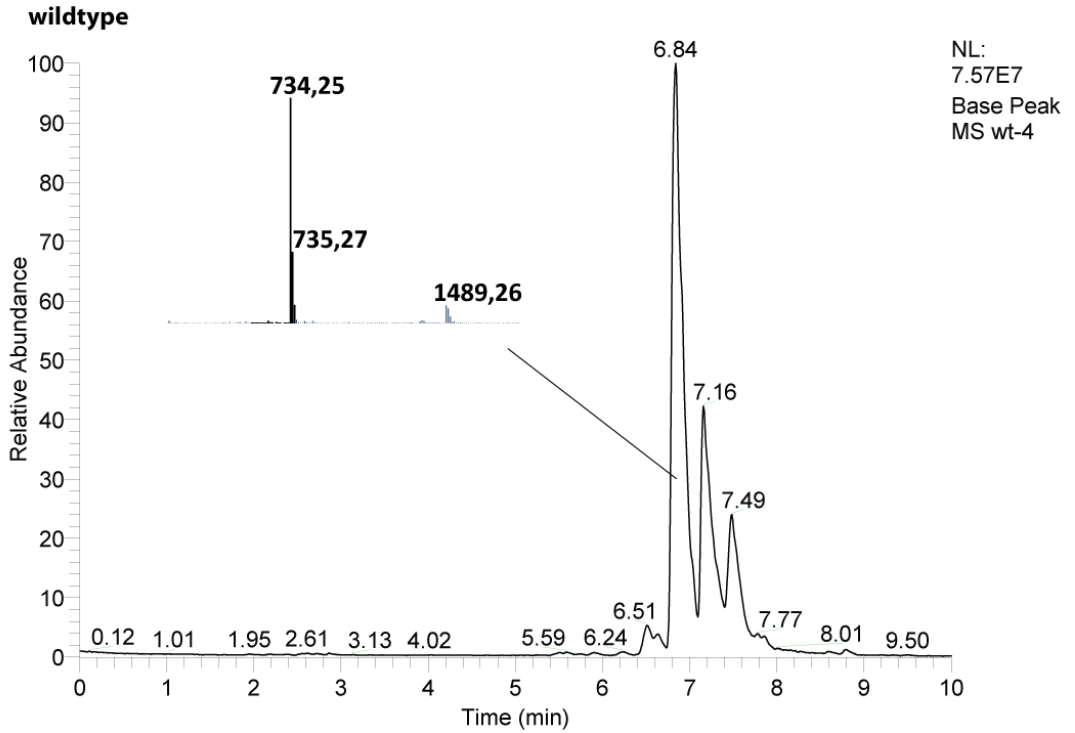
Generation of a Δ AT6 mutant of *S. erythraea* was done by sub-cloning the disruption cassette from pKSSU96 into an XbaI-HindIII-digested pKC1139 (shuttle vector containing an apramycin resistance gene and the temperature-sensitive Streptomyces origin of replication from pSG5)¹² The resulting plasmid pKSSU129 was transformed into *E. coli* ET12567/pUZ8002 and introduced into *S. erythraea* NRRL-B-24071 cells by conjugation. Transconjugants were selected on ABB13 medium overlaid with an Apramycin, Hygromycin and Phosphomycin-containing soft agar. Strains in which a single cross-over between pKSSU129 and the *S. erythraea* chromosome had occurred were selected by cultivation of a

Hyg^RApr^R transconjugant at 40°C (the non-permissive temperature for the pSG5 based replicon) in the presence of the two antibiotics. After 3 rounds of propagation in the absence of antibiotics at 30°C (to allow for the second crossover) and 42°C (to exclude plasmid-backbone from the cells) Hyg^R Apr^S colonies with double crossover were identified by replica-plating of single clones onto on LB-agar plates, containing Hygromycin, with and without Apramycin (50 µg/ml). The loss of erythromycin production in the mutant strain was shown by analysis of fermentation broth via a zone of inhibition assay using *B. subtilis* as test-culture and ESI-MS analysis of fermentation extracts.

Supplementary Figure 4: erythromycin-deletion in *S. erythraea*. Biological testing for erythromycin-negative *S. erythraea* strains using an agar diffusion assay with *B. subtilis* DSM10 as indicator strain. (wild type = *S. erythraea* NRRL-B-24071, Mutant = *S. erythraea* ΔAT6hyg^R)



Supplementary Figure 5: erythromycin-deletion in *S. erythraea*. HPLC/ESI-MS analysis of the crude ethyl acetate extracts of a cultivation of *S.erythraea* in SM3 medium. The chromatogram shows the production of erythromycin by the wild type and the non-functional *S. erythraea* variant lacking the ability to produce erythromycin.

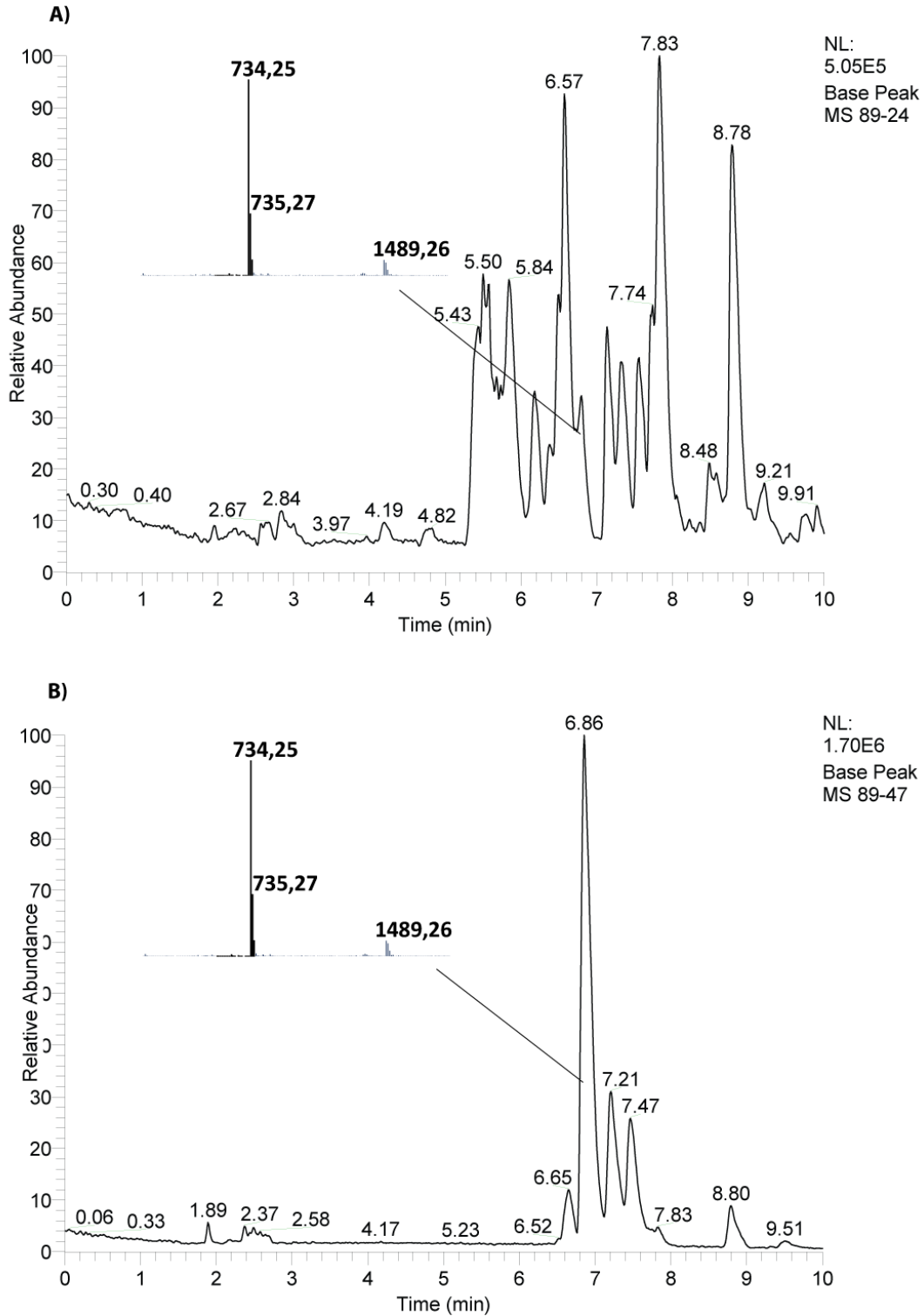


5. Restoration of *S. erythraea* to produce erythromycin

The DEBS3-encoding plasmid pKSSU89 (a non-replicative plasmid based on the integrative vector pSET152 derived from the bacteriophage ϕ C31 which allows high efficiency integration into the chromosome of a recipient actinomycete bacterium into the ϕ C31 integration site and contains the DEBS3-encoding *eryAIII* under control of the strong actI-actIIorf4 promoter) was transformed into *E. coli* ET12567/pUZ8002 and conjugated into *S. erythraea* Δ AT6hyg^R.

To quantify the effect of the mutasis procedure itself onto erythromycin production levels, 60 independent clones of transconjugants were selected and the erythromycin production levels detected and compared to the wildtype (see Supplementary Figure 6).

Supplementary Figure 6: Clone-to-clone variations in *S. erythraea*. LC/MS chromatograms of the ethyl acetate extracts of a cultivation of the restored wildtype (*S. erythraea* Δ AT6hyg^R/pKSSU89) in SM3 medium showing the boundaries of clone to clone variations. A) Low productivity levels at ~10% of the wild type level. B) High productivity level at around 50% of the non-modified wild type.



6. Site-directed mutagenesis of AT6

The alterations of the selected residues were accomplished by oligonucleotide-mediated mutagenesis and overlap-extension PCR. Briefly, mutagenesis was achieved by performing PCR with designed oligonucleotide primers that include the desired mutation in their sequence and flanking oligonucleotides 9 and 10 in 96-well PCR plates in an Eppendorf cycler with the following program: 3 min denaturation at 99°C, 5 cycles of 20 s at 99°C, annealing for 15 s at 60°C and 30 s extension at 72°C, 25 cycles of 15 s 99°C 30 s at 72°C, and a final extension of 240 s at 72°C. The *EcoRV* digested plasmid pKSSU89 was used as template. The PCR products were *DpnI* digested, purified and precipitated using SureClean (Bioline, German) and redissolved in water. The two overlapping fragments were fused together in a subsequent extension reaction. The inclusion of outside primers 9 and 10 in the extension reaction allowed the amplification of the fused product by PCR: 3 min at 99°C, 25 cycles of 20 s at 99°C and 40 s of 72°C, 240 s of 72°C. The final PCR products were gel-purified and cloned into *ScaI* linearized pKSSU96 via SLIC-MIX¹⁶. Insert-containing clones were identified by blue-white screening, colony PCR using oligonucleotides 1 and 2, and analysis of isolated plasmids. Identity of the plasmids was confirmed by DNA sequencing. The DEBS3-encoding plasmids with a single amino acid exchange were transformed into *E. coli* ET12567/pUZ8002 and then conjugated into *S. erythraea* ΔAT6hyg^R.

Supplementary Table 1: Oligonucleotides used in this study.

1	ATCACGGGCGCGGATGTGGCCGTGG
2	ACAGCCGCGCCAGCGACACC
3	TTCTCGACTGCGCTGGTCAGCGACTCC
4	CGGTCCCCGTCTTCGCCGAG
5	GCGGGTCGTCGGGCCCGGTGCTGCTGGGTTCGGTGAAGTACTAAATCCAGATCCTTGACCCGAGTTGCAA ACCCTCACTGATCCG
6	CGCCTTCCAGGTCCACCGGCGTGGTGGCCAGCGGTGCCAGTACTACAAATTCTTCCAAGTATCTGCGCG GATCGATCCTTGCCGAGCTG
7	TGGGTCGTTGCGTGACGG
8	AGGCGACGAACGCCTCGC
9	TTACGGCAAGTCGCGCGGGTTCGTCGGGCCCGGTGCTGCTGGGTTCGGTG
10	AAGCCGCCTTCCAGGTCCACCGGCGTGGTGGCCAGCGGTGCCAGTCCG

Supplementary Table 2: Mutagenic oligonucleotides for targeted mutagenesis of DEBS AT6. XXX denotes the position of point-mutations leading to amino acid exchanges in the target protein. In position of XXX are the codons for the 19 different amino acid residues, taking the codon usage of *S. erythraea* into account (see Supplementary Table 3).

Pro94fwd	TCGCCGAGGGCGTTGCGGCTXXXGGTGCCACCACCGGAAC
Pro94rev	TTCCGGTGGTGGCACCCXXXAGCCGCAACGCCCTCGGCGACCGC
Gly95fwd	AGGGCGTTGCGGCTCCCXXXGCCACCACCGGAACCGCCTC
Gly95rev	AGGCGGTTCCGGTGGTGGCXXXGGGAGCCGCAACGCCCTCGGC
Glu154fwd	TTCTCGGCCTCCXXXGTGCTGGAGCAGCGTCCGGAC
Glu154rev	GTCCGGACGCTGCTCCAGCACXXXGGAGGCCGAGAACCCGGCC
Pro157fwd	AAGTGTGGAGCAGCGTXXXGACGCGCCGTCGCTGGAGC
Pro157rev	GCTCCAGCGACGGCGCTCXXXACGCTGCTCCAGCACTTCGGAG
Ser174fwd	AGCCGGTGTGTTTCXXXGTGATGGTGTGCTGTCGCGCGGC
Ser174rev	AGCCGCGCCAGCGACACCATCACXXXGAACAACACCGGCTG

Val193fwd	AGTCAGCCCCTCGGCCXXXATCGGCCATTCGCAGGGCGAG
Val193rev	TCGCCCTGCGAATGGCCGATXXXGGCCGAGGGGCTGACTCCG
Ile194fwd	AGCCCCTCGGCCGTCXXXGGCCATTCGCAGGGCGAG
Ile194rev	TCGCCCTGCGAATGGCCXXXGACGGCCGAGGGGCTGAC
Gln198fwd	TCGGCCGTCATCGGCCATTCGXXXGGCGAGATCGCCGCCGCGG
Gln198rev	ACCGCGGCGGCGATCTCGCCXXXCGAATGGCCGATGACGGCCG
Asn262fwd	TCCGTCGCGGGCGGTCXXXTCCCCGTCTCGGTCGTG
Asn262rev	ACGACCGAGGACGGGGAXXXGACCGCCGCGACGGAG
Ser263fwd	TCGCGGGCGGTCAACXXXCCGTCTCGGTCGTGGTCTC
Ser263rev	AGACCACGACCGAGGACGGXXXGTTGACCGCCGCGACGGAGA
Tyr297fwd	AAGACGCTCCCGGTGGACXXXGCCTCGACTCCCGCCAC
Tyr297rev	TGGCGGGAGTGCAGGGCXXXTCCACCGGGAGCGTC
Ser299fwd	ACGCTCCCGGTGACTACGCCXXXCACTCCCGCCACGTTCGAGGAG
Ser299rev	ATCTCTCGACGTGGCGGGAGTXXXGGCGTAGTCCACCGGGAGCG
Arg336fwd	ACGCTGCACGGCGAACGGXXXGACGGCGCCGACATGGGTCTC
Arg336rev	ACCCATGTCGGCGCCGTCXXXCCGTTTCGCCGTGCAGCG
Asp337fwd	ACGGCGAACGGCGCXXXGGCGCCGACATGGGTCCG
Asp337rev	ACCCATGTCGGCGCCXXXGCGCCGTTTCGCCGTGCA
Val295Gly_fwd	CGCGCCAAGACGCTCCCGGGCGACTACGCCTCGCACTCC
Val295Gly_rev	GGAGTGCAGGCGTAGTCGCCCGGGAGCGTCTTGCGCGC
Val295Ala_fwd	CGCGCCAAGACGCTCCCGGCCGACTACGCCTCGCACTCC
Val295Ala_rev	GGAGTGCAGGCGTAGTCGGCCGGGAGCGTCTTGCGCGC
Val295Leu_fwd	CGCGCCAAGACGCTCCCGTTGGACTACGCCTCGCACTCC
Val295Leu_rev	GGAGTGCAGGCGTAGTCCAACGGGAGCGTCTTGCGCGC

Supplementary Table 3: Codons for the 20 proteinogenic amino acids used in the mutagenic oligonucleotides.

Amino acid	fwd-codon	rev-codon
Ala	gcc	ggc
Arg	cgc	gcg
Asn	aac	gtt
Asp	gac	gtc
Cys	tgc	gca
Gln	cag	ctg
Glu	gag	ctc
Gly	ggc	gcc
His	cac	gtg
Ile	atc	gat
Leu	ttg	caa
Lys	aag	gtt
Met	atg	cat
Phe	ttc	gaa
Pro	ccg	cgg
Ser	tcg	cga
Thr	acc	ggt
Trp	tgg	cca
Tyr	tac	gta
Val	gtc	gac

II. Fermentation experiments with *Saccharopolyspora erythraea*

1. General procedure

Cultivation of *S. erythraea* was carried out in the 24-well plate system Duetz¹⁷. Pre-cultures were cultivated for 48 h in 2 ml TSB at 30°C/180 rpm in an orbital shaker with 5 cm deflection. The 3 ml main culture in SM3 medium was inoculated with 1/20 pre-culture and cultivated for 5 days at 30°C/180rpm. 4 clones of each mutant were used. The *S. erythraea* wild type and the restored wild type were cultivated as control.

2. Feeding of 2-Propargylmalonyl-SNAC (compound 3) to the DEBS3 Variants

For feeding experiments, the main culture was supplemented with 10 mM 2-propargylmalonyl-SNAC. The *S. erythraea* wild type and the restored wild type were supplemented with the same concentration to serve as controls.

3. Analysis of fermentation products

For characterization via LC-ESI-MS the fermentation broth was extracted with 2 volumes of ethyl acetate overnight at 19°C. The solvent phase was evaporated and the residue re-dissolved in 0.5 ml methanol. 5 µl were used for analysis.

HPLC/ESI-MS: The separations were carried out on an Accela HPLC-System (consisting of pump, autosampler, column oven and PDA detector) coupled online to an Orbitrap mass spectrometer equipped with a LTQ XL linear ion trap (Thermo Electron Corporation, Dreieich, Germany) using the standard electrospray ionization source. Parallel UV detection occurred at 210 and 254nm. All solvents were LC-MS grade (Chromasolv, Sigma-Aldrich, Munich, Germany). 5 µl of each sample were injected via an autosampler (T = 10 °C) onto a CC12514 Nucleor C18 Gravity column (3 µm particle size; Macherey-Nagel Germany) using a flow rate 500 µl/min. A linear gradient starting with 80% Solvent A / 20% Solvent B for one minute and increasing to 0% Solvent A / 100% Solvent B in 10min. After that the column was washed with 0% Solvent A / 100% solvent B for 5min and re-equilibrated to starting conditions for additional 5min (solvent A water containing 0.1% formic acid, solvent B acetonitril containing 0.1% formic acid). For mass spectrometric detection the electrospray ionization was carried out in positive (Erythromycin) ionization mode using a source voltage of 4 kV. The capillary voltage was set to 18V, the capillary temperature to 275°C, and the tube lens voltage to 115V. Spectra were acquired in full scan centroid mode with a mass-to-charge range from 200 to 2000.

Fragmentation for LC-ESI-MS/MS analysis was accomplished via collision induced dissociation (CID) at 35%. The following masses were fragmented:

m/z= 734 (erythromycin, C₃₇H₆₈NO₁₃⁺)

m/z= 720 (2-desmethylethromycin, C₃₆H₆₆NO₁₃⁺)

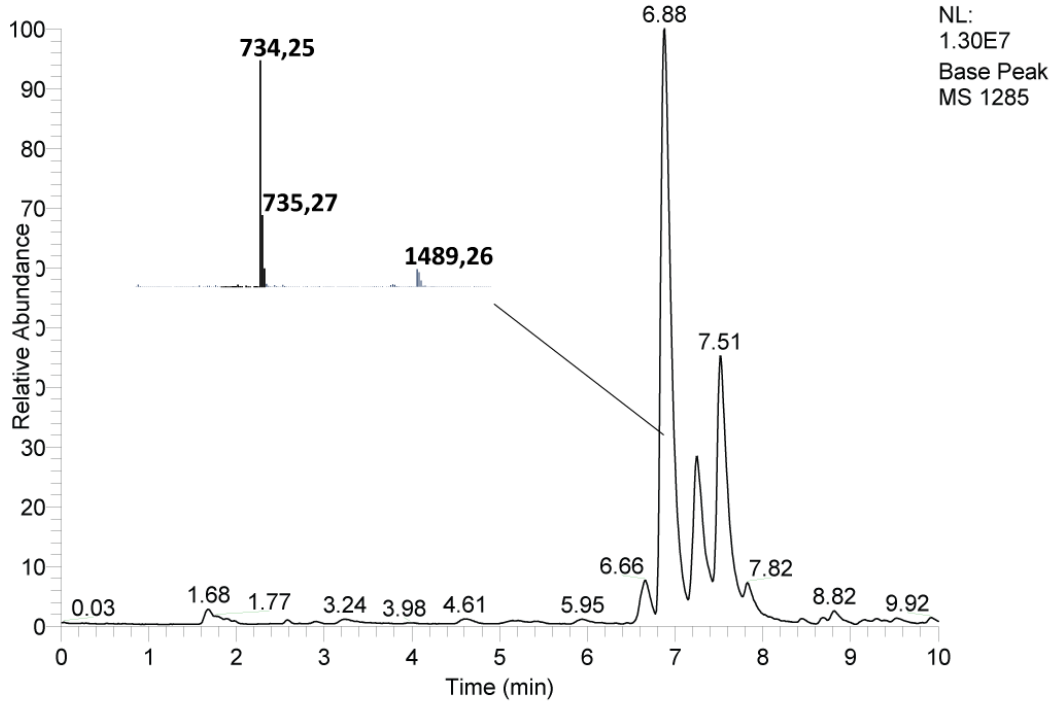
m/z= 758 (2-propargylethromycin, C₃₉H₆₈NO₁₃⁺)

Supplementary Table 4: Residual wild-type activity as judged by LC-MS-detection of erythromycin A in the 266 individual AT6-variants. The averaged activity in % relative to the unmanipulated NRRL-wild-type strain is given. n.d. = not determined

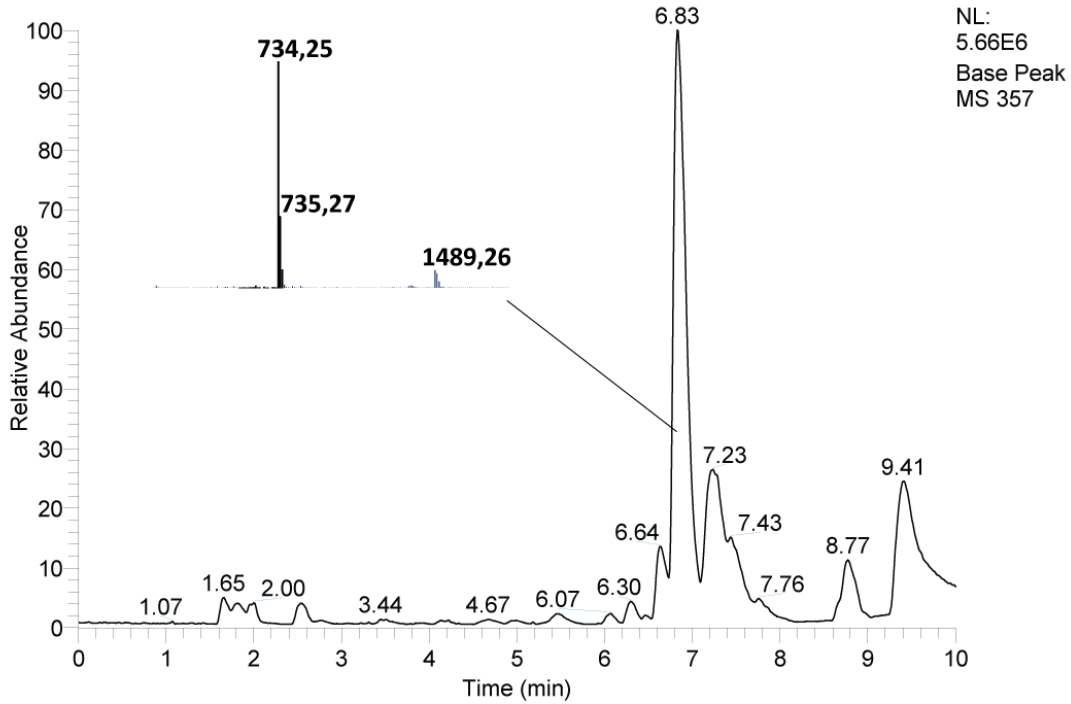
	Pro94	Gly95	Glu154	Pro157	Ser174	Val193	Ile194	Glu198	Asn262	Ser263	Tyr297	Ser299	Arg336	Asp337	
nonpolar / hydrophobic	Ala	28	4	12	12	0	23	1	25	5	0	3	25	45	38
	Val	19	44	50	0	38		2	1	11	47	9	43	12	8
	Met	35	45	21	0	0	11	30	5	7	39	0	0	0	n.d.
	Ileu	45	0	11	5	6	9	8	28	<1	<1	15	0	0	51
	Ile	39	22	1	14	48	20		0	4	22	5	28	49	n.d.
	Pro		0	16		14	36	0.3	28	6	21	8	45	5	16
	Trp	0	22	0	0	31	31	0	1	8	0	48	0	0	20
	Phe	1	8	0	41	25	0	30	0	2	26	16	65	26	3
basic	Lys	12	0	50	46	0	10	0	4	2	33	0	51	3	n.d.
	Arg	<1	0	35	38	17	25	8	24	11	0	1	0		0
	His	6	0	26	9	0	3	19	1	2	36	17	0	0	0
polar / neutral	Tyr	0	0	51	22	20	<1	0	0	3	14		0	38	41
	Thr	<1	46	18	0	33	11	54	10	13	26	2	0	0	0
	Gln	20	41	35	24	25	42	0		0	0	0	13	30	0
	Gly	23		35	43	23	8	0	43	49	6	0	0	24	1
	Ser	0	7	48	50		0	19	25	0		0		0	17
	Cys	2	32	48	0	0	44	9	0	47	40	6	0	25	<1
	Asn	<1	13	6	39	41	24	0	0		27	0	29	20	n.d.
acidic	Glu	17	32		18	44	39	3	0.4	1	24	3	18	33	20
	Asp	23	15	0	15	0	13	24	2	3		1	1	0	

4. LC-ESI-MS analysis of the DEBS3-AT6 variants studied by MD-simulations

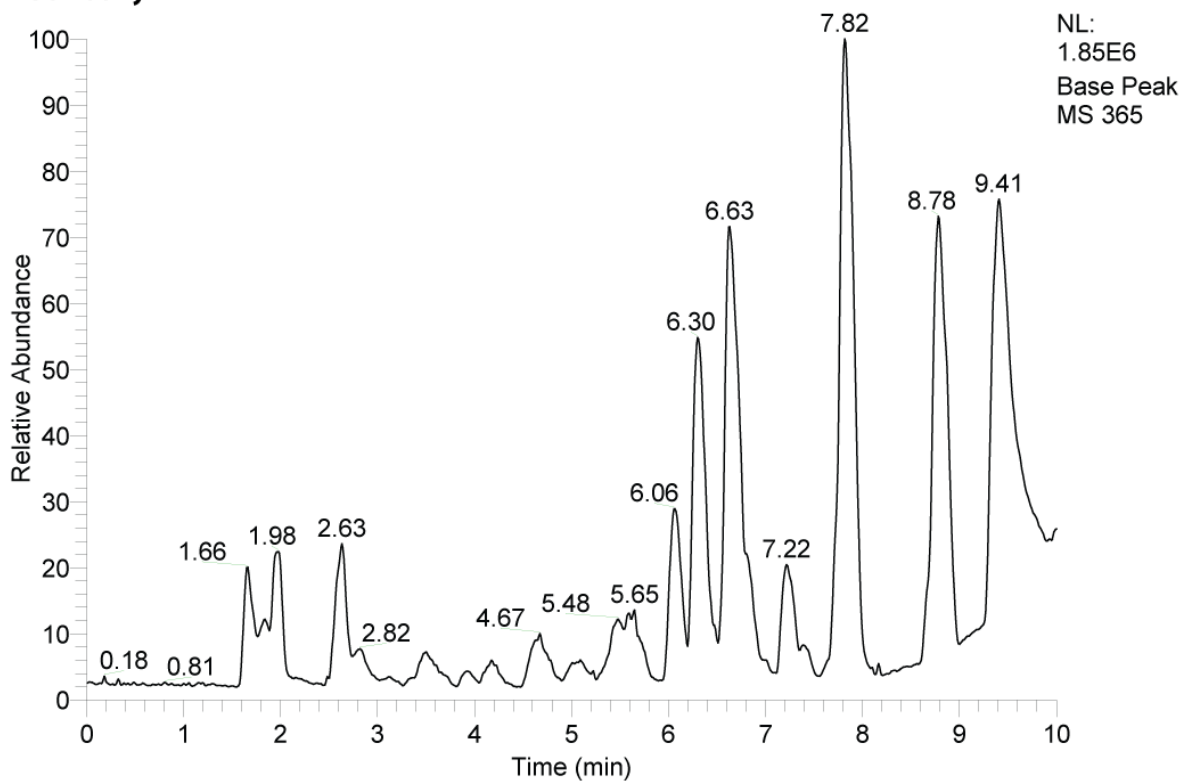
Ser299Val



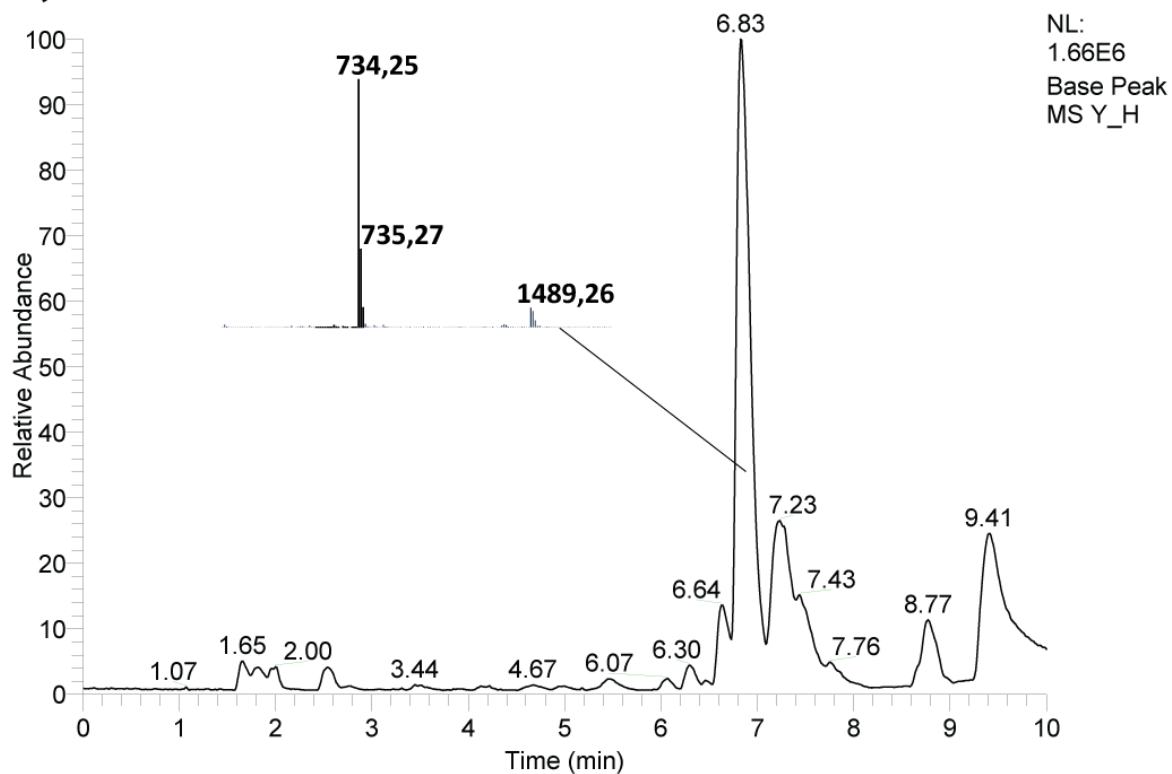
Ser299Phe



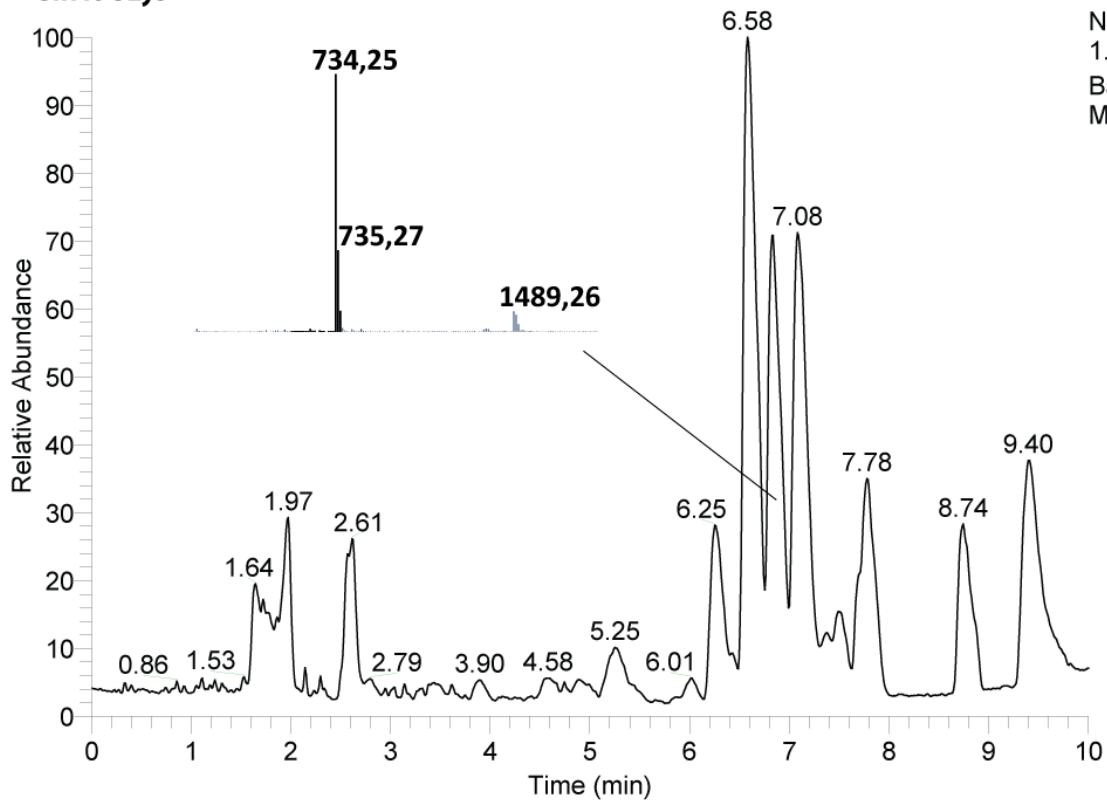
Ser299Tyr



Tyr297His

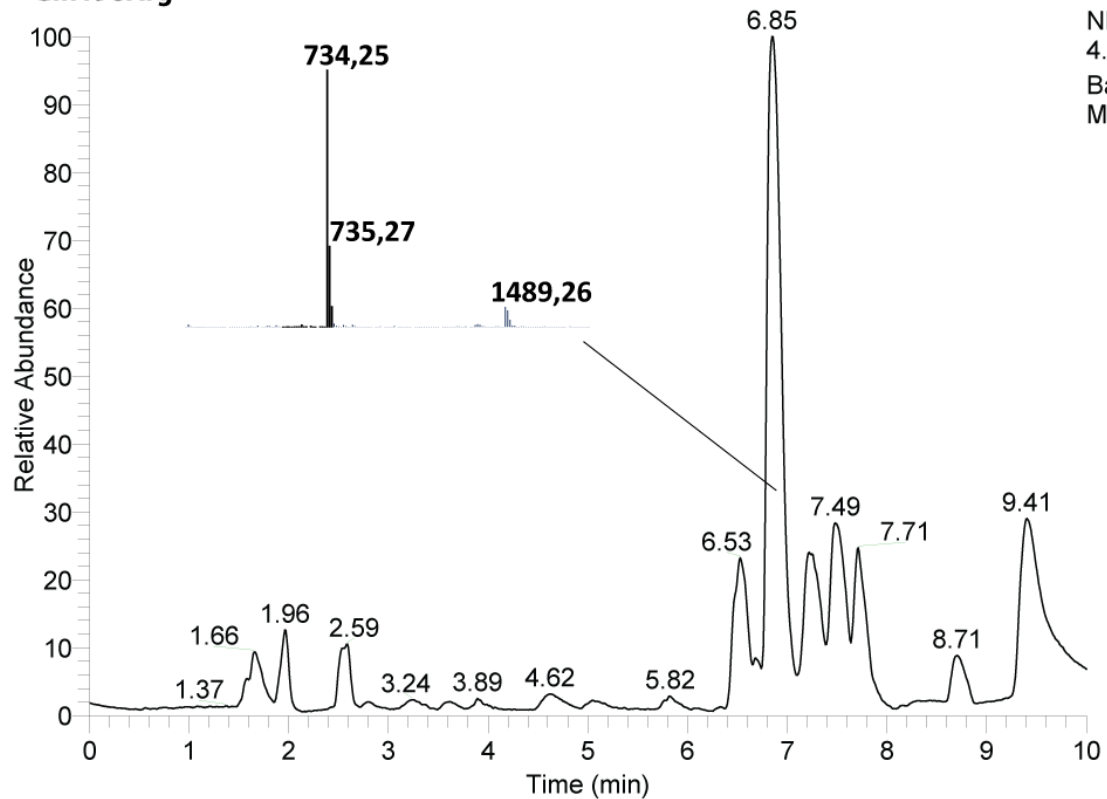


Gln198Lys



NL:
1.68E6
Base Peak
MS 187

Gln198Arg



NL:
4.79E6
Base Peak
MS 241

5. Further Characterization of $m/z = 720$ (2-desmethylethromycin)

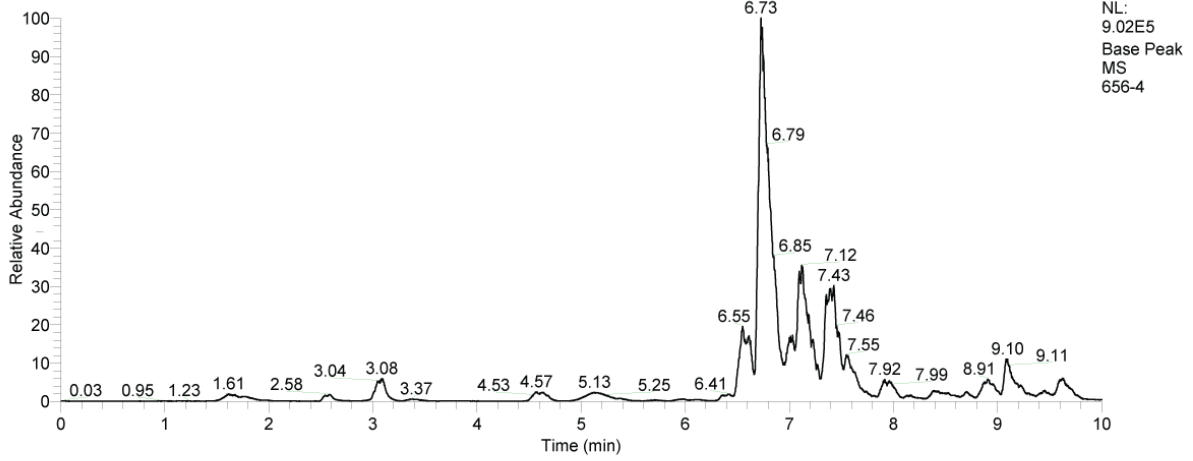
High resolution mass: $[M+H]^+ = 720.45292$, $C_{36}H_{66}O_{13}N = 720.45287$, 0.07341 ppm

Supplementary Table 5: MS/MS-fragmentation of 2-desmethylethromycin.

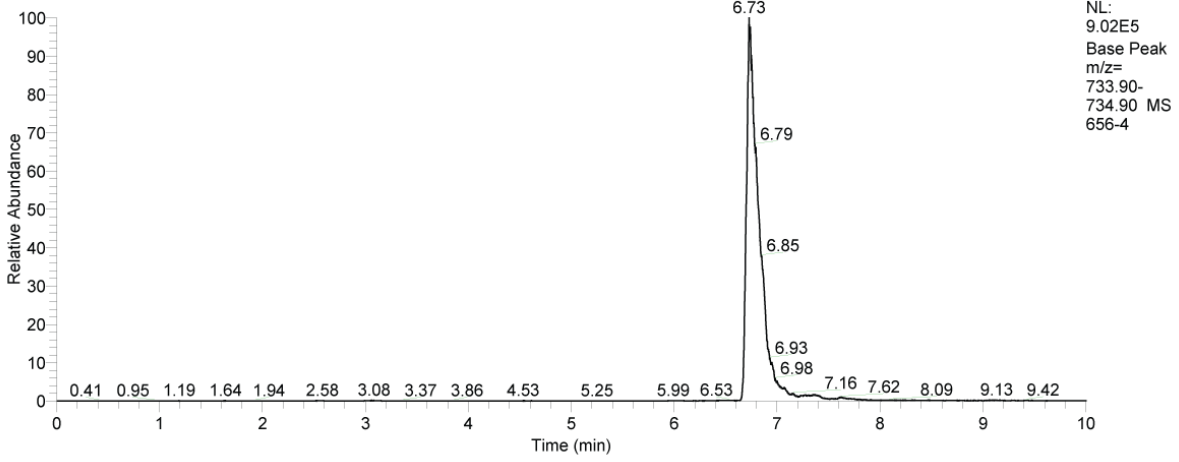
fragments	m/z	m/z
	theoretically	found
$C_{36}H_{66}NO_{13}^+$	720.4529	720.45
$C_{36}H_{64}NO_{12}^+$	702.4423	702.16
$C_{36}H_{62}NO_{11}^+$	684.4317	684.37
$C_{28}H_{52}NO_{10}^+$	562.3586	562.28
$C_{28}H_{50}NO_9^+$	544.3480	544.14
$C_{28}H_{48}NO_8^+$	526.3374	526.11
$C_{28}H_{46}NO_7^+$	508.3269	508.23
$C_{25}H_{40}NO_6^+$	450.2850	450.04

6. Analysis of Feeding-Studies - Val295Ala

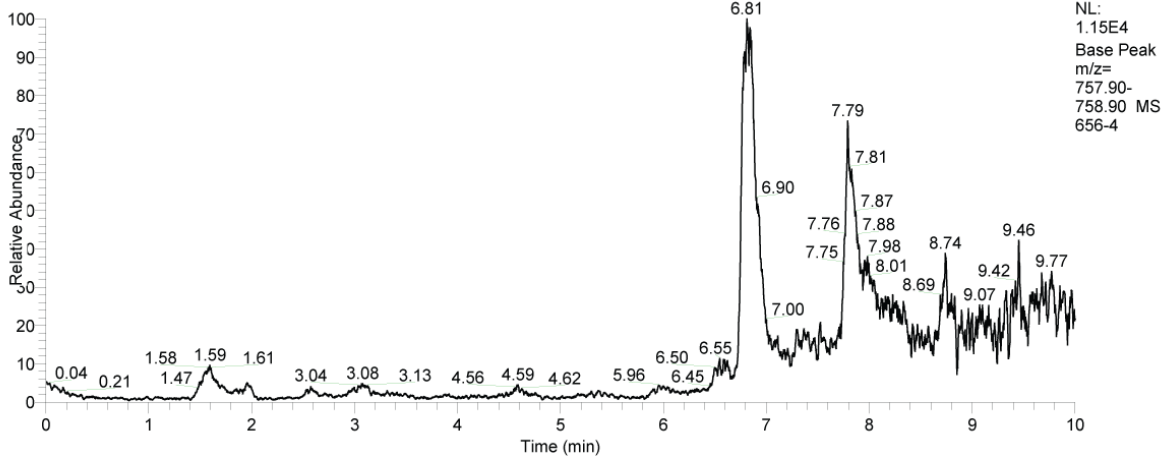
RT: 0.00 - 10.00 SM: 7B



RT: 0.00 - 10.00 SM: 7B



RT: 0.00 - 10.00 SM: 7B



High resolution mass: $[M+H]^+ = 758.46822$, $C_{36}H_{66}O_{13}N = 758.46852$, -0.39578 ppm

Supplementary Table 6: MS/MS fragmentation of the new erythromycin A derivative 2-propargylerythromycin as produced by the AT6 Val295Ala variant upon being presented with 2-propargylmalonyl-SNAC.

fragments	m/z	m/z
	theoretically	found
$C_{39}H_{66}NO_{12}^+$	740,4580	740,48
$C_{39}H_{64}NO_{11}^+$	722,4474	722,34
$C_{31}H_{54}NO_{10}^+$	600,3742	600,44
$C_{31}H_{52}NO_9^+$	582,3637	582,29
$5C_{31}H_{50}NO_8^+$	564,3531	564,32
$C_{31}H_{48}NO_7^+$	546,3425	546,34
$C_{28}H_{42}NO_6^+$	488,3007	488,40

III. Computational Details

Homology modeling of the acyltransferase domain of the sixth module of 6-deoxyerythronolide synthase (DEBS-AT6) was performed using the I-Tasser server.¹⁸ The acyltransferase domains of modules 3 and 5 of DEBS share a high sequence identity with the query sequence (> 40 % of primary structure sequence identity) and therefore were considered as top templates.^{19,20} The resulting DEBS-AT6 model was used as starting point for docking the (2*S*)-methylmalonyl-coenzyme A (MMCoA) substrate into AT6. The docking procedure was performed using Glide²¹ as implemented in the Schrödinger molecular modeling platform.²² Protein coordinates were pre-processed for docking and protonation states of titratable residues were assigned using the Protein Preparation Wizard as provided in the Schrödinger Maestro environment.²³ For the docking calculations the Extra-Precision (XP) rigid docking protocol was used and ten thousand poses were kept in the initial phase of the docking. A maximum of 100 conformations with the best binding energies was retained for the final analysis. The best resulting model was selected based on the predicted free binding energy and the conservation of key interactions that support the specificity of the enzyme.¹⁹

Next, the models for the wild type protein and selected mutants were solvated with a box of water of $80 \times 105 \times 85$ Å dimensions and the system's charge was neutralized using the VMD 1.9 software.²⁴ The solvated systems were subjected to a 100 ps NVT molecular dynamics (MD) simulation with the MMCoA and protein atoms frozen. After that, a 100 ps NPT MD simulation was carried out with only the backbone atoms of the protein fixed. Finally, a 30 ns production MD simulation was performed. All MD simulations were done using the NAMD 2.7 program with the CHARMM 22 force field.^{25,26} The parameters for the MMCoA residue were generated using the Swissparam server²⁷ and compared with the results obtained from the geometry optimization of MMCoA in gas phase (B3LYP/SVP level of theory).^{28,29} The bonds distances and angles obtained with both force field and quantum mechanical methods are in very good agreement and the distribution of the atomic charges shows similar trends in both cases. QM/MM optimizations of three randomly picked MD snapshots were performed with the program ChemShell v3.2^{30,31} which is a modular package that allows the combination of several QM and MM codes. We used Turbomole 5.10³² for the QM calculations and DL_POLY³³ as driver of the CHARMM22 force field. The QM part which includes all atoms of MM-CoA was described using the BP86 density functional with empirical dispersive energy correction (BP86-D2)^{34,35} and the SVP basis set from the Turbomole basis set library. Open valencies at the QM/MM border were saturated using hydrogen link atoms. To avoid overpolarization of the QM region at the boundary, a charge shift scheme was applied.^{36,37} An electrostatic embedding scheme as implemented in Chemshell 3.2 was used.³⁸ The optimization was performed with the HDLC optimizer.³⁹ The active region was formed by the amino acids forming the binding pocket

(Pro111, Gly112, Gln113, Gln169, Ser197, Arg222, Leu226, Met235, Ser237, Asn262, Lys291, Val295, His377, Pro378, Val379, Leu380, Ala383, His398 and Arg399) except the mutated residues 198, 297 and 299. All atoms within the active region were allowed to move in each optimization step. The optimization was finished when the maximum gradient component was below 0.00045 a.u.

IV. Analysis of the wild type system

For a better understanding, we classify the regions within the MMCoA residue as following (see Supplementary Information Table 7):

-Thioester region: includes all atoms from the carboxyl group of MMCoA to its thioester group (discussed in the main text).

-Isopropyl region or middle region: from the aliphatic carbon atom bonded to the sulfur atom of the thioester group to the isopropyl group.

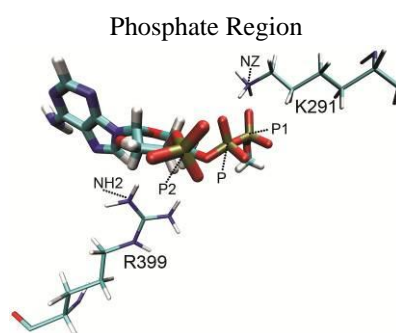
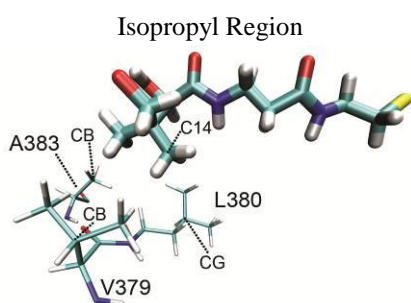
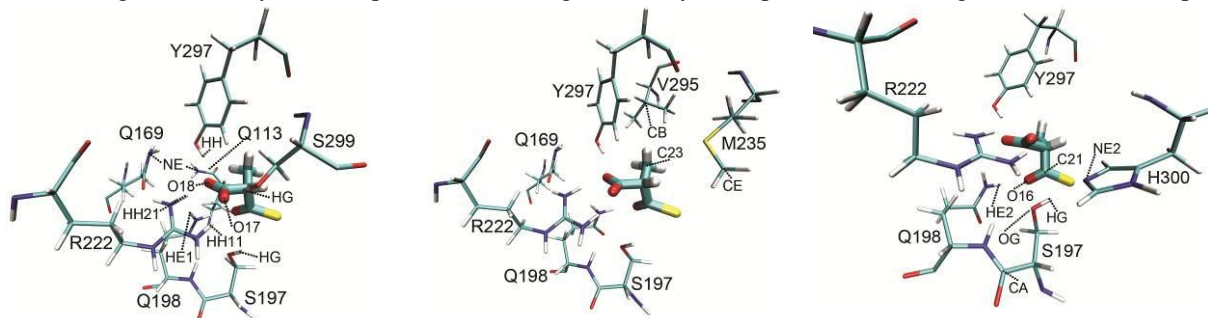
-Phosphate region: from the phosphate groups of MMCoA to the adenine group.

Isopropyl and phosphate regions

Few interactions are conserved between the isopropyl region of MMCoA and the AT6 binding pocket (Supplementary Information, Table S8). This can be related to the fact that coenzyme-A must leave the cavity of AT6 once the nucleophilic attack by Ser197 has taken place, to allow the proper transfer of the methylmalonyl residue to the ACT domain. Asn262, Gln112, His196 and Ser237 create a polar environment around the amide groups of the isopropyl region while the non-polar isopropyl moiety of MMCoA is located in a non-polar region formed by Pro111, Val379, Leu380 and Ala383 (Figure 3c, main text). Although the phosphate region of MMCoA is exposed to the solvent, its conformational flexibility is restricted by the interactions of Lys291 and Arg399 with the phosphate groups.

Supplementary Table 7: Relevant distances (Å) between MMCoA and the amino acids from the binding pocket (QM/MM optimizations and MD simulations). The general agreement between the QM/MM and MD distances corroborates the validity of the results from the MD simulations.

Thioester Region (Carboxylate Group) Thioester Region (Methyl Group) Thioester Region (Thioester Group)



Wild type				
Distance (Å)	MD Values	Snapshot 1	Snapshot 2	Snapshot 3
Thioester Region (Carboxylate Group)				
S299(HG)-O17	1.76 ± 0.14	1.63	1.73	1.61
R222(HH11)-O17	1.87 ± 0.21	2.05	1.69	2.56
R222(HH21)-O18	2.50 ± 0.31	2.53	2.16	2.77
Q198(HE1)-O17	3.68 ± 0.34	3.49	3.46	4.01
Q198(HE1)-O18	3.36 ± 0.34	3.65	2.39	3.33
Q169(NE)-O18	3.37 ± 0.52	3.44	4.22	2.77
Q113(NE)-O18	4.36 ± 0.89	5.47	5.11	3.2
Y297(HH)-O18	1.72 ± 0.14	1.73	1.73	1.85
S197(HG)-O17	N/A	N/A	N/A	N/A
Thioester Region (Methyl Group)				
M235(CE)-C23	4.57 ± 0.54	5.51	4.27	4.25
V295(CB)-C23	4.89 ± 0.44	4.87	4.83	5.28
Thioester Region (Thioester Group)				
Q198(HE2)-O16	1.98 ± 0.23	1.81	1.86	1.79
S197(HG)-O16	3.53 ± 0.57	3.76	3.35	3.26
S197(HG)-H300(NE2)	2.13 ± 0.36	1.92	2.00	1.93
S197(OG)-C21	3.69 ± 0.34	3.66	3.28	3.61
S197(CA)-C21	5.86 ± 0.32	5.8	5.42	5.79
Isopropyl Region				
A383(CB)-C14	5.84 ± 1.16	5.86	6.08	8.65
L380(CG)-C14	5.06 ± 0.41	5.15	4.86	5.93

V379(CB)-C14	4.98 ± 0.39	5.16	5.19	6.13
Phosphate Region				
K291(NZ)-P	4.47 ± 1.01	4.06	3.74	3.91
K291(NZ)-P1	3.96 ± 0.85	3.85	3.64	3.64
R399(NH2)-P2	3.66 ± 0.13	3.70	3.67	3.65

Q198R				
Distance (Å)	MD Values	Snapshot 1	Snapshot 2	Snapshot 3
Thioester Region (Carboxylate Group)				
S299(HG)-O17	1.79 ± 0.15	1.82	1.62	1.86
R222(HH11)-O17	1.95 ± 0.26	2.05	2.04	2.00
R222(HH21)-O18	2.49 ± 0.27	2.60	2.50	2.61
Q198(HE1)-O17	3.87 ± 0.14	3.83	3.79	3.79
	[R198(HH12)-O17]			
Q198(HE1)-O18	1.76 ± 0.12	1.61	1.74	1.69
	[R198(HH12)-O18]			
Q169(NE)-O18	3.63 ± 0.42	3.68	3.95	3.94
Q113(NE)-O18	N/A	N/A	N/A	N/A
Y297(HH)-O18	1.78 ± 0.24	1.70	1.71	1.62
S197(HG)-O17	N/A	N/A	N/A	N/A
Thioester Region (Methyl Group)				
M235(CE)-C23	4.94 ± 0.57	5.19	4.96	5.11
V295(CB)-C23	4.87 ± 0.34	4.36	5.41	4.31
Thioester Region (Thioester Group)				
Q198(HE2)-O16	3.86 ± 0.30	3.46	3.55	3.33
	[R198(HH22)-O16]			
S197(HG)-O16	3.70 ± 0.43	3.86	3.56	3.85
S197(HG)-H300(NE2)	1.98 ± 0.19	1.88	1.88	1.86
S197(OG)-C21	3.89 ± 0.30	3.8	3.36	3.43
S197(CA)-C21	5.90 ± 0.30	6.20	5.75	5.78
Isopropyl Region				
A383(CB)-C14	7.55 ± 1.04	8.61	8.56	8.02
L380(CG)-C14	5.13 ± 0.87	4.70	4.75	5.11
V379(CB)-C14	4.89 ± 0.57	4.96	5.14	5.36
Phosphate Region				
K291(NZ)-P	4.00 ± 0.67	3.94	4.00	3.62
K291(NZ)-P1	4.28 ± 1.01	3.63	3.92	3.57
R399(NH2)-P2	3.72 ± 0.27	3.70	3.72	3.80

Q198K				
Distance (Å)	MD Values	Snapshot 1	Snapshot 2	Snapshot 3
Thioester Region (Carboxylate Group)				
S299(HG)-O17	1.75 ± 0.14	1.81	1.72	1.60
R222(HH11)-O17	1.87 ± 0.22	1.94	1.75	1.75
R222(HH21)-O18	2.40 ± 0.31	2.79	2.04	1.95
Q198(HE1)-O17	3.62 ± 0.17	4.79	4.75	3.62
	[K198(HZ1)-O17]			
Q198(HE1)-O18	1.75 ± 0.12	2.63	2.57	2.69
	[K198(HZ1)-O18]			
Q169(NE)-O18	4.14 ± 0.60	3.93	5.06	4.69
Q113(NE)-O18	N/A	N/A	N/A	N/A
Y297(HH)-O18	N/A	N/A	N/A	N/A

S197(HG)-O17	N/A	N/A	N/A	N/A
Thioester Region (Methyl Group)				
M235(CE)-C23	5.11 ± 0.56	4.50	4.67	5.10
V295(CB)-C23	5.18 ± 0.47	6.99	4.66	4.84
Thioester Region (Thioester Group)				
Q198(HE2)-O16	2.11 ± 0.31	4.62	3.00	2.87
	[K198(HZ1)-O16]			
S197(HG)-O16	3.77 ± 0.63	2.80	4.22	5.09
S197(HG)-H300(NE2)	2.17 ± 0.57	2.18	1.88	1.88
S197(OG)-C21	3.87 ± 0.38	4.05	3.89	4.55
S197(CA)-C21	6.12 ± 0.36	5.93	6.30	6.89
Isopropyl Region				
A383(CB)-C14	6.59 ± 0.67	6.28	6.85	6.01
L380(CG)-C14	5.59 ± 0.49	6.05	6.17	5.52
V379(CB)-C14	4.52 ± 0.40	4.45	4.55	5.22
Phosphate Region				
K291(NZ)-P	3.86 ± 0.18	3.98	3.57	3.71
K291(NZ)-P1	3.79 ± 0.17	3.93	3.61	3.66
R399(NH2)-P2	3.62 ± 0.12	3.80	3.57	3.62

Y297H				
Distance (Å)	MD Values	Snapshot 1	Snapshot 2	Snapshot 3
Thioester Region (Carboxylate Group)				
S299(HG)-O17	1.76 ± 0.14	1.87	1.51	1.86
R222(HH11)-O17	1.79 ± 0.18	1.91	1.79	2.34
R222(HH21)-O18	2.24 ± 0.34	2.52	2.48	3.04
Q198(HE1)-O17	3.88 ± 0.40	3.69	3.96	3.61
Q198(HE1)-O18	2.95 ± 0.37	2.81	3.07	3.20
Q169(NE)-O18	3.45 ± 0.55	2.77	2.85	3.36
Q113(NE)-O18	N/A	N/A	N/A	N/A
Y297(HH)-O18	3.66 ± 0.36	2.95	3.68	3.87
	[H297HE2-O18]			
S197(HG)-O17	N/A	N/A	N/A	N/A
Thioester Region (Methyl Group)				
M235(CE)-C23	5.11 ± 0.64	5.25	4.55	5.01
V295(CB)-C23	4.86 ± 0.39	4.92	5.80	6.01
Thioester Region (Thioester Group)				
Q198(HE2)-O16	2.01 ± 0.22	1.85	1.66	2.20
S197(HG)-O16	3.74 ± 0.69	2.56	2.99	3.48
S197(HG)-H300(NE2)	2.06 ± 0.24	2.16	2.08	2.11
S197(OG)-C21	4.34 ± 0.44	4.29	3.95	4.54
S197(CA)-C21	6.48 ± 0.36	6.25	5.92	6.49
Isopropyl Region				
A383(CB)-C14	6.87 ± 1.32	6.52	3.94	6.00
L380(CG)-C14	5.53 ± 0.89	6.62	6.79	5.78
V379(CB)-C14	5.09 ± 0.62	4.76	4.55	4.91
Phosphate Region				
K291(NZ)-P	4.12 ± 0.63	3.75	7.38	3.71
K291(NZ)-P1	3.86 ± 0.47	3.72	6.08	3.85
R399(NH2)-P2	3.63 ± 0.13	3.64	3.60	3.61

S299V				
Distance (Å)	MD Values	Snapshot 1	Snapshot 2	Snapshot 3
Thioester Region (Carboxylate Group)				
S299(HG)-O17	N/A [V299-O17]	N/A	N/A	N/A
R222(HH11)-O17	1.72 ± 0.11	1.63	1.64	1.75
R222(HH21)-O18	1.71 ± 0.10	1.63	1.72	1.68
Q198(HE1)-O17	3.45 ± 0.25	3.28	3.33	3.36
Q198(HE1)-O18	2.02 ± 0.23	1.76	1.98	1.65
Q169(NE)-O18	4.44 ± 0.45	4.31	4.22	4.28
Q113(NE)-O18	N/A	5.45	N/A	N/A
Y297(HH)-O18	4.70 ± 0.62	4.19	4.27	4.28
S197(HG)-O17	1.81 ± 0.36	1.73	1.86	1.70
Thioester Region (Methyl Group)				
M235(CE)-C23	4.84 ± 0.71	4.25	4.26	4.32
V295(CB)-C23	6.31 ± 0.65	6.01	6.31	5.90
Thioester Region (Thioester Group)				
Q198(HE2)-O16	2.98 ± 0.34	2.92	2.75	2.75
S197(HG)-O16	3.44 ± 0.32	3.58	2.84	3.42
S197(HG)-H300(NE2)	4.00 ± 0.34	4.14	3.94	3.88
S197(OG)-C21	3.64 ± 0.25	3.62	3.56	3.67
S197(CA)-C21	6.00 ± 0.28	6.11	6.05	6.12
Isopropyl Region				
A383(CB)-C14	7.98 ± 1.33	7.54	10.60	10.42
L380(CG)-C14	6.49 ± 1.25	7.17	8.50	8.97
V379(CB)-C14	4.67 ± 0.60	4.63	4.95	5.12
Phosphate Region				
K291(NZ)-P	3.85 ± 0.24	3.84	3.67	3.83
K291(NZ)-P1	3.80 ± 0.27	3.51	3.78	3.86
R399(NH2)-P2	3.69 ± 0.14	3.66	3.60	3.61

S299F					
Distance (Å)	MD Values	Snapshot 1	Snapshot 2	Snapshot 3	MD Values (MCoA)
Thioester Region (Carboxylate Group)					
S299(HG)-O17	N/A [F299-O17]	N/A	N/A	N/A	N/A
R222(HH11)-O17	1.69 ± 0.13	1.60	1.69	1.67	1.99 ± 0.64
R222(HH21)-O18	1.76 ± 0.18	1.71	1.62	1.90	1.87 ± 0.25
Q198(HE1)-O17	N/A	3.25	3.13	N/A	N/A
Q198(HE1)-O18	N/A	2.30	1.90	N/A	N/A
Q169(NE)-O18	4.77 ± 0.86	3.76	5.70	5.50	3.52 ± 0.54
Q113(NE)-O18	5.70 ± 0.92	6.12	6.08	5.65	N/A
Y297(HH)-O18	1.76 ± 0.14	3.71	3.78	1.76	1.80 ± 0.51
S197(HG)-O17	1.69 ± 0.14	1.56	1.60	1.64	1.70 ± 0.20
Thioester Region (Methyl Group)					
M235(CE)-C23	4.15 ± 0.48	4.21	4.13	3.70	N/A
V295(CB)-C23	6.12 ± 0.94	7.74	7.07	5.54	N/A
Thioester Region (Thioester Group)					
Q198(HE2)-O16	N/A	2.52	2.68	N/A	N/A
S197(HG)-O16	3.36 ± 0.39	3.82	3.51	2.67	3.28 ± 0.50
S197(HG)-H300(NE2)	N/A	N/A	N/A	N/A	N/A
S197(OG)-C21	3.76 ± 0.27	4.14	3.74	3.32	3.68 ± 0.32

S197(CA)-C21	6.08 ± 0.31	6.49	5.87	5.81	6.07 ± 0.29
Isopropyl Region					
A383(CB)-C14	7.81 ± 2.08	8.01	8.05	7.71	7.48 ± 0.59
L380(CG)-C14	6.60 ± 1.76	4.72	4.82	10.07	7.06 ± 0.47
V379(CB)-C14	4.32 ± 0.58	3.91	4.31	6.06	4.12 ± 0.28
Phosphate Region					
K291(NZ)-P	3.80 ± 0.22	3.71	3.70	4.05	3.85 ± 0.19
K291(NZ)-P1	4.95 ± 0.63	5.07	5.45	5.88	3.79 ± 0.20
R399(NH2)-P2	3.69 ± 0.12	3.74	3.73	3.72	3.74 ± 0.38

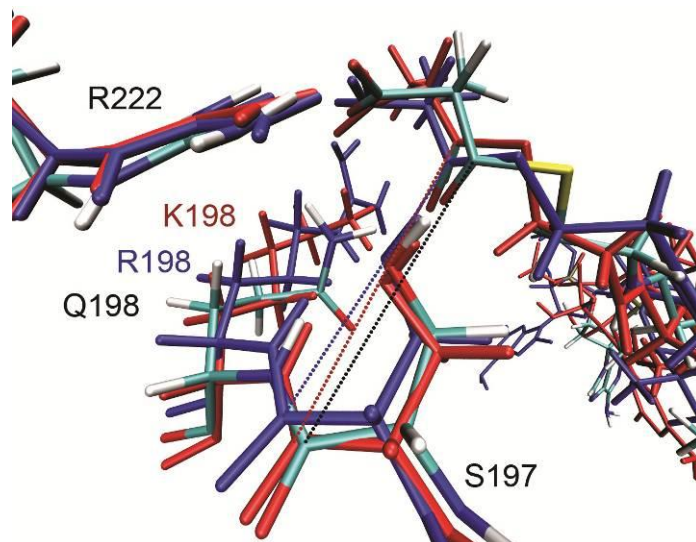
S299Y				
Distance (Å)	MD Values	Snapshot 1	Snapshot 2	Snapshot 3
Thioester Region (Carboxylate Group)				
S299(HG)-O17	N/A [Y299-O17]	N/A	N/A	N/A
R222(HH11)-O17	2.14 ± 0.50	2.59	1.63	2.44
R222(HH21)-O18	3.76 ± 0.40	3.92	4.45	3.76
Q198(HE1)-O17	2.99 ± 0.30	2.80	2.93	2.55
Q198(HE1)-O18	3.87 ± 0.32	4.16	4.46	4.12
Q169(NE)-O18	5.95 ± 1.07	7.17	7.1	8.09
Q113(NE)-O18	5.14 ± 1.31	5.39	4.78	5.20
Y297(HH)-O18	2.55 ± 1.30	N/A	3.17	4.1
S197(HG)-O17	N/A	N/A	N/A	N/A
Thioester Region (Methyl Group)				
M235(CE)-C23	3.91 ± 0.27	3.84	4.22	3.67
V295(CB)-C23	7.79 ± 0.72	6.48	9.16	7.28
Thioester Region (Thioester Group)				
Q198(HE2)-O16	2.02 ± 0.30	1.88	1.90	1.74
S197(HG)-O16	4.16 ± 0.69	4.56	5.23	4.42
S197(HG)-H300(NE2)	2.26 ± 0.52	2.22	1.86	2.36
S197(OG)-C21	4.05 ± 0.44	4.29	4.77	4.38
S197(CA)-C21	5.86 ± 0.39	5.98	5.75	5.95
Isopropyl Region				
A383(CB)-C14	7.78 ± 0.83	7.43	7.03	8.18
L380(CG)-C14	6.87 ± 0.61	7.26	7.35	7.34
V379(CB)-C14	4.05 ± 0.30	4.25	5.47	3.79
Phosphate Region				
K291(NZ)-P	4.17 ± 0.97	3.87	4.99	4.86
K291(NZ)-P1	4.05 ± 0.88	3.82	4.06	3.95
R399(NH2)-P2	3.68 ± 0.16	3.70	3.64	3.76

V. Analysis of AT6-variants

1. Glutamine 198

Mutation of Gln198 for arginine leads to a mutant (Q198R) that retains biological activity. Mutation of the same residue for lysine results in a mutant (Q198K) with no biological activity. Our MD simulations show that in both cases the interaction patterns between the binding pocket and MMCoA are similar to the wild type, except for three interactions involving the thioester region of MMCoA (Supplementary Information Table 7). We discussed previously that Gln198 is placed below the plane defined by the carboxylate group of MMCoA. In the case of the substitution by arginine the large rigid lateral chain of this amino acid makes this arrangement impossible and a side by side interaction is preferred (Supplementary Information Figure 7). The more flexible lateral chain of lysine allows this residue to be accommodated below the plane defined by the carboxylate group of MMCoA. This behavior is reflected by the HE2₁₉₈•••O16 distance (1.98 ± 0.23 Å for Gln, 3.86 ± 0.30 Å for Arg and 2.11 ± 0.31 Å for Lys) that takes similar values in the wild type and in Q198K but it is larger for Q198R. Also the interactions with the carboxylate group of MM-CoA are affected: for the wild type the distance HE2₁₉₈•••O18 is 3.36 ± 0.34 Å but for the mutants the values are 1.76 ± 0.12 Å for Q198R and 1.75 ± 0.12 Å for Q198K. Moreover, the CA₁₉₇•••C21_{MM-CoA} distance (Figure 3B) seems to be sensitive to the mutation of Gln198 by lysine which can be related to the loss of biological activity in Q198K (CA₁₉₇•••C21_{MM-CoA} distances for the wild type, Q198R and Q198K: 5.86 ± 0.32 , 5.90 ± 0.30 and 6.12 ± 0.36 Å respectively).

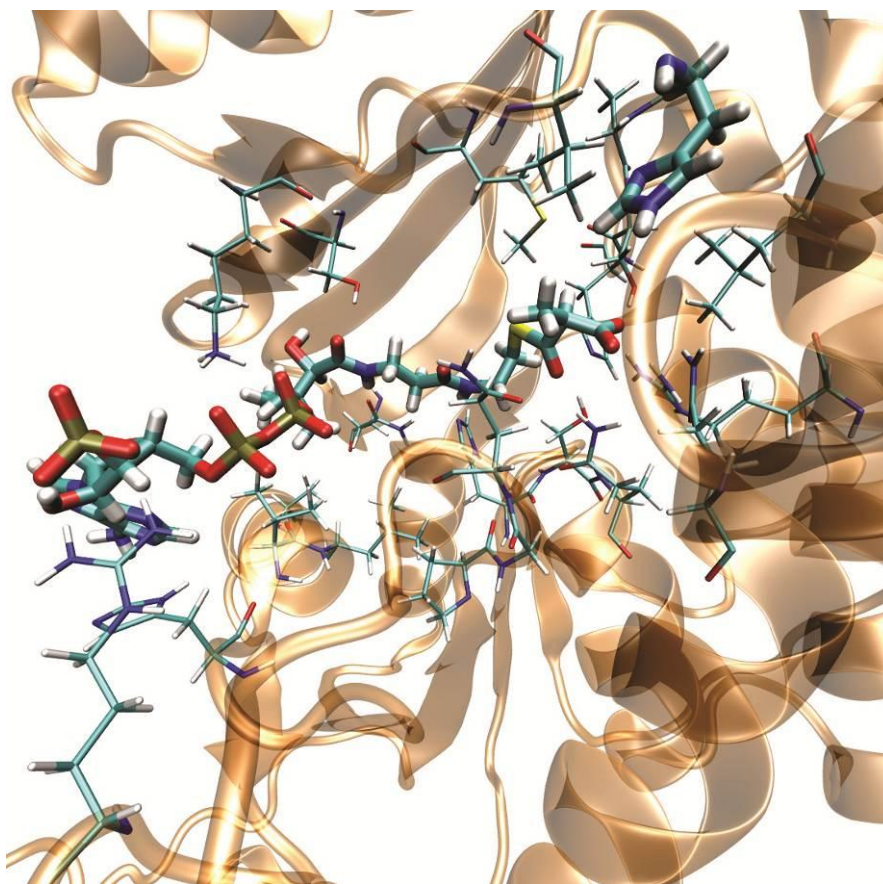
Supplementary Figure 7: Comparison of mutations at position 198. Relative positions of S197 and R222 with respect to Q198 (WT): the mutated systems R198 and K198 are shown in blue and red, respectively



2. Tyrosine 297

The effect of the loss of the hydroxyl group of Tyr and the reduction of the size of the lateral chain in the Y297H mutation (Supplementary Information Figure 8) is discussed in the main text. The remaining interaction pattern in the binding pocket of Y297H is basically the same than in the wild type model. For example, the HE2₁₉₈•••O16_{MMCoA} distance is 2.01 ± 0.22 Å and the HG₁₉₇•••NE2₃₀₀ distance is 2.06 ± 0.24 Å which allow a proper activation of Ser197 and MMCoA. The methyl group of MMCoA is surrounded by Met235 and Val295 while the isopropyl group is accommodated in the cavity formed by Val379, Leu380 and Ala383. In the phosphate region of MMCoA the Lys291 and Arg399 residues interact with the phosphates groups of MMCoA.

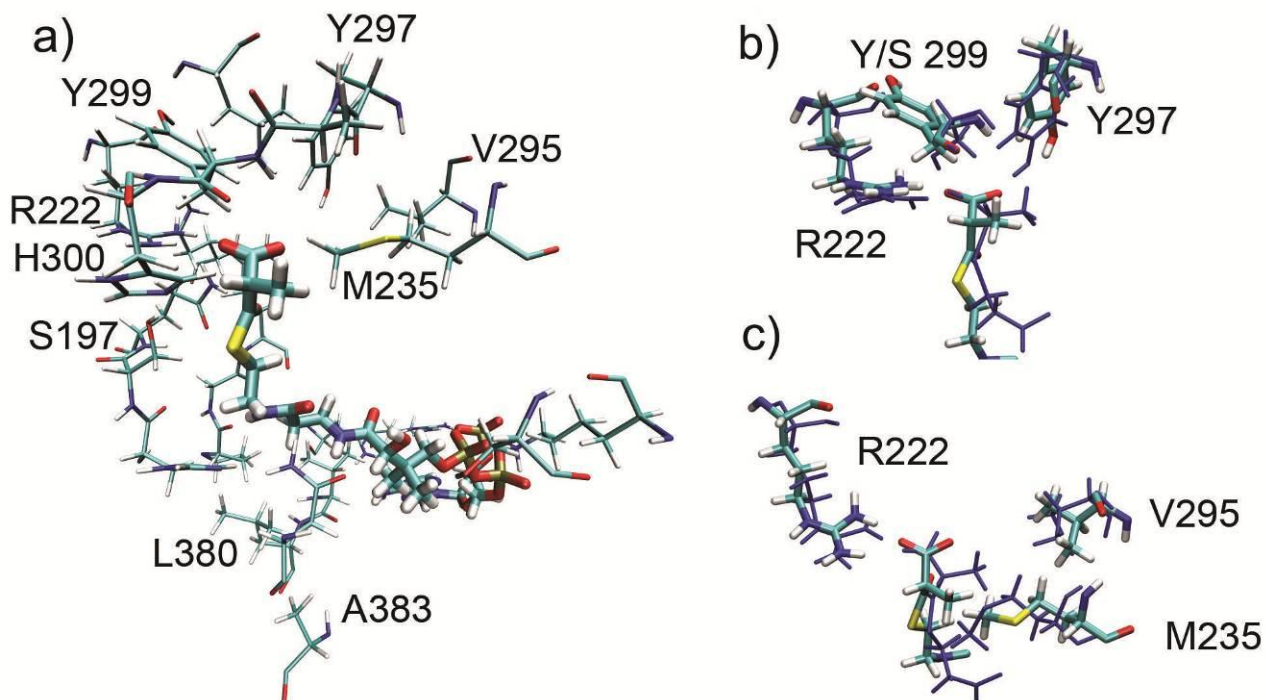
Supplementary Figure 8: Effect of the mutagenesis Y297H. H297 is placed between Val295 and Leu226.



3. Serine 299

As mentioned before, Ser299 was mutated for the Val, Phe and Tyr amino acids. Mutants containing Val and Phe keep some degree of biological activity. In the case of the S299F mutation the substrate specificity is diminished and the protein is able to accept MMCoA and malonyl-coenzyme A (MCoA). The mutation S299Y inactivates the enzyme. The correlation between loss of activity and the size of the amino acid size is discussed in the main text. We found that the relative position of Gln198 with respect to MMCoA was also affected. For the mutant S299V the $HE_{2198} \cdots O_{16_{MMCoA}}$ distance is $2.98 \pm 0.34 \text{ \AA}$, in the wild type the value is $1.98 \pm 0.23 \text{ \AA}$. The MD simulations of mutation S299F which lowers the specificity of the protein have shown a reduction of the space available to accommodate the MMCoA inside the binding pocket. For instance, the distance between the methyl group of MMCoA and Met235 is reduced from $4.57 \pm 0.54 \text{ \AA}$ in the wild type to $4.15 \pm 0.48 \text{ \AA}$ in S299F. Also the orientation of the carboxylate group of MMCoA is quite altered in variant S299Y. For variants S299V and S299F values for distances $HH_{11_{222}} \cdots O_{17}$ and $HH_{21_{222}} \cdots O_{18}$ are similar to the wild type but for S299Y values are larger ($2.14 \pm 0.50 \text{ \AA}$ and $3.76 \pm 0.40 \text{ \AA}$).

Supplementary Figure 9: Mutation S299Y. a) The binding pocket becomes wide and unstructured, b) and c) overlays between the wild type (blue) and S299Y.



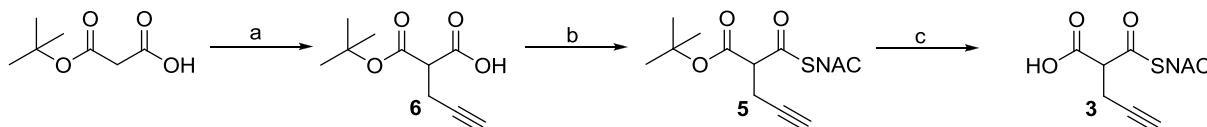
VI. Synthesis of Malonic acid derivatives for feeding studies

1. General methods

Unless otherwise noted, materials for chemical synthesis were obtained from commercial suppliers (Sigma-Aldrich, Alfa Aesar, Fluka, Acros) in the highest purity available and used without further purification. Dry solvents were purchased from Sigma-Aldrich, stored over molecular sieves and used as supplied. Solvents used for extraction and chromatography were purchased from Thermo Fisher Scientific. Flash chromatography was carried out using Acros silica gel 60 (35-70 μm mesh). Thin layer chromatography (TLC) was performed on aluminiumbacked, precoated silica gel (60 F-245) from Merck with Cyclohexane/EtOAc or DCM/MeOH mixtures as mobile phases. Spots were detected by staining with KMnO_4 staining solution (5.0 g KMnO_4 , 33 g K_2CO_3 , 10 ml 5% aqueous NaOH in 500 ml H_2O) and subsequent heat treatment.

NMR spectra were recorded using a Varian Mercury 400 (400 MHz, ^1H ; 100 MHz, ^{13}C) spectrometer and calibrated using residual undeuterated solvent as an internal reference. High-resolution mass spectra were recorded at *LTQ Orbitrap* with *Accela* HPLC-System (column Hypersil Gold, length 50 mm, inside diameter 1 mm, particle size 1.9 μm , ionization method: Electrospray Ionization). Products were characterized by NMR (^1H , ^{13}C) and HRMS.

2. Synthetic procedures



Supplementary Scheme 1: Synthesis of 2-Propargylmalonic acid-SNAC ester 3. Reagents and conditions: a) LDA, Propargylbromide, THF, -78°C , 43%; b) SNAC, CDI, THF, RT, 82%; c) TiCl_4 , DCM, qant.; LDA= Lithium diisopropylamide; THF= Tetrahydrofurane; SNAC= *N*-Acetylcysteamine; CDI= Carbonyldiimidazole; RT= room temperature; DCM= Dichloromethane

Synthesis of Lithiumdiisopropylamine (LDA)

2 ml (14.23 mmol) diisopropylamine (freshly distilled over NaH) were mixed with 5 ml abs. THF under argon protection. The solution was cooled to -78°C . Then 9.78 ml (15.65 mmol) *n*-butyllithium (1.5 M solution in hexane) were added dropwise and the reaction mixture was stirred for 40 min at -78°C . After 40 min the white suspension was allowed to reach room temperature before immediately using the resulting clear solution.

Synthesis of *N*-acetylcysteamin (SNAC)

11.4 g (100 mmol) cysteamine hydrochloride, 25.2 g (300 mmol) NaHCO₃ and 5.6 g (100 mmol) KOH were added to 500 ml of bidest. H₂O. After everything was dissolved 9.5 ml (100 mmol) acetic anhydride were added dropwise. After stirring at room temperature for 2 h, the light rose solution was quenched to pH = 4 with conc. HCl and extracted three times with 100 ml EtOAc. The combined organic layers were washed with 100 ml sat. NaHCO₃-solution and 100 ml sat. NaCl-solution and dried over Na₂SO₄ to obtain 7.56 g (61%) of the desired product as clear oil.⁴⁰

R_f: 0.42 (DCM/MeOH 9:1); **HRMS**: calculated for: 120.04776 [M+H⁺] C₄H₁₀ONS; found: 120.04730 [M+H⁺] C₄H₁₀ONS; **¹H-NMR**: (400 MHz, CDCl₃-d₁) δ = 1.34-1.38 (t, J= 8.4Hz, 1.97 (s, 3H, CH₃), 2.60-2.66 (m, 2H, CH₂), 3.36-3.40 (m, 2H, CH₂), 6.33 (bs, 1H, NH); **¹³C-NMR**: (101MHz, CDCl₃-d₁) δ = 23.3 (CH₃), 24.6 (CH₂), 42.6 (CH₂), 170.4 (C_q, AcNHR)

Synthesis of 2-(^tbutoxycarbonyl)pent-4-ynoic acid (5)

3 g (18.73 mmol) 3-(^tButoxy)-3-oxopropanoic acid (freshly purified by column chromatography before use) was dissolved in 20 ml abs. THF under argon. The solution was cooled to -78°C. Slowly, 39.33 mmol of freshly prepared LDA were added and stirred for 15 min and 2.813 g (2.099 ml, 18.92 mmol) Propargylbromide were added dropwise. After the addition of propargylbromide in THF, the slightly yellow reaction mixture was allowed to reach RT over 18 h. The brown reaction mixture was cooled to 0°C and quenched with 5 ml saturated NH₄Cl solution before the evaporation of the organic solvent. The resulting slurry was diluted with 50 ml saturated NaHCO₃-solution and washed with 50 ml of EtOAc. The organic layer was extracted twice with 50 ml of saturated NaHCO₃-solution. The combined water phase was acidified to pH = 1 with conc. HCl and extracted three times with 50 ml EtOAc, dried over Na₂SO₄ and the crude product was purified by column chromatography (Cyclohexane/EtOAc 95:5) to obtain 1.605 g (43%) of the desired product as slightly yellow oil.

R_f: 0.25 (EtOAc/Cyclohexane 8:2, two times development); **HRMS**: calculated for: 221.07843 C₁₀H₁₅O₄Na, [M+Na⁺]; found: 221.07845 C₁₀H₁₅O₄, [M+Na⁺]; **¹H-NMR**: (400 MHz, CDCl₃-d₁) δ = 1.46 (s, 9H, ^tBu), 2.04 (t, J= 2.6 Hz, 1H, CH), 2.17-2.18 (s, 1H, CH_{Propargyl}), 2.94-2.95 (d, J= 2.6 Hz, 2H, CH₂_{Propargyl}); **¹³C-NMR**: (101MHz, CDCl₃-d₁) δ = 23.1 (CH₂_{Propargyl}), 28.1(CH₃, ^tBu), 57.2 (CH), 72.2 (CH_{Propargyl}), 78.8 (C_q_{Propargyl}), 83.8 (C_q, ^tBu), 167.7 (C_q, COO^tBu), 174.4 (C_q, COOH)

Synthesis of ^tbutyl 2-(((2-acetamidoethyl)thio)carbonyl)pent-4-ynoate (6)

3.368 g (17.00 mmol) **5** was dissolved in 30 ml abs. THF under argon. Subsequently, 3.031 g (18.7 mmol) CDI and 622 mg (5.10 mmol) DMAP were added at 0°C and the mixture stirred for 60 min at 0°C before 2.633 g (22.09 mmol) SNAC were added dropwise. The reaction mixture was stirred for another 30 min at 0°C and then for 18 h at RT. The solvent was removed *in vacuo*

and the residue was suspended in 50 ml 1M HCl. The suspension was extracted three times with 50 ml of EtOAc. The combined organic layers were collected, dried over Na₂SO₄ and purified by column chromatography (DCM/MeOH 99:1) to obtain 4.163 g (82%) of the desired product as slightly yellow oil.

R_f: 0.69 (DCM/MeOH 9:1); **HRMS**: calculated for: 300.12641 C₁₄H₂₂O₄NS, [M+H⁺], 322.10835 C₁₄H₂₂O₄NSNa, [M+Na⁺], 317.15295 C₁₄H₂₅O₄N₂S; found: 300.12664 C₁₄H₂₂O₄NS, [M+H⁺], 322.10861 C₁₄H₂₂O₄NSNa, [M+Na⁺], 317.15325 C₁₄H₂₅O₄N₂S, [M+NH₄⁺]; **¹H-NMR**: (400 MHz, CDCl₃-d₁) δ = 1.47 (s, 9H, ^tBu), 1.96 (s, 3H, CH₃), 2.01-3.03 (t, J= 2.7 Hz, 1H, CH_{Propargyl}), 2.74-2.76 (ddd, J= 7.6, 2.7, 0.6 Hz, 2H, CH_{2 Propargyl}), 3.09-3.12 (m, 2H, CH_{2 SNAC}), 3.43-3.47 (m, 2H, CH_{2 SNAC}), 3.69-3.73 (t, J= 7.6 Hz, 1H, CH), 5.87 (bs, 1H, NH); **¹³C-NMR**: (101MHz, CDCl₃-d₁) δ = 18.8 (CH_{2 Propargyl}), 23.3 (CH₃), 27.9 (CH₃, ^tBu), 29.1(CH_{2 SNAC}), 39.5 (CH_{2 SNAC}), 59.6 (CH), 70.7 (CH_{Propargyl}), 79.9 (C_{q Propargyl}), 83.3 (C_q, ^tBu), 166.3 (C_q, ^tBu), 170.5 (C_q, CONHR), 194.4 (C_q, COS_{NAC})

Synthesis of 2-(((2-acetamidoethyl)thio)carbonyl)pent-4-ynoic acid (3)

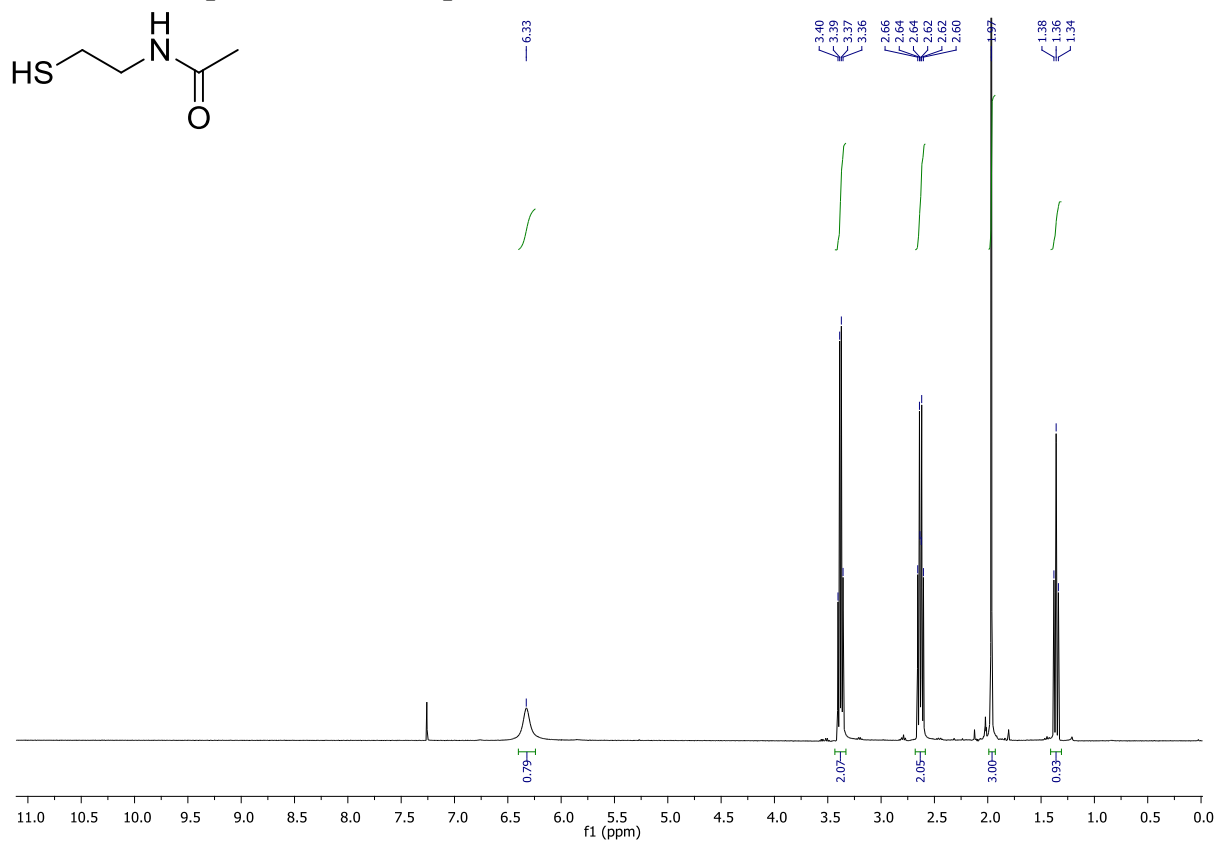
349 mg (1.17 mmol) **6** was dissolved in 30 ml abs. DCM under argon. At 0°C 442 mg (255 μl, 2.34 mmol) TiCl₄ was added dropwise. The dark brown reaction mixture was stirred for 30 min at 0°C, then for another 6 h at room temperature. After 6 h (DC-control) the reaction mixture was quenched with 12 ml of the sodium carbonate-based feeding buffer at 0°C to reach a calculated final concentration of 0.1 M of product. DCM was evaporated at RT and the remaining white slurry was transferred to 50 ml polypropylene tubes. After centrifugation for 4 min to remove TiO₂ the resulting clear solution was sterile filtered and used directly for feeding experiments.

R_f: 0.18 (MeOH/DCM 1:9); **HRMS**: calculated for: 244.06381 C₁₀H₁₄O₄NS, [M+H⁺]; found: 244.06402 C₁₀H₁₄O₄NS, [M+H⁺]; **¹H-NMR**: (400 MHz, D₂O-d₂) δ = 1.90 (s, 3H, CH₃); 2.36 (m, 2H, CH_{2 Propargyl}); 2.75-2.76 (m 1H, CH); 3.02-3.06 (m, 2H, CH_{2 SNAC}), 3.29-3.35 (m, 2H, CH_{2 SNAC}), 3.71-3.75 (t, J= 7.6 Hz, 1H, CH_{Propargyl})

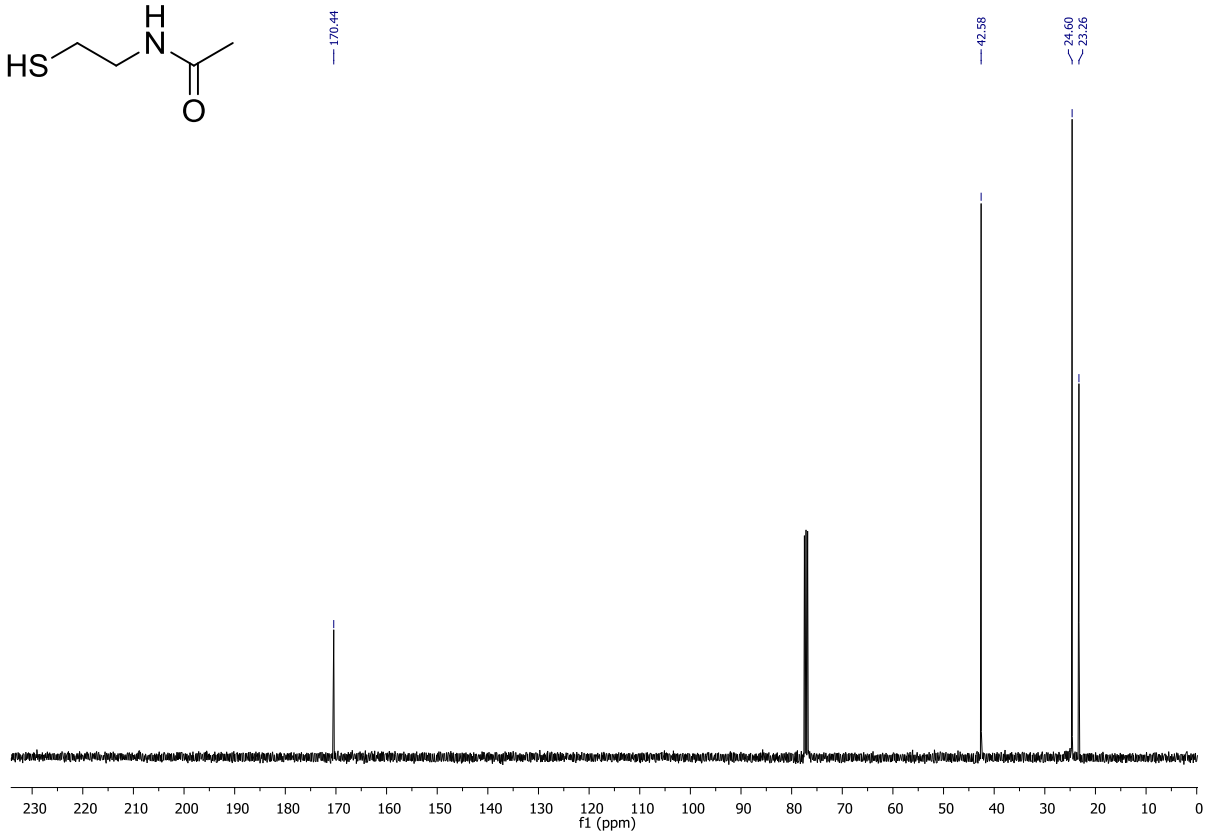
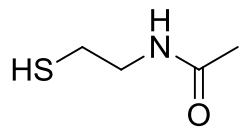
Preparation of the feeding buffer

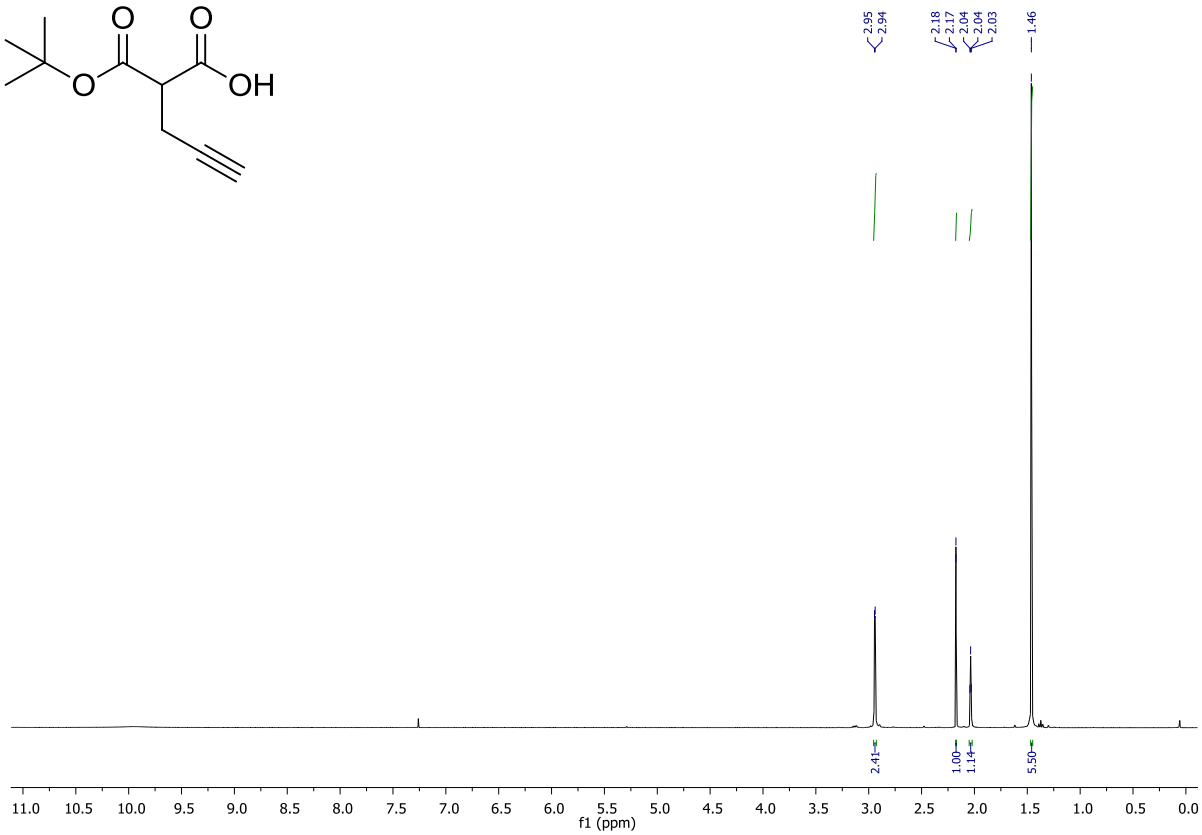
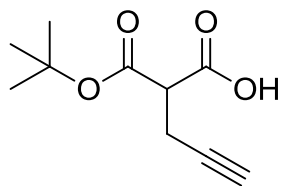
5 g Glucose, 50 g Glucidex IT29 (Roquette), 83.83 g NaHCO₃, 186 mg Na₂CO₃ were dissolved in 1 l of Millipore water to obtain 1 M carbonate feeding buffer at pH= 8.

3. NMR-Spectra of the compounds

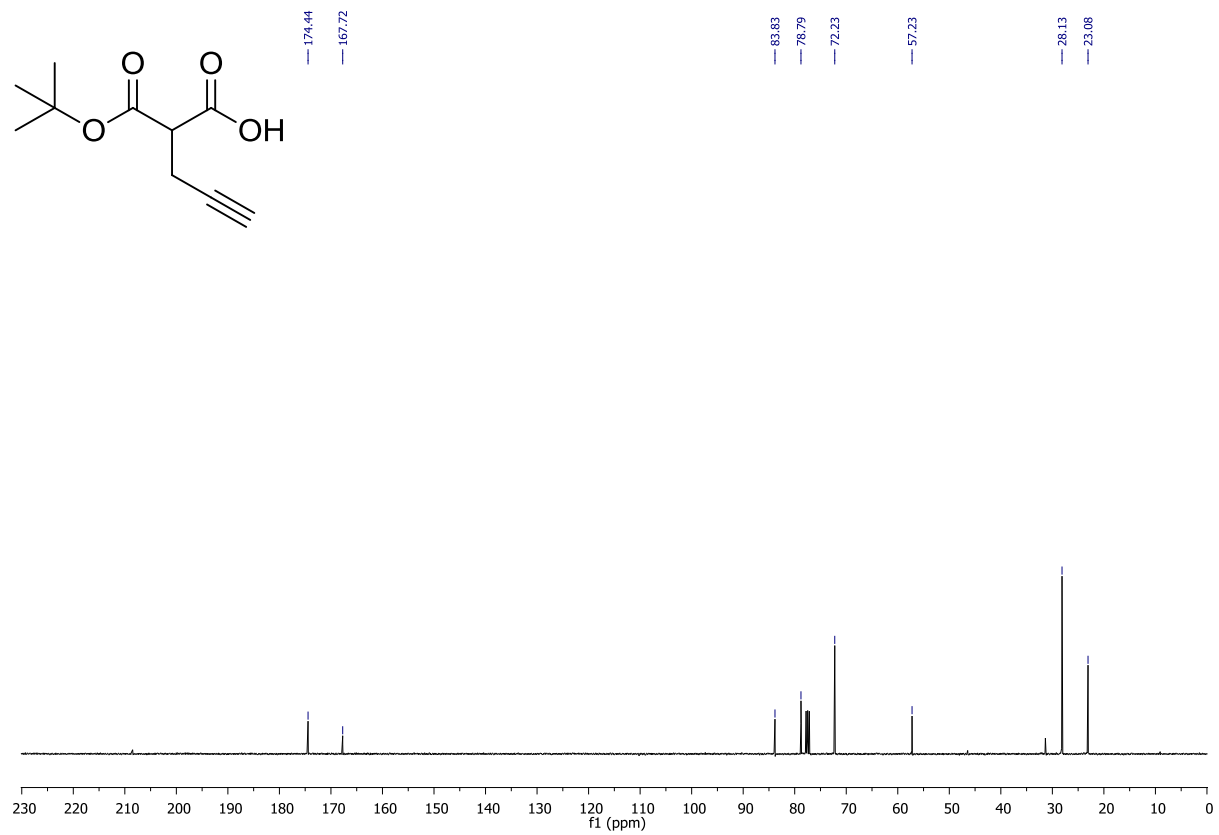
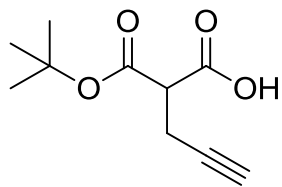


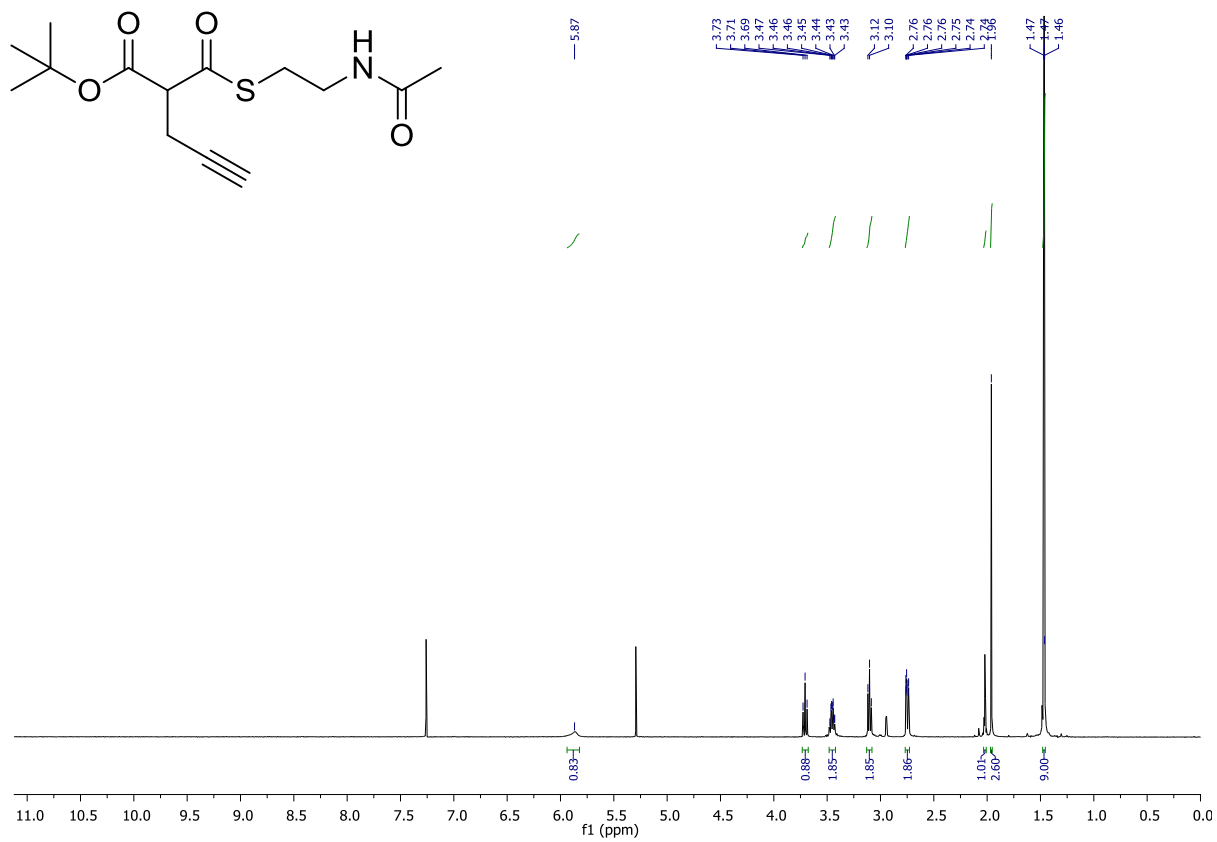
¹H NMR- and ¹³C-NMR spectra of SNAC in CDCl₃-d₁



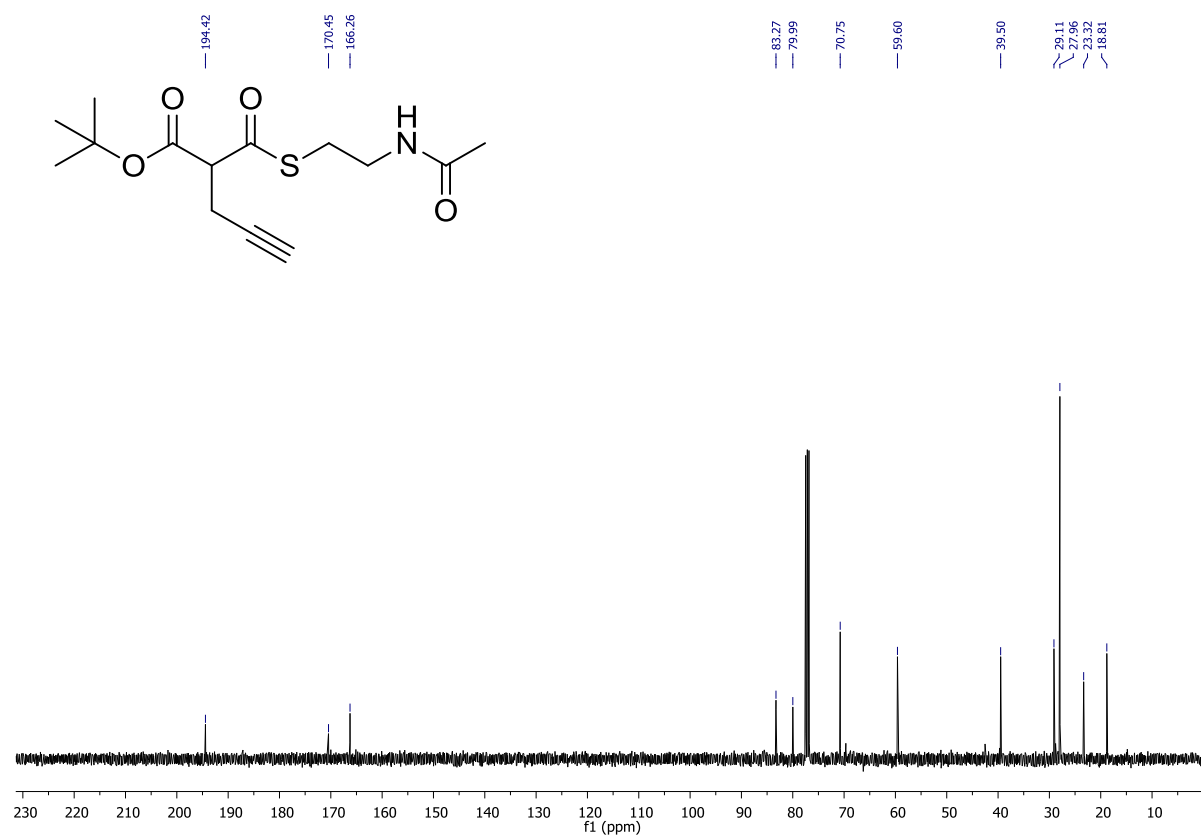


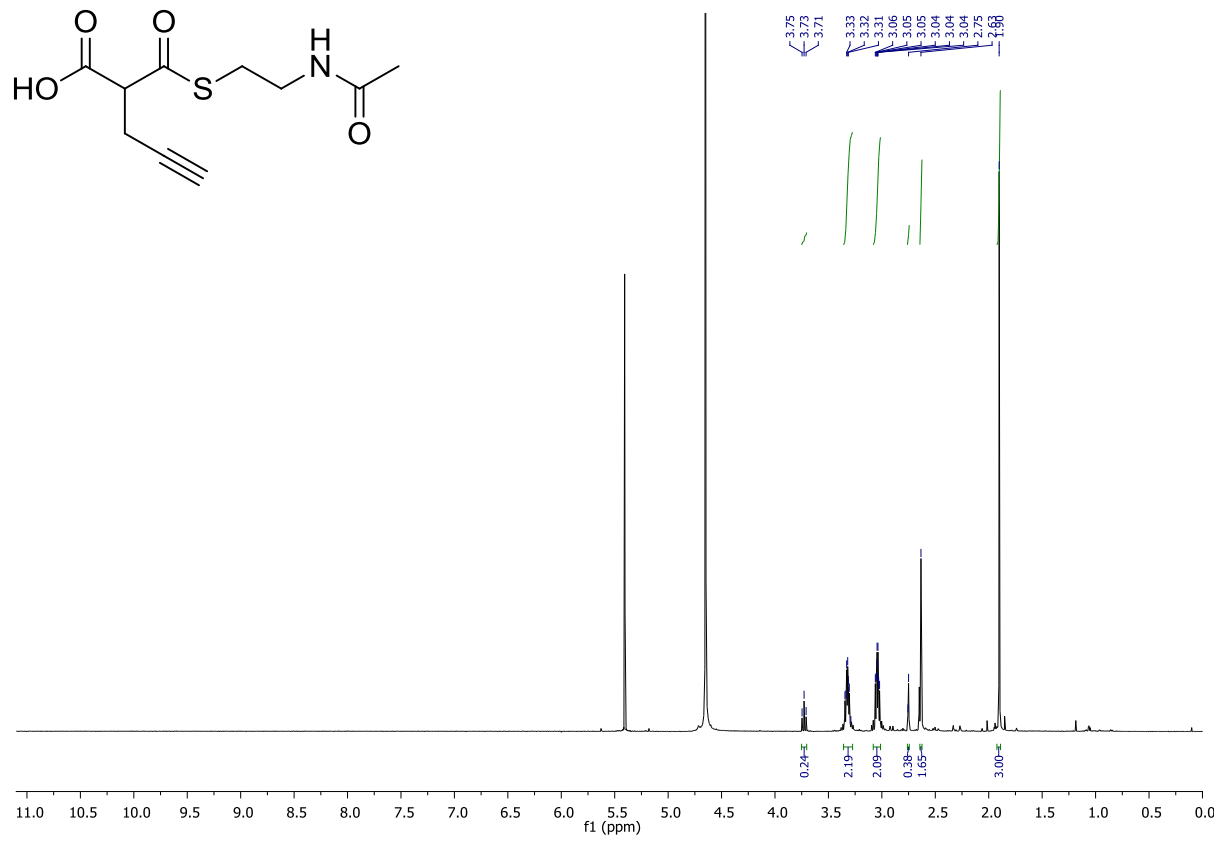
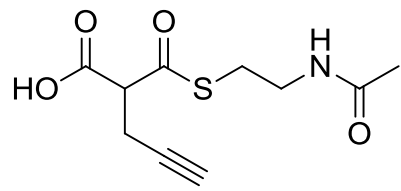
^1H - and ^{13}C -NMR spectra of **5** in $\text{CDCl}_3\text{-d}_1$





¹H- and ¹³C-NMR spectra of **6** in CDCl₃-d₁





¹H-NMR spectrum of 3 in D₂O-d₂

VII. References

- (1) Anand, S.; Prasad, M. V. R.; Yadav, G.; Kumar, N.; Shehara, J.; Ansari, M. Z.; Mohanty, D. *Nucleic Acids Res.* **2010**, *38*, W487.
- (2) Notredame, C.; Holm, L.; Higgins, D. G. *Bioinformatics* **1998**, *14*, 407.
- (3) Ridley, C. P.; Lee, H. Y.; Khosla, C. *Proc Natl Acad Sci U S A* **2008**, *105*, 4595.
- (4) Kopp, M.; Irschik, H.; Gemperlein, K.; Buntin, K.; Meiser, P.; Weissman, K. J.; Bode, H. B.; Muller, R. *Mol BioSystems* **2011**, *7*, 1549.
- (5) Kakavas, S. J.; Katz, L.; Stassi, D. *J. Bacteriol.* **1997**, *179*, 7515.
- (6) Bergeret, F.; Gavalda, S.; Chalut, C.; Malaga, W.; Quémard, A.; Pedelacq, J.-D.; Daffé, M.; Guilhot, C.; Mourey, L.; Bon, C. *J. Biol. Chem.* **2012**.
- (7) Liu, Y.; Hazzard, C.; Eustáquio, A. S.; Reynolds, K. A.; Moore, B. S. *J. Am. Chem. Soc.* **2009**, *131*, 10376.
- (8) Laureti, L.; Song, L.; Huang, S.; Corre, C.; Leblond, P.; Challis, G. L.; Aigle, B. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 6258.
- (9) Sambrook, J.; Russel, D. W. *Molecular Cloning - A laboratory manual*; CSH Press: New York, 2001; Vol. 3.
- (10) Fitzgerald, N. B.; English, R. S.; Lampel, J. S.; Vanden Boom, T. J. *Appl. Environ. Microbiol.* **1998**, *64*, 1580.
- (11) Wu, K.; Chung, L.; Revill, W. P.; Katz, L.; Reeves, C. D. *Gene* **2000**, *251*, 81.
- (12) Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical streptomyces genetics*; John Innes Foundation, 2000.
- (13) Ranganathan, A.; Timoney, M.; Bycroft, M.; Cortés, J.; Thomas, I. P.; Wilkinson, B.; Kellenberger, L.; Hanefeld, U.; Galloway, I. S.; Staunton, J.; Leadlay, P. F. *ChemBiol* **1999**, *6*, 731.
- (14) Blondelet-Rouault, M. H.; Weiser, J.; Lebrhi, A.; Branny, P.; Pernodet, J. L. *Gene* **1997**, *190*, 315.
- (15) Gust, B.; Challis, G. L.; Fowler, K.; Kieser, T.; Chater, K. F. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1541.
- (16) Kushnir, S.; Sundermann, U.; Schulz, F. *Asian J. Biotechnol.* **2012**, *under revision*.
- (17) Duetz, W. A.; Ruedi, L.; Hermann, R.; O'Connor, K.; Buchs, J.; Witholt, B. *Appl. Environ. Microbiol.* **2000**, *66*, 2641.
- (18) Roy, A.; Kucukural, A.; Zhang, Y. *Nat. Protoc.* **2010**, *5*, 725.
- (19) Tang, Y.; Chen, A. Y.; Kim, C.-Y.; Cane, D. E.; Khosla, C. *ChemBiol.* **2007**, *14*, 931.
- (20) Tang, Y.; Kim, C.-Y.; Mathews, I. I.; Cane, D. E.; Khosla, C. *Proc. Natl. Acad. Sci. USA.* **2006**, *103*, 11124.
- (21) Glide, version 5.5, Schrödinger, LLC, New York, NY, **2009**.
- (22) Schrödinger Suite 2009 Protein Preparation Wizard; Epik version 2.0, Schrödinger, LLC, New York, NY, 2009; Impact version 5.5, Schrödinger, LLC, New York, NY, 2009; Prime version 2.1, Schrödinger, LLC, New York, NY, **2009**.
- (23) Maestro, version 9.0, Schrödinger, LLC, New York, NY, **2009**.
- (24) Humphrey, W.; Dalke, A.; Schulten, K. *J. Mol. Graphics* **1996**, *14*, 33.
- (25) Mackerell, A. D., Jr; Feig, M.; Brooks, C. L., III *J. Comput. Chem.* **2004**, *25*, 1400.
- (26) Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R. D.; Kale, L.; Schulten, K. *J. Comp. Chem.* **2005**, *26*, 1781.
- (27) Zoete, V.; Cuendet, M. A.; Grosdidier, A.; Michielin, O. *J. Comp. Chem.* **2010**, *32*, 2359.
- (28) Lee, C.; Yang, W.; Parr, R. G. *Phys. Rev. B* **1988**, *37*, 785.
- (29) Schäfer, A.; Horn, H.; Ahlrichs, R. *J. Chem. Phys.* **1992**, *97*, 2571.
- (30) ChemShell, a Computational Chemistry Shell, see www.chemshell.org

- (31) Sherwood, P.; de Vries, A. H.; Guest, M. F.; Schreckenbach, G.; Catlow, C. R. A.; French, S. A.; Sokol, A. A.; Bromley, S. T.; Thiel, W.; Turner, A. J.; Billeter, S.; Terstegen, F.; Thiel, S.; Kendrick, J.; Rogers, S. C.; Casci, J.; Watson, M.; King, F.; Karlsen, E.; Sjøvoll, M.; Fahmif, A.; Schäfer, A.; Lennartz, C. *J. Mol. Struct. (TEOCHEM)* **2003**, *632*, 1.
- (32) Ahlrichs, R.; Bär, M.; Häser, M.; Horn, H.; Kölmel, C. *Chem. Phys. Lett.* **1989**, *162*, 165.
- (33) Smith, W.; Forester, T. R. *J. Mol. Graphics* **1996**, *14*, 136.
- (34) Grimme, S. *J. Comput. Chem.* **2004**, *25*, 1463.
- (35) Becke, A. D. *J. Chem. Phys.* **1992**, *97*, 9173.
- (36) de Vries, A. H.; Sherwood, P.; Collins, S. J.; Rigby, A. M.; Rigutto, M.; Kramer, G. J. *J. Phys. Chem. B* **1999**, *103*, 6133.
- (37) Antes, I.; Thiel, W. *ACS Symp. Ser.* **1998**, *712*, 50.
- (38) Bakowies, D.; Thiel, W. *J. Phys. Chem.* **1996**, *100*, 10580.
- (39) Billeter, S. R.; Turnera, A. J.; Thiel, W. *Phys. Chem. Chem. Phys.* **2000**, *2*, 2177.