## **Supporting Information File**

for

# A non-canonical peptide synthetase adenylates 3-methyl-2oxovaleric acid for auriculamide biosynthesis

Daniel Braga<sup>1,2</sup>, Dirk Hoffmeister<sup>1</sup> and Markus Nett\*<sup>3</sup>

Address: <sup>1</sup>Friedrich-Schiller-Universität Jena, Department Pharmaceutical Microbiology at the Hans-Knöll-Institute, Winzerlaer Strasse 2, 07745 Jena, Germany, <sup>2</sup>Friedrich-Schiller-Universität Jena, Junior Research Group Synthetic Microbiology at the Hans-Knöll-Institute, Adolf-Reichwein-Strasse 23, 07745 Jena, Germany and <sup>3</sup>Department of Biochemical and Chemical Engineering, Technical Biology, Technical University Dortmund, Emil-Figge-Strasse 66, 44227 Dortmund, Germany

Email: Markus Nett - markus.nett@bci.tu-dortmund.de

\*Corresponding author

## **Complete experimental details**

# Experimental details

Bacterial strains, culture conditions, and general methods	S3
Bioinformatics	S3
Generation of hexahistidine-tagged constructs	S3
Production and purification of the recombinant proteins	S4
Figure S1. SDS-PAGE of the purified recombinant AuIA variants	S5
Protein sequences (without the hexahistidine tag)	S6
In vitro ATP-[ <sup>32</sup> P]pyrophosphate exchange assay	S8
References	S8

#### **General methods**

**Bacterial strains, culture conditions, and general methods.** *E. coli* was routinely grown at 37 °C on lysogenic broth (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L) amended with kanamycin (100 µg/mL) when appropriate. Strain XL-1 blue was used for cloning and preparation of plasmid DNA, whereas strain KRX was used in the heterologous production of the recombinant NRPS. Standard molecular biology procedures followed described protocols [1]. DNA purification, restriction, and ligation were performed in accordance with the instructions of the manufacturers. Kits and enzymes were purchased from NEB, Promega, Thermo Fisher Scientific, and Zymo Research. Eurofins Genomics performed the DNA sequence verification. Chemicals were acquired from Sigma-Aldrich, BD, VWR, and Carl Roth. [<sup>32</sup>P]pyrophosphate was purchased from PerkinElmer.

**Bioinformatics.** In silico analyses of the nonribosomal code and domain disposition were performed with NRPSpredictor2 [2] and PKS/NRPS [3]. Amino acid sequences of the adenylation domains pertaining to AuIA (ABX05054.1), CesA (ABD14711.1), CesB (ABK00633.1), PksJ (P40806.3), and PyrG (AEF33080.1) were aligned using ClustalW algorithm by Geneious v.9.1.5 (Biomatters, New Zealand) in order to verify the presence of a lysine residue in the last position of AuIA nonribosomal code.

**Generation of hexahistidine-tagged constructs.** Genomic DNA of *H. aurantiacus*  $114-95^{T}$  [4] served as template to amplify *aulA*. The amplification of the sequence encoding the full-length protein (A<sub>1</sub>-A<sub>2</sub>-KR-PCP) was performed with the oligonucleotide primers oDB88/89 (5'-GCT CCG GCG GAT CCA TGG AAA TGG ATT TTC AGA GT-3' and 5'-GCA TCG CTG TCG ACT TAC CCT AAT CCT GCT TCA AG-3' respectively). The sequence encoding the truncated protein (A<sub>2</sub>-KR-T)

was amplified with oligonucleotides oDB89/90 (5'-CCA TAG AAT TCA TGT TGT CAA CTC AGG CCA AGC-3'). 20 µL-reactions consisted of 0.2 mM (each) dNTP, 20 pmol (each) oligonucleotide primer, 2 mM MgCl<sub>2</sub>, and 0.4 U Phusion® High Fidelity DNA polymerase in the supplied buffer, and 100 ng genomic DNA as template. Thermal cycling parameters were: 30 s at 98 °C; 33 cycles of 98 °C for 10 s, 55 °C for 15 s and 72 °C for 150 s, followed by a terminal hold 5 min at 72 °C. Amplicons of full and partial *aulA* were ligated into pET28a(+) to generate pDB039 (10,813 bp) and pDB042 (9,181 bp), respectively. The resulting plasmids encode the respective *N*-terminal hexahistidine fusions.

**Production and purification of the recombinant protein.** For heterologous production of the recombinant AuIA variants, *E. coli* KRX (Promega) was used as host and grown in 400 mL LB medium supplemented with 100 µg/mL kanamycin. After reaching an OD<sub>600</sub> = 0.7, gene expression was induced by adding 0.1% (w/v) L-rhamnose, followed by further cultivation at 16 °C for 18 hours. Cells were harvested by centrifugation (3,200*g*, 50 min, 4 °C) and suspended in lysis buffer (20 mM imidazole, 50 mM sodium phosphate, 300 mM sodium chloride, pH 8.0). Cells were disrupted with a Sonopuls ultrasonic sonifier (Bandelin). The lysate was centrifuged (17,000*g*, 30 min, 4 °C) to remove cellular debris. The proteins were purified by metal affinity chromatography using an Äkta FPLC (GE Healthcare) equipped with a HisTrap HP column (GE Healthcare), subjecting the sample to a stepwise imidazole gradient (20–500 mM) in lysis buffer. Proteins were desalted on a PD-10 column (GE Healthcare) and eluted with reaction buffer (100 mM PIPES, pH 7.0, 5 mM MgCl<sub>2</sub>, 125 nM EDTA). Purification was confirmed by SDS-PAGE.

S4



**Figure S1:** SDS-Polyacrylamide gel of the purified recombinant AuIA. The electrophoretic run was performed in 12% Laemmli gels at 35 mA.

- (1) Protein ladder
- (2) AulA (A1-A2-KR-PCP, 1852 aa, 203.1 kDa)
- (3) Truncated AulA (A<sub>2</sub>-KR-PCP, 1308 aa, 143.8 kDa).

### Protein sequences (without the hexahistidine tag)

AuIA (A<sub>1</sub>-A<sub>2</sub>-KR-T)

MEMDFQSIQRRFATAVEQRSSQAALRYHDQVVSYHELAEHAQRIASGLANQQVGV NTNVAIQLTNPIDVCSTILATLLLGARYALLSPNLAKLRLQQVLARQQFVLVGSAASN NLAANYIEFEQLANSELAEITPHSATAESLIGLSLASNPSGLIEAGQLSQTNLLSFIDFN LSKAKVSFQQSLWLGEEFNDFSAFASLATLASGGTLRFSTLETLDHDLDEQAQTLM LTLATLGQLFAQQSALPKVRHILSSGEGLLDGEALKQQLKQQQTAWHNYYGFPAFQ LLTVVGANTQTQTASQIHSGKPVPHTQALILDQHKQLAPIGLTGELYVAGAGVFAGF EQAQLNAERFIASPFAADTQLYQTRYLARWQDDGRLSISGSLDSTIELAATPILLQEV ERLLEQHPAIVECCIVRRITLSNTEQLTGFVVAKQRVEPSEILNYLEDQLDCQLANLG LIQVDQLPRTADGQLDRQQLAQTNLLDNRQIADLQAQLQQAANGAELAVVAQPILPV SSPLHIDDLVPMVETSNFGTSQRTISEQPVEMLSTQAKPALAVGPPLIKAEQAPLTLA EALVLAAKHYPEHGISYIEADGKALFQSYAALLADAEAVLAGLRAAGLKHGQHVVLQ FAHNEPFVVAFWACMLGGFTAVPLALPNSSDPNNPAVSKLYNTWQTLEQPLIVSEQ ASFSLLQRIFNGLGVVKPAIQITEQLRQHQPDQQHQHLAPQDSALLLFTSGSTGLPK GVELSHHNIISRSKASAQHNRFDHNDVSLNWMPLDHVGGIVMFHVHDVCLGCRQIQ AKTDYILEDPVRWLDLLEQYRATITWSPNFAYALINDQHERVNSRRRNLSSLRFILNG GEGINKQTALNFLGLLQAHGLPATAMHPAYGMSETSSGISSSDQLVLGATTGFHEL DQASLTGVIQPASADSIGVAFVEVGAPLPGVSLRIVNTNNQLLSEDLIGRLQIQGPTIT AGYYRNPELNREVFTDDGWFTTGDLAFLHQGRLTIAGREKDVIIINGINYHNHEIEAL VETIEGVEVSYTAACSVPSKHTGGTESLVIFYVSKSAEFDQQLAQINQIREVVVQKIGI NPSYVLPVAKSDIPKTAIGKIQRSQLSQRFINGEFSSITKPIDLALANQQTLPRWFFSK QWQPVSKRHNSALLKPSYAIFSDDTTLARELIDVLEQHHRDWVLISAGETFSQQGQ HYTINLHDPEHYHQIAATLAATNIHDYVHLYSCDLPSEIEHVGDLAAAQYRGTYSLLF LTQALAKQKLSQASLTVVSQRSHAINQSDQVIYAAAPIHGLLKTMPLEIDWLSCQHV DLDAASATTNSQQIYYELAQPKPSAEVAYRAGQRLVPQLVEAEMAQSSPVESPLVK

S6

GGLYLVTGGLGGIGSQFARWLLQNYNARLLITGSTELPLGSDWAKHLGTDSSLSKR LRAYKDLIDISNDVHYQAVDITDSAQLARLINDAEQRWNQPLAGVFHFAGAGNLAYH WTVMDHHWITNESLATFEMMFAPKVYGTWALQRALSQRPELPIVAMSSINSFFGGA TFSAYSAANSFLDSFMLHQRQTSHPKALCLNWTQWDNIGMSLNNPQQIRSLSAER GYNVIGLQQGLQSLLAGISQNQYPLLMIGLNADSPALRQHLAVSQPLQQRINLYTTH QHGPLSHDRYRQLANSYFGSATLEWYRVAELPRTSSGAIDLAALGQLDATNQQTAL DQPTNIIEEQLVSIWQEILGKPKIGIHDNFFALGGHSLLATQLVSRLRDGFNLEVRLYQ LFAAPTIAELANCIAELQLEQIDSAEMDALLAELEGLSEAELEAGLG

## Partial AuIA (A<sub>2</sub>-KR-T)

MLSTQAKPALAVGPPLIKAEQAPLTLAEALVLAAKHYPEHGISYIEADGKALFQSYAA LLADAEAVLAGLRAAGLKHGQHVVLQFAHNEPFVVAFWACMLGGFTAVPLALPNSS DPNNPAVSKLYNTWQTLEQPLIVSEQASFSLLQRIFNGLGVVKPAIQITEQLRQHQP DQQHQHLAPQDSALLLFTSGSTGLPKGVELSHHNIISRSKASAQHNRFDHNDVSLN WMPLDHVGGIVMFHVHDVCLGCRQIQAKTDYILEDPVRWLDLLEQYRATITWSPNF AYALINDQHERVNSRRRNLSSLRFILNGGEGINKQTALNFLGLLQAHGLPATAMHPA YGMSETSSGISSSDQLVLGATTGFHELDQASLTGVIQPASADSIGVAFVEVGAPLPG VSLRIVNTNNQLLSEDLIGRLQIQGPTITAGYYRNPELNREVFTDDGWFTTGDLAFLH QGRLTIAGREKDVIIINGINYHNHEIEALVETIEGVEVSYTAACSVPSKHTGGTESLVIF YVSKSAEFDQQLAQINQIREVVVQKIGINPSYVLPVAKSDIPKTAIGKIQRSQLSQRFI NGEFSSITKPIDLALANQQTLPRWFFSKQWQPVSKRHNSALLKPSYAIFSDDTTLAR ELIDVLEQHHRDWVLISAGETFSQQGQHYTINLHDPEHYHQIAATLAATNIHDYVHLY SCDLPSEIEHVGDLAAAQYRGTYSLLFLTQALAKQKLSQASLTVVSQRSHAINQSDQ VIYAAAPIHGLLKTMPLEIDWLSCQHVDLDAASATTNSQQIYYELAQPKPSAEVAYRA GQRLVPQLVEAEMAQSSPVESPLVKGGLYLVTGGLGGIGSQFARWLLQNYNARLLI TGSTELPLGSDWAKHLGTDSSLSKRLRAYKDLIDISNDVHYQAVDITDSAQLARLIND

S7

AEQRWNQPLAGVFHFAGAGNLAYHWTVMDHHWITNESLATFEMMFAPKVYGTWA LQRALSQRPELPIVAMSSINSFFGGATFSAYSAANSFLDSFMLHQRQTSHPKALCLN WTQWDNIGMSLNNPQQIRSLSAERGYNVIGLQQGLQSLLAGISQNQYPLLMIGLNA DSPALRQHLAVSQPLQQRINLYTTHQHGPLSHDRYRQLANSYFGSATLEWYRVAEL PRTSSGAIDLAALGQLDATNQQTALDQPTNIIEEQLVSIWQEILGKPKIGIHDNFFALG GHSLLATQLVSRLRDGFNLEVRLYQLFAAPTIAELANCIAELQLEQIDSAEMDALLAE LEGLSEAELEAGLG

**In vitro ATP-[<sup>32</sup>P]pyrophosphate exchange assay.** All reactions were run in triplicates at 30 °C. The reactions were composed of reaction buffer (described above), 1 mM individual substrates, 100 nM purified enzyme and 0.1 μM [<sup>32</sup>P]pyrophosphate (50 Ci/mmol). The reaction volume was 100 μL. The reactions were stopped after 30 min and further processed as described [5]. To establish optimum conditions for enzymatic activity, 3-methyl-2-oxovaleric acid served as a substrate in reactions carried out at varied temperatures (4–56 °C) and pH values (6.0–9.0) using PIPES- and TRIS-buffers. Radioisotope exchange was quantified on a PerkinElmer TriCarb 2910TR scintillation counter.

#### References

1. Sambrook, J.; Russell, D. W., *Molecular Cloning: A Laboratory Manual.* 3 ed.; Cold Spring Harbor Laboratory Press: 2001.

2. Röttig, M.; Medema, M. H.; Blin, K.; Weber, T.; Rausch, C.; Kohlbacher, O., *Nucleic Acids Res* **2011**, *39* (Web Server issue), W362-7.

3. Bachmann, B. O.; Ravel, J., Methods Enzymol 2009, 458, 181-217.

4. Schieferdecker, S.; Domin, N.; Hoffmeier, C.; Bryant, D. A.; Roth, M.; Nett, M., *Eur J Org Chem* **2015**, 3057-3062.

5. Schneider, P.; Weber, M.; Rosenberger, K.; Hoffmeister, D., *Chem Biol* **2007**, *14* (6), 635-44.